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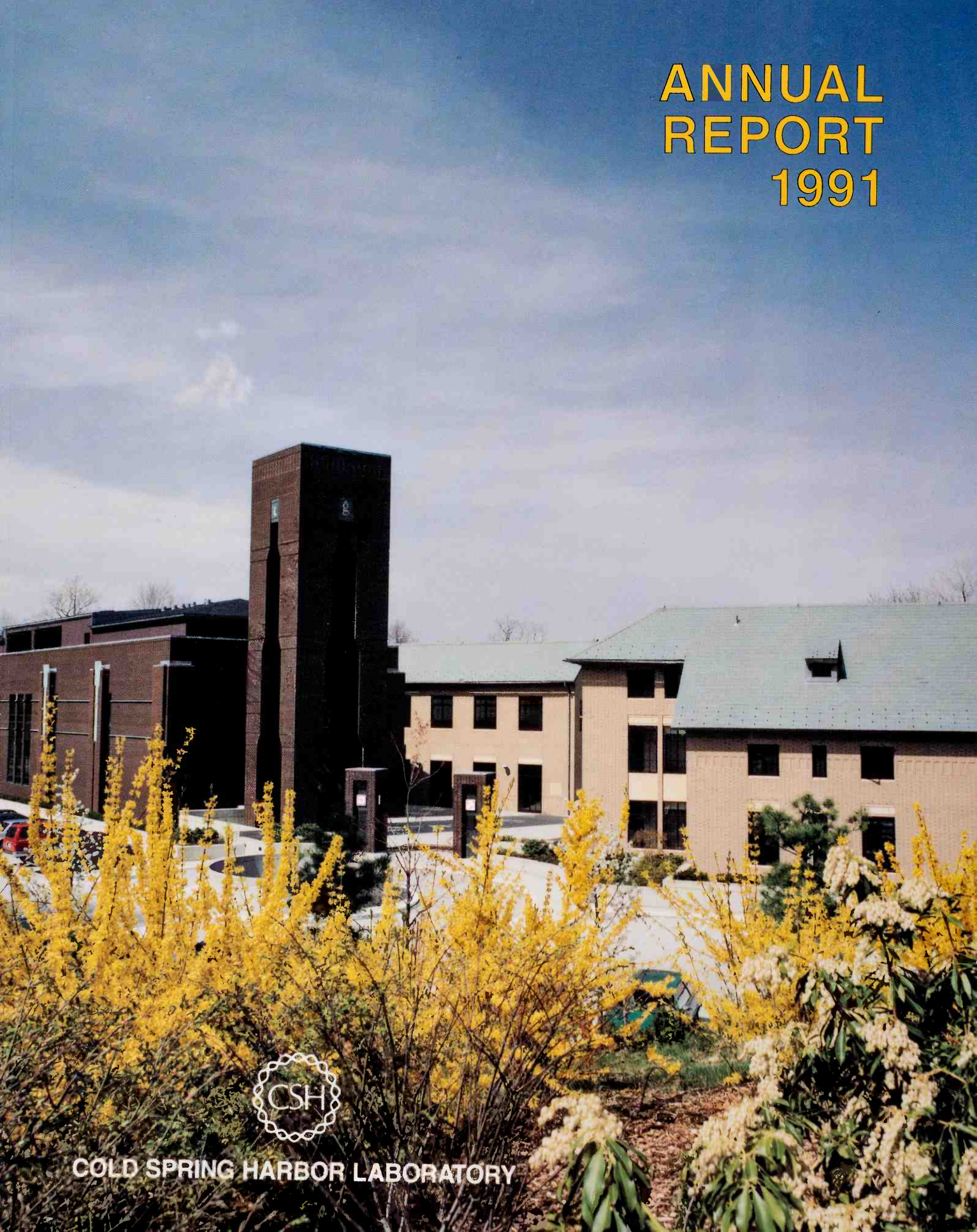

COLD SPRING HARBOR LABORATORY

ANNUAL REPORT 1991

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ANNUAL REPORT 1991

Cold Spring Harbor Laboratory
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Front cover: The new Beckman Neuroscience Center at Cold Spring Harbor Laboratory, dedicated May 3, 1991. (Photograph by Margot Bennett.)

Back cover: One of the Adirondack-style guest cabin complexes adjacent to the Neuroscience Center. (Photo by Margot Bennett.)

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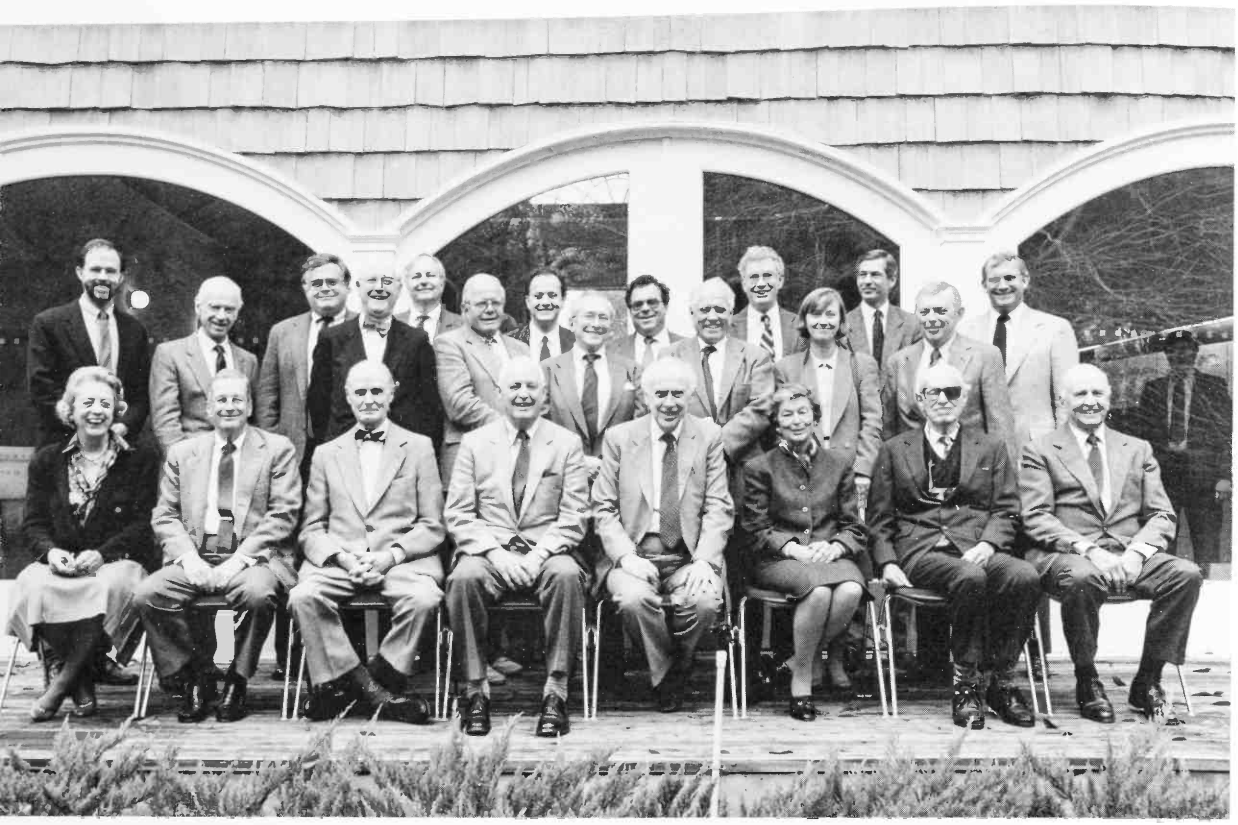

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FABIAN BACHRACH

Oliver Russell Grace
(1909–1992)

On January 16, 1992, we suffered a grievous loss with the passing of Oliver Russell Grace. In his 82 years, Oliver lived at least three lives: he was a dedicated, loving husband and father; a successful businessman; and a generous and thoughtful philanthropist. It was in this last capacity that the Laboratory and I initially became acquainted with him. My first visit with him was in 1977, soon after he and his wife Lorraine moved to Oyster Bay. They had long been friends with Franz and Betty Schneider who brought us all together for a Sunday lunch at the Piping Rock Club. The Schneiders knew of Oliver's dedication to cancer research and wanted to interest him in our Laboratory, of which Betty was then a trustee. Later, his fiscal, intellectual, and moral support would help make possible the substantive changes that have so enlarged this Laboratory's potential to promote science.

Oliver's generosity and intelligence go back well before his association with the Laboratory. In fact, they go back at least six generations. The Grace family genealogy, which traces to William the Conqueror, credits John Grace of Sheffield (1706–1780) with being "the ancestor of that branch of the family whose superior business capacities, rare political integrity, and far-reaching generosity acquired fame and fortune in North and South America." His great-grandson, James Grace, lived in Ireland, having married a well-to-do woman named Ellen Russell. They emigrated from Ireland to Peru during the potato famine of the 19th century. The oldest of their sons, William Russell, and the youngest, Michael Paul, came to the United States and went into business together in the firm of Bryce, Grace, and Company, which would ultimately become W.R. Grace and Company, a major New York trading and shipping firm. W.R. Grace had a reputation for being tough, fair, and concerned for the underpriviledged. On these strengths, he was twice elected mayor of New York City, serving from 1880 to 1882 and 1884 to 1886.

Another brother, Morgan S. Grace, Count of the Holy Roman Empire, was trained as a physician and went off to Auckland to the New Zealand war. He became surgeon general of New Zealand and was awarded the prestigious Cross of St. Michael and St. George. Morgan fathered ten children, one of whom was Oliver's father, Morgan H. Grace. The younger Morgan Grace was born in Wellington, New Zealand, and emigrated to the United States, where his eldest son, Oliver Russell Grace, was born in 1909, in Great Neck, New York. Unlike his younger siblings, Oliver had dual citizenship because his father had not yet relinquished his allegiance to the Queen.

Oliver's childhood was a happy one and he adored his parents. His mother, in particular, was enthusiastic about her son's sports endeavors and was the one who taught him to play bridge, a game he continued to enjoy throughout his life. He went to school at St. Paul's for one year, but found the other boys cruel and was not happy there. He transferred to Phillips Academy at Andover, Massachusetts, where he flourished. He was a bright and sociable student. At the age of 13, he got into a game involving slingshots and rocks and took an irreparable blow to his eye, which had to be removed. He lived out his childhood and early adulthood with a glass eye, which did not stop him from enjoying tennis and sailing, but which later prevented his serving in the military during World War II. Instead, Oliver served without pay in Washington, D.C. on the War Production Board, which was responsible for allocating scarce resources during the war.

At one point in his schooling, Oliver's grades began to slip. The headmaster called the boy in saying, "When we see somebody of your caliber falling behind in his work, we smell a rat." It came out that he had fallen in with a card-playing group at school and was spending his study time playing bridge instead of

studying algebra. While on academic probation for this episode, Oliver proceeded to win a school prize for having the most greatly improved grades in his class. He finished Andover two years early.

Oliver matriculated at Yale at age 16. He was young for his class and was a late-bloomer, and so must have seemed still a boy among his collegiate peers. Nevertheless, he did well at Yale, graduating with honors in 1930. He took a job at Grace National Bank, a subsidiary of W.R. Grace and Co. run by his father's cousin, Joseph Grace. It was here that Oliver began to learn the complexities of finance, managing trusts and investments for the Grace bank. In addition to managing the investments of the bank's clients, he began investing his own money and accumulating his own fortune. He left in 1936 because, as he put it, he realized that "working for a bank put unacceptble limits on economic freedom." He joined his childhood friend Duncan Sterling, Jr., in an investment firm with Sterling's father. Sterling, Grace & Co. became a major force in New York investing and secured Oliver's ability to take on major philanthropic projects.

At the age of 24, Oliver married Anne Chilton McDonnell. They had three daughters, Helen, Anne, and Ruth, and were married ten years, divorcing in 1944. In 1949, Oliver married Lorraine Graves, with whom he would have four children and to whom he would be married 42 years, until his death. He was a loving and caring father, taking particular pleasure in initiating his two sons Oliver, Jr., and John very early into the world of business and finance. Later, they went into business for themselves and, in the family tradition, work on some projects together. When only eight, Oliver, Jr., would listen with rapt attention to his father explain details of business and finance that no one else in the family was interested in.

Despite the lack of depth perception resulting from the loss of his eye, he was an excellent tennis player and sailor and won several championships at the numerous clubs to which he belonged. Here he frequently played tennis at the Piping Rock Club in Locust Valley and the even nearer to his home Cove Neck Tennis Club. In his later years, he sailed at the Sewanaka Yacht Club on Center Island, having earlier spent many summers in the Hamptons and Martha's Vineyard sailing with his children.

In both his business and his personal life, Oliver was a man of principle, and one who respected the principles of others. He had a reputation as a tough, fair businessman, with a flair for identifying undervalued stocks and burgeoning experimental industries. He was one of the first to invest in geothermal energy, owning at one point one million acres of geothermal federal land. Oliver initiated exploratory operations on this land and later owned a geothermal vegetable dehydration plant and a geothermal mushroom-growing facility. In 1964, Oliver was one of the first Americans to invest in Japan, setting a very lucrative trend. His business ventures ranged widely, from Long Island real estate (he once owned Roosevelt Field), to an oil concession in the Ecuadorian Andes, to a Florida bus company, to trading in stocks and bonds, to a Panamanian shrimp fleet.

Tough as he was, Oliver's kindness was famous among his family and friends. He was always concerned about others troubles—even when he disapproved of those troubles. Lorraine was reminded of a father of the Old West when Oliver rode horses with his children—if one wanted to ride off through the bushes, Oliver rode along with them and scooped them up when they fell. He was always riding at their side, a little behind but keeping up.

Lorraine and Oliver were totally devoted to one another, and she often influenced him in subtle ways that affected his business life. It was she who convinced him to replace the glass eye with an eyepatch. Lorraine and Oliver made

the patches at home, and they became an instant trademark. Oliver would remark to her that the patch increased his recognition on Wall Street so dramatically that people he did not recall addressed him by name. The Graces moved into an apartment on New York's Upper East Side, where Lorraine still lives. Their weekdays were spent in New York, but they also had a house in Greenwich, Connecticut, where they spent weekends. In 1976 they sold this house and bought a house in Oyster Bay that had been built by James Alfred Roosevelt, uncle of Theodore. Named *Yellowbanks*, the house was built a few years before 1882 Sagamore Hill, the Theodore Roosevelt home. *Yellowbanks* was passed along to sons for three generations, but when, in 1974, James Alfred's grandson, John Kean Roosevelt, died, the Graces purchased the lovely home that looked westward over Oyster Bay.

In 1953, the Graces' New York apartment became the site of a series of portentous meetings that gathered together groups of unconventional doctors bringing photographic slides and cancer patients. The woman who first brought them together was Helen Coley Nauts, a childhood friend of Oliver Grace. Her passionate interest was a then-unorthodox form of treatment for cancer, first conceived by her father Dr. William B. Coley. Coley discovered that small amounts of certain toxins found in bacteria could stimulate the immune system into fighting off certain cancerous tumors. He died without his ideas gaining wide acceptance, but his daughter felt his findings were prescient. Without formal medical training, she digested thousands of medical articles as well as her father's writings, synthesizing his concepts and publishing her own articles in medical journals. She was already a professional-caliber scholar on immunological cancer research when, in 1953, she first enlisted Oliver's help in forming the Cancer Research Institute (CRI).

Those early meetings in the Grace livingroom were the beginnings of a far-seeing medical institute. With the exception, however, of the prevailing treatment for superficial bladder cancer, major clinical applications of the Coley approach have yet to be realized. But through the research done to understand its basis, the relationship between the immune system and cancer has become a well-respected and fertile area of basic research, giving rise to such mainstream fields of study as the function of cytokines, intercellular immune system messenger molecules.

Oliver's support of Helen, adding his name and prestige to her cause and bringing many other respected names to the board, helped to quickly establish CRI as a very serious, dedicated effort. Oliver later worked closely with Dr. Lloyd Old, who became chairman of CRI's Scientific Advisory Council, and whose scientific daring led in the mid 1970s to the discovery of the important cytokine tumor necrosis factor.

Oliver personally supported several programs that are now integral to the CRI operation. He backed their core Fellowship Program, which supports postdoctoral students in immunological and cancer research. He was also interested in public education. He pushed for the establishment of an "800" number, which the public could use to obtain information about cancer or its treatment or to donate support for cancer research. Skeptics initially criticized this idea, but it has turned out to be a valuable link between the Institute and the public. Today, CRI is a major funder of cancer and immunology research. Known in philanthropy circles for their administrative efficiency, a full 85% of their more than $5 million annual budget goes toward program support. In 1991, CRI funded 96 postdoctoral fellows, 20 established investigators, and 21 institutional grants. They also sponsored two workshops and three scientific conferences.

This long-standing interest in cancer research prepared Oliver for a call in the early 1980s from his friend Ed Pulling. Pulling was the vice-chairman of our board as well as chairman of the Long Island Biological Association, our community support group then mounting a drive to raise funds for a new auditorium in which to hold our ever-swelling meetings. Oliver liked the plans and soon made the major gifts making possible the Oliver and Lorraine Grace Auditorium, with its imposing outside terrace carved out of the hillside to the north. Later, he supported the Laboratory in other ways, most notably in establishing the Oliver R. Grace Professorship for Cancer Research of which I am the first recipient. He joined the Laboratory's board of trustees in 1983 and immediately became one of its most active members, later serving as the board's secretary as well as a member of the board's committees.

Everyone who knew him well remembers Oliver Grace as a man of integrity, intelligence, discipline, and compassion. From his family life, to his Wall Street transactions, to his philanthropy with the Cancer Research Institute and Cold Spring Harbor Laboratory, Oliver Grace lived up to the Latin motto of his ancient Irish lineage: *Concordant Nomine Acta*—"Our deeds bear out our name."

In 1990, we celebrated his 80th birthday at our annual trustee dinner at Robertson House. At that event, I took great pleasure in presenting him with a rare reprint of my original 1953 paper with Francis Crick on the double helix. Then Oliver seemed in peak form, a condition that suddenly changed for the worse when he suffered a seizure several weeks later. This cruel blow did not stop him, and he pursued every possible medical lead that might hasten his recovery. Happily, his strength returned, and on the day before he died in his sleep, he had not only played tennis, but was also on the dance floor in Palm Beach.

His death leaves a gaping hole in the lives of his wife Lorraine and his children—Anne, Helen, Ruth, Lori, Wendy, Oliver, Jr., and John. I personally feel the deep lament that comes from the loss of an invaluable friend and supporter.

James D. Watson

Elinor Montgomery
(1937–1992)

Elinor Montgomery, who died much too prematurely at the age of 54 on March 18, 1992, was a compassionately intelligent woman, embodying so well the heritage of public service and decency that long has been the hallmark of Oyster Bay's North Shore families. Her father Alexander M. White was the noted investment banker and supporter of Harvard University, and her mother Posy was a LIBA member for more than 25 years, serving on both the LIBA and Laboratory boards. I first appreciated Elinor's warmth and wit at the Symposium dinner parties given each year by her mother at her family's Georgian home to whose grounds had been moved a schoolhouse at which Walt Whitman had taught.

Becoming a member of LIBA herself in 1970, Elinor's goals became even more deeply intertwined with the Laboratory's when in 1972 her daughter Alexandra died of leukemia, instilling in her a strong emotional desire to support cancer research. That year she not only joined the board at Memorial Sloan-Kettering Cancer Center, but also learned firsthand about cancer research through working in Joseph Burchenal's laboratory at Sloan-Kettering. Also linking her both to Cold Spring Harbor Laboratory and to Memorial Sloan-Kettering was the deep friendship initiated then with our trustee Bayard Clarkson, who headed the Hematology Section of Memorial Hospital while also doing research on leukemia.

In 1988, Elinor joined our board, hopefully for a long period of service that would equal her mother's. The year before, she had dedicated a large gift to our Second Century Fund in honor of her mother, establishing the Posy White Fund. In Elinor's words, the Fund was intended to encourage a young postdoctoral scientist to "take the plunge and make a contribution in the field of science." Elinor knew that the opportunities to find better cancer cures and treatments were to be found through basic research.

She devoted great amounts of time and energy to spreading the word about the Laboratory, encouraging her friends to join LIBA and to contribute to the Posy White Fund. She was particularly valuable in expanding the Laboratory's support base to include more people from New York City, where she lived and had many friends and contacts.

As a member of the Laboratory board, Elinor served on the DNA Learning Center committee, combining her commitment to molecular biology and cancer research with her long-standing interest in education. She taught at the Brearly School in Manhattan for ten years during the 1970s and 1980s and was a trustee of the Thatcher School and of Miss Porter's school, her alma mater. She was also chairman of the board of the School Volunteer Program in New York City, a not-for-profit organization with more than 1000 members. The volunteers go into New York City elementary schools to give special courses in reading, math, and other subjects. For relaxation, Elinor loved to sing and be with her family, spending the summers at her family's residence on an island off the Maine coast.

Cruelly, she was diagnosed with breast cancer all too soon after coming on our board. Unfortunately, the best medicine we have today did not stop the cancer's spread. Despite all of the major advances made so far in cancer research, we still have a long road ahead. Characteristically, Elinor did not falter as her condition worsened but remained vibrant and optimistic. In particular, Liz and I will always cherish the lovely Lewis Carroll-inspired poem she read two years ago at her home to mark our wedding anniversary.

She leaves bereaved her husband George and her three surviving children, George, Jonathan, and Diana. With her passing, the Laboratory has lost a compassionate advocate, caring supporter, and devoted friend.

James D. Watson

DIRECTOR'S REPORT

It is now impossible for any one individual to effectively appreciate all of the scientific accomplishments of this century. Almost all that is now learned in universities has been discovered since 1900, and the brains of even the best of my scientific friends are inadequate to store all of the facts that we deem important. Nowhere does this rush of achievement so overwhelm than in the study of the gene, the fundamental unit of heredity that is transmitted from one generation to the next through the chromosomes. An active pursuit of the gene and its extraordinary implications for all of biology, as well as for human existence, has been going on since the start of the century.

In evaluating the immensity of what has already been accomplished, we can divide the achievements in genetics during this century into six separate intellectual movements. The first was the Mendel-Morgan era in which genetic crosses were used to establish the existence of genes and their linear arrangement along chromosomes. Following this was a much shorter second era that began in 1944 with the announcement by Avery, MacCleod, and McCarty that DNA carried genetic information and culminated in 1953 with the finding by Francis Crick and me of the double helical structure of DNA. This finding immediately initiated the search for the way in which the genetic information of DNA is used to determine the order of amino acids in proteins. This third movement, led by Francis Crick and George Gamow, needed only the 13 years between 1953 and 1966 to find "the genetic code," the rules by which the information conveyed by the sequence of the four different nucleotides along DNA molecules is translated into the information conveyed by the order of the 20 different amino acids within the individual polypeptide chains of proteins.

Overlapping the search for the genetic code was the Lwoff-Monod-Jacob-initiated movement that used genetics to learn how gene functioning is controlled. This era, which effectively began in the early 1960s, was initially dominated by the elegant genetic analysis of bacteria and their viruses, the phages. From these experiments, largely done in Paris at the Institut Pasteur, came the concept that specific genes interact with sets of specific molecules that can positively or negatively affect the rate at which their respective genetic messages are read. The identification of these controlling molecules as proteins was first demonstrated in the mid 1960s at Harvard through Gilbert and Müller-Hill's isolation of the lactose repressor and by Ptashne's isolation of the bacteriophage λ repressor. Today, a very large number of gene regulatory proteins are known not only in microorganisms, but also in higher organisms where their differential functioning in time is known to control the order and development of multicellular organisms from fertilized eggs. Molecular genetic analysis is now providing the keys to unlock the once totally mysterious phenomena of embryological development.

A decade later in the laboratories of Paul Berg and Stanley Cohen at Stanford University, the recombinant DNA era began in which the rules underlying gene

expression are successfully used to move functional genes from one organism to another, in some cases between life forms as different as the yeasts and human. Magnifying manyfold the impact of these gene manipulation experiments was the independent development by Gilbert and Maxam at Harvard and Sanger at Cambridge of accurate and rapid procedures for sequencing very long DNA molecules. Thus, not only could individual genes be isolated (cloned), but their exact structures (sequences of bases) could also be determined, thereby revealing the amino acid sequences of the proteins encoded by the respective cloned genes. Out of this era of genetic manipulation has arisen modern biotechnology by which living organisms are genetically modified to become factories for the efficient production of new drugs, specialty chemicals, and food products.

Now we are just entering the sixth major era of genetics—the genome era—in which the totality of the genetic information of a given virus, organelle, or organism is worked out. Already, the complete DNA sequences of a number of important smaller genomes are known, thus allowing the identification of all of the respective protein-coding segments carried by many viral chromosomes as well as by the chromosomes of numerous mitochondria and several chloroplasts. The establishment of these genomes has already proven very valuable, for they generally reveal the existence of previously unknown genes. The complete sequence of the HIV virus, for example, alerted AIDS researchers to the existence of several small genes (proteins) that were unlikely to be discovered in any other way. Equally valuable have been the sequences of the mitochondrial and chloroplast genomes, which revealed that many vital mitochondrial and chloroplast proteins are synthesized outside mitochondria (chloroplasts) using genetic information provided by conventional nuclear genes.

As of now, no complete genome of any cell is yet known. Even the smallest cellular genomes represent DNA molecules whose sizes have been considered until very recently far too big to be sequenced within the climate of contemporary science. For example, the single DNA molecule that comprises the bacterium *Escherichia coli* genome contains 4.5 million base pairs. Our initial hesitation to move on to cellular genomes, particularly to the very large human genome, which encompasses some 3 billion base pairs, was largely based on its potential cost. When the proposal was first made in 1985 to sequence the complete human genome (henceforth known as the "Human Genome Project" or "HGP"), the cost of sequencing was approximately $10 per base pair. So virtually no one then wanted an objective whose final cost might be several times the annual budget of the National Institutes of Health (NIH).

Initially, only the Department of Energy (DOE) took the plunge, arguing early in 1986 that the technological advances needed to reduce the cost of sequencing to a manageable level could be achieved under their auspices using their high-tech national laboratories, which were soon to be re-oriented toward the public good as opposed to military purposes. By then, it was becoming more and more clear that the ability to manipulate and study DNA sequences was going to have far-reaching economic implications, with biotechnology most likely having an importance in the 21st century equal to that which the chemical industry has played in the late 20th century. Playing a leading role in the HGP was thus perceived as an essential step in retaining the United States' dominant role in biotechnology.

An equally compelling reason to place the HGP as a high scientific priority was the emergence in the early 1980s of human genetics as a major intellectual discipline. Until then, human genetics was inherently an observational as opposed to experimental discipline. Only rarely did the bearers of genetic disorders

marry among themselves, thereby effectively making conventional Mendelian analysis impossible. So except for those disease genes that preferentially expressed themselves in males and so were assigned to the X chromosome, the location of most disease genes remained unknown. This barren picture suddenly changed after the now celebrated 1980 Botstein, White, Scolnick, and Davis proposal that high-quality human genetic maps could easily be made using as genetic markers the frequently found natural variations (polymorphisms) of DNA sequences within homologous DNA segments from different humans. Within five years, the first comprehensive genetic maps of these polymorphic markers had been constructed by research groups led by Helen Donis-Keller in Boston, Ray White in Salt Lake City, and Jean Dausset in Paris, making possible the mapping of a number of important human disease genes to specific chromosomal regions. Cystic fibrosis, for example, was assigned to chromosome 7, and Huntington's disease was mapped to chromosome 4.

Important as these findings were in opening up first-generation genetic diagnostic tests for these diseases, genetic mapping is only the first step in the isolation (cloning) of the disease gene itself—the ultimate objective of most human genetics research. With the disease gene in hand, the means become available to search out the function of its respective protein. For example, the isolation of the gene responsible for cystic fibrosis immediately revealed that its corresponding protein product was a membrane protein regulating the transport of ions. On the way to ultimate cloning must be the isolation of the DNA encompassing the chromosomal region pinpointed by the genetic linkage analysis. Toward this end, not only must all of the human DNA be cloned as discrete pieces, but these DNA fragments must also be assembled into overlapping fragments (contigs) that go from one end of a given chromosome to another. With such physical maps in hand, the task facing a scientist wishing to clone a disease gene of interest becomes much, much simpler than if he or she had to find *de novo* the DNA segments located between the flanking genetic markers. Even with the DNA in hand, however, the finding of the desired disease gene is not necessarily routine. In many cases, large sections of DNA must be sequenced to spot the changes that cause the disease.

Although considerable progress had already been made by 1986 to accelerate the construction of contig maps, as well as to improve the resolution of the genetic map, it was very clear that the sums of money available were vastly inadequate to ensure the cloning of many important disease genes whose chromosomal locations could by then only be roughly pinpointed by genetic analysis. The human genetics community thus soon became a strong partisan for an HGP, particularly one led by the NIH, their traditional funding source. By mid 1987, so encouraged by key NIH supporters, the United States Congress voted $18 million for fiscal 1988 to support the HGP. At the same time, $10 million was appropriated for the DOE to start its own HGP, principally within the DOE-operated national laboratories such as Los Alamos, Berkeley, and Livermore.

In so acting, Congress believed it was initiating an HGP that would encompass a 15–20-year period and eventually cost some $3 billion (in 1988 dollars). These were the time intervals and sums of money considered in three separate evaluations of the potential usefulness of the HGP carried out in 1986–1987 by the committees organized by the National Academy of Science, the Office of Technology Assessment of the United States Congress, and the DOE. In all three reports, the HGP was divided into two overlapping phases. In the first phase, most efforts should be directed toward the making of high-resolution genetic and physical maps and the development of more efficient ways to sequence DNA.

Particularly important would be the lowering of sequencing costs, since these would eventually consume most of the needed money. When they had been lowered to some 50 cents per base pair, then the second phase of massive sequencing could begin. Most likely, full-blown sequencing of human DNA would not start until the mid 1990s. On the other hand, there were strong medical reasons for a sense of urgency about completing as soon as possible the needed genetic and physical mapping. Only with these tasks finished could the serious hunts for many important disease genes commence.

I was then arguing that a project this big needed an active director, as opposed to being run by a committee, and so was not surprised when in May 1988, the director of the NIH, James Wyngaarden, asked me to lead the NIH component of the HGP. After being reassured that I could simultaneously remain the director of Cold Spring Harbor Laboratory, I accepted his offer as of October 1, 1988, and began to live a life in which I had to carry the burdens of two full-time jobs. In taking on the HGP position, I focused on four immediate objectives. (1) The building up of a high-level NIH genome bureaucracy capable of functioning smoothly when I was at Cold Spring Harbor. Here I was well served by Jim Wyngaarden, who urged me to appoint as my deputy director Elke Jordan, then holding a high-level position at the Institute for General Medical Science. (2) The appointment of a program advisory committee that commanded great respect in the outside worlds of human and molecular genetics. Here it was important to have the presence of several major scientists not perceived as partially motivated by wanting more genome monies for their own labs. (3) The active recruitment into human genome research of the many more bright young scientists who would be needed both to develop new genome technologies and to supervise production-level acquisition of genetic maps, physical maps, and DNA sequences. (4) The establishment of a proactive Human Genome Ethics program aimed at anticipating potential misuses of genetic knowledge and so reassure the American people that the HGP would improve, not impoverish, the quality of the lives of our citizens bearing genetic diseases.

From the start, I tried as much as possible to avoid micromanagement, letting my staff and advisers know that they were there to make real decisions not just to follow orders laid down from the top. The multiplicity and breadth of decisions that needed to be made obviously far exceeded my competence, and I knew I had been chosen to lead the effort in part because I did not have many preconceived ideas as to how we should proceed at the tactical level. My primary task was to focus on long-term strategic planning, working to be sure that the means would be available for final victory. Not only did we need top brains, but also the monies to pay for their staffs and the instruments they would operate. I thus had to be a credible witness when I appeared before Congress to argue for the continually needed real increase in our annual budget that would let us together with the DOE soon reach a combined appropriation of some $200 million a year.

Here I was helped by the fact that I did not have real doubts as to whether we had a realistic chance of winning on time. Giving me that confidence were two major scientific breakthroughs that preceded my appointment. The first was the development in Maynard Olson's laboratory in St. Louis of the YAC (Yeast Artificial Chromosome) vector for use in the cloning of DNA segments approaching one half million base pairs in length. Given human DNA pieces that big, assembling them into contigs that duplicated their order within human chromosomes should be an achievable goal within a decade at most. Equally important was the emergence of the PCR (polymerase chain reaction) technique of Kary Mullis,

which took off like a flash following its 1986 announcement and which ever-increasingly is being used as a way to clone desired DNA fragments. Now it is virtually impossible to conceive of the HGP proceeding without the help of PCR. Completing the task of finding all the human genes will be some 10 to 100 times more efficient because of PCR.

Even with these powerful techniques, however, the tasks ahead would be daunting, and there was never any doubt in my mind that we would need to create "genome centers" where a variety of intellectual disciplines would be utilized to develop even more cost-effective ways to make maps and explore potentially powerful new ways to sequence DNA. Although the concept of multimillion-dollar-funded centers, containing 20–30 people, raised the hackles of the many scientists who feared their eventual demise in a future ever dominated by "Big Science," there was no other way to proceed. The only real question was "how big" was big. Here our concern was that most centers should be large enough to complete the physical map of an average-sized chromosome within several years of hard work.

It was never thought, however, that we should have as many American mapping centers as 24, that being the number of human chromosomes. To start with, we believed that genome centers in other nations would come into being. We also anticipated further technological breakthroughs that would permit single centers to complete high-resolution maps of several chromosomes. These expectations are now in the process of being realized. Suddenly, several end-to-end maps are nearing completion in the UK and France as well as in the United States. In fact, one chromosome has already been completed. The physical map of the human Y chromosome has just been effectively completed in David Page's laboratory at the Whitehead Institute. Most importantly, the French made the decision to create a super, 200-person laboratory capable of simultaneously making low-resolution maps of all of the human chromosomes. Their gamble is now paying off, particularly aided by their ability to make YACs with human DNA inserts averaging 1 million (mega) base pairs, approximately twice the size of YACs made here or in England. Happily, they intend to share their resources with other nations, which should greatly reduce the time for the American laboratories to complete their intended high-resolution maps. Now there is every reason to believe that the mapping phase of the HGP should effectively be completed by 1996.

How to proceed toward production-level DNA sequencing was always much less clear than was the path toward production-level mapping. Should we aim for complete automation in which machines replace human beings as the key ingredient for day-to-day success? Instruments for sequencing had already been developed by the late 1980s at Caltech and Heidelberg, but there was no consensus as to whether the first-generation efforts could ever be improved to sequence at an acceptable cost. Moreover, the largest DNA sequences then known (several herpesviruses) contained less than a quarter of a million base pairs, more than 100 times fewer than the smallest human chromosome. Clearly, we had to induce some key scientists to take on megabase-pair goals. At NIH, we made the proviso that the pilot project should focus not only on finding genes, but also on technological improvements aimed at bringing sequencing costs toward the 50 cents per base pair goal. In addition, we stated our desire to fund the sequencing of smaller model organisms, such as *E. coli* (4.5 megabases), *Saccharomyces cerevisiae* (15 megabases), the roundworm *C. elegans* (100 megabases), and *Drosophila* (125 megabases). Here we believed that the se-

quencing of these model organisms would not only provide training for the much harder task of sequencing human genes, but also help in identifying the functions of many human genes.

These pilot projects are just now beginning to yield real sequences (genes), as well as provide hints as to which sequencing procedures are likely to work. Contrary to the pessimism of just two years ago, we already know that sequencing machines should replace much of conventional manual sequencing and that very likely they can be improved to the point where their introduction into production-level supersequencing laboratories will bring the final costs to an acceptable level. These data come from the joint UK-American sequencing project on *C. elegans* led by John Sulston (Cambridge) and Bob Waterston (Washington University). In this their second year of funding, each laboratory is likely to sequence some 400,000 base pairs while confidently aiming for 1 million base pairs next year. During the next year, the decision should be made whether or not to move from the development phase to pure production-line sequencing. I hope the answer will be in the affirmative, opening up the possibility of our knowing the complete genetic instructions of *C. elegans* soon after the turn of the century. The intellectually venerable *Drosophila* world now also wishes to enter the genome era and is working to prepare all of the *Drosophila* DNA as overlapping cloned fragments ready for sequencing. Whether the eventual sequencing unit occurs in two to several superlaboratories on both sides of the Atlantic or whether the task could be parceled out to several hundred now existing *Drosophila* laboratories remains an open question.

A cottage-industry-type approach certainly can work for the smaller model organisms up through the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, each of whose genomes comprise about 15 million base pairs. This approach applied to *S. cerevisiae* through a 35-member European-community-funded consortium has already generated the complete sequence of chromosome 3 (approximately 315,000 base pairs). Moreover, within several more years the gene contents of chromosomes 2, 10, and 11 also may be known. No efforts were made toward technological improvements, the argument being made that the pure knowledge generated justified paying several dollars a base pair. Not enough *S. pombe* laboratories exist, however, for the cottage-industry approach to succeed, and here a high-tech approach makes sense, with the possibility that both genomes may also be known even before the turn of the century. The initial look at chromosome 3 suggests a 7000–8000-gene number for *S. cerevisiae;* what the *S. pombe* number is remains to be seen. Also unknown is how many of their genes are similar and have common functions. Conceivably by knowing both genomes, we shall have discovered members of most of the gene families that make up the human genome.

Equally exciting should be the genomes of well-studied bacteria such as *E. coli*. Already, some 35% of its genome has been sequenced in the course of everyday science endeavors. To fill in the remaining gaps, both a cottage-industry approach in Japan and a big-laboratory effort run by Fred Blattner in Wisconsin have been started. The latter effort started before the suitability of machine sequencing had been demonstrated, leading to gel running and reading production lines dominated by student labor. This approach has taken far more time than anticipated to get into high gear, with only several hundred thousand base pairs having been sequenced over more than two years. Even with these initial setbacks, the fact that the *E. coli* genome has only 4.5 megabases of DNA makes it likely that we shall know its complete sequence by the end of 1995 at the latest. Moreover, work has already started in several independent

laboratories on the even smaller 1-megabase-sized mycoplasmas, the smallest of all bacteria. It is thus possible that we shall know the complete genetic instructions for several forms of single-cell existence within at most four years. We must be cautious, however, not to underestimate the difficulty of assigning functions to a genome-sized set of instructions. Less than one third of both the *E. coli* and *S. cerevisiae* genes are essential, and many decades, if not centuries, will pass before we can assign specific functions to virtually all of the *E. coli* and yeast genes.

Even with this caveat, there certainly remains every reason to push on as fast and sensibly as possible toward sequencing the complete human genome. Only in this way are we likely to find all of the human genes. Merely sequencing the expressed regions of DNA by making cDNA copies of their respective messenger RNA products is likely to severely underestimate the gene number as well as leave us ignorant of many rarely used genes that nonetheless have important biological roles. Even so, having in hand large libraries of human expressed sequences, each member mapped to its corresponding DNA region, will vastly simplify the process of delineating the borders of individual genes. The time required for this cDNA component of the HGP, however, will necessarily be dwarfed by the total sequencing tasks. Toward this end, production-level DNA sequencing factories, each aimed at sequencing 10–25 megabases per year, must be set up. Consolidation of these efforts into only a few worldwide sites might eventually make sense, but now it seems premature to predict what the HGP will look like more than five years ahead. In any event, the question remains of how to finance the setting up of the supersequencing laboratories. In Europe, governmental (foundation) monies are likely to eventually get them going as well as provide funds for sequencing itself. Here in the United States, the route we shall take remains open, with the likelihood being that sequencing companies, perhaps in some cases partly owned by nonprofit research institutions or universities, will come into existence. The rate at which they are formed obviously will depend on the amount of federal monies specifically targeted for sequencing.

We must now work hard to increase public, and with that Congressional, support for the HGP. Almost paradoxically, because its mission relates to all of human disease, it does not yet enjoy the strong backing of organizations like the American Heart Association founded to encourage research aimed at specific diseases. So conceivably, the most effective way to keep the HGP on its needed upward course is for Congress to hear more directly and often from its supporters among our nation's leading biomedical scientists. This was how the HGP gained its initial support from Congress, and we should not underestimate the respect that Congress continues to have for those who have made major advances in the past. Congress needs our advice as much as we need their help. They want to know what is best for our country, not for the individual self interests of its scientists. So those of us who feel so strongly about what the HGP can do for our futures must take the time to see that our views are heard. Otherwise, those who oppose and even hate the HGP may be perceived as the voice of true science. Fortunately by now, the HGP is a truly international effort, and the major programs now being developed outside our borders can be seen as a testament to the deep importance of the HGP to all of human kind.

In so moving toward the identification of all human genes, we must never forget the ethical, legal, and social consequences of this new knowledge. That so many human characteristics are largely determined by our genes is inherently unsettling to the vast majority of our citizens who would like to believe that we are all born with equal chances for meaningful lives. But such equality does not exist, and the throws of the genetic dice invariably lead to all too many cases of genet-

ic injustice. DNA replication will never be a perfect copying process. Only through the continuous creation of new mutant genes through mistakes in DNA replication can the evolution occur that allows species to adapt to the ever-changing biological and physical environments of our earth. But in generating individuals with increased survival potential, evolution also necessarily leads to individuals severely impaired by newly created deleterious mutant genes.

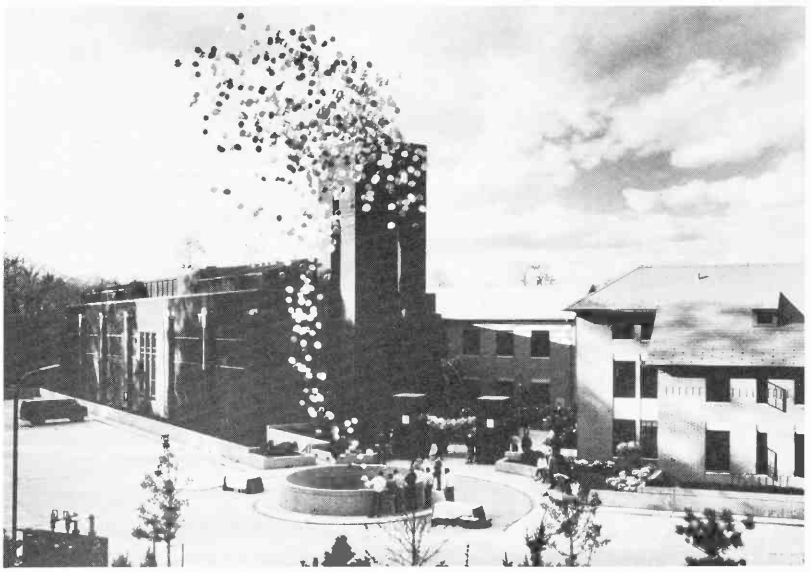
As a compassionate society, however, we must never forget, and thus abandon, the victims of genetic injustice. To do so would abrogate the moral values that are the hallmarks of a civilized human society. We must thus not consider ourselves off the moral hook when we have passed laws to ensure genetic privacy and so prevent future discrimination on the basis of genetic heritage. Such laws will only truly work if we are part of societies in which the more fortunate share their resources with those who are less so. As long as we accept the fact that significant numbers of human beings belong to perpetual underclasses based on poverty, race, or nationality, we might well also learn to live with an ever-growing genetic underclass that we feel impotent to help.

The forthcoming explosion of genome knowledge will thus bring us not only the power to vastly improve the human condition, but also the obligation to reaffirm that successful human beings are more than well-fed, adequately clothed, and decently housed savages.

HIGHLIGHTS OF THE YEAR

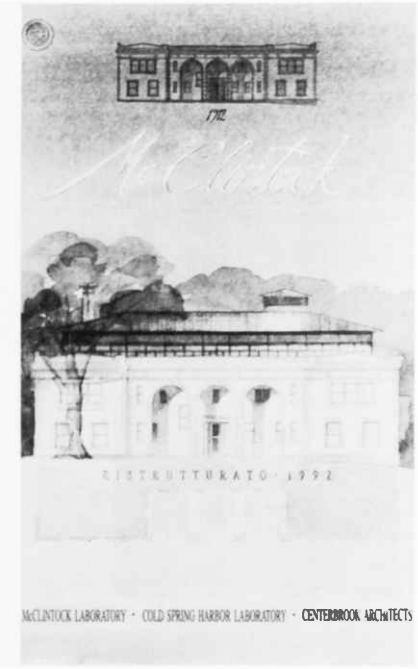
Neuroscience Center Dedicated, Inhabited

The Laboratory inaugurated its search for the seat of memory this year at the ribbon-cutting and dedication ceremonies for the new Neuroscience Center. On April 26, Arnold Beckman cut the ribbon at the entrance to the Arnold and Mabel Beckman Laboratory, following the first Laboratory trustees meeting to be held in its beautiful new Plimpton seminar room. During a reception with members of the board, a portrait of Mr. Beckman was unveiled in the lobby on the main floor, and recently we received a portrait of Mrs. Beckman that now graces a facing column. One week later, on May 3, the Neuroscience Center was dedicated. Christened in high style, with balloons, a brass band, and a keynote speech from U.S. Representative Bob Mrazek, the center symbolizes the beginning of the Laboratory's "second century." It includes three structures: the Arnold and Mabel Beckman Laboratory for research and teaching; Dolan Hall, a 60-room residence for visiting scientists and Laboratory guests; and Hazen Tower, an instant Laboratory landmark with its one-ton brass bell, helical staircase, and the DNA initials, a, c, t, and g, adorning each of the sides. Designed by Centerbrook Architects of Essex, Connecticut, the center projects a modern, state-of-the-art appearance while maintaining architectural harmony with other Laboratory buildings. The end of the ceremony was marked by a cloud of colored balloons rising to the main plaza through the air shaft from the garage below and the tolling of the Hazen Tower bell. Our Public Affairs Department produced a splendid book-



let for the occasion, the most extensive of the nine brochures they have produced since 1979. It features many photographs as well as essays on the architecture of the Neuroscience Center, on the future facing Neurobiology, and on the past history of neurobiology teaching and research at Cold Spring Harbor.

By summer's end, Beckman Laboratory had begun to fill with scientists. The third floor contains laboratories dedicated to identifying and characterizing genes related to learning and memory in the fruitfly *Drosophila*. Research on the main floor will focus on chemical signals in the vertebrate brain, and the ground floor houses the W.M. Keck Structural Biology Laboratory where scientists will explore protein structure and function, focusing in large part on proteins involved in cell signaling processes or in gene expression. The center is filling rapidly and will ultimately house a total of 75 scientists.



McClintock Campaign

As the Second Century campaign winds down, one of its most important remaining objectives is the $4 million drive to raise funds for the renovation of McClintock Laboratory. A committee of investment bankers from the community will lead the way to complete this key fund-raising priority. We are already more than one quarter of the way toward our goal, with contributions totaling more than $1.4 million raised. We wish to express our thanks to Lita Hazen, the Starr Foundation, Michel David-Weill, and Margo Walker for getting the campaign off to an encouraging start.

Funds raised from the McClintock campaign will go toward transforming the 1914 building into a state-of-the-art laboratory for cell cycle and gene sequencing research. Three quarters of the monies will go toward renovations of the Laboratory. These include structural renovations and the addition of a third floor to the building. The remaining funds will be used to purchase laboratory equipment.

The first floor of McClintock Laboratory once boasted a dirt-floor pen for housing sheep when the building was known as the "Animal House." Once renovated, the first floor will be home to a gene-sequencing laboratory, focusing on deciphering genes related to cancer. The second floor will become a laboratory for research on the cell cycle, the set of events responsible for the duplication of the cell. The third floor will house offices, a library, and a seminar room with one of the Laboratory's most spectacular views of the harbor.

The erstwhile inhabitants of McClintock Laboratory have moved to other buildings. David Helfman and Daphna Bar-Sagi continue their cancer-related research in Demerec Laboratory, and David Beach and Robert Franza have been given temporary laboratory space in the Neuroscience Center. Beach is pursuing his research on the cell cycle and will return to McClintock when renovations are completed. Franza, who works on questions relating to AIDS and cancer, will set up shop in Jones Laboratory following renovations there.

LIBA: 1924–1991

The Laboratory's oldest supporting institution, the Long Island Biological Association, was formed in 1924 by a group of concerned neighbors to reinvigorate the summer program at Cold Spring Harbor and provide it with financial backing. LIBA took over the administration of the Biological Laboratory (Bio Lab) from the Brooklyn Institute of Arts and Sciences, sharing the current Laboratory grounds

with the Carnegie Institution of Washington's Department of Genetics. For 38 years, LIBA ran the Bio Lab, continuing the summer courses and establishing research programs in genetics, neurophysiology, and endocrinology.

When Milislav Demerec became director in 1941, the focus of the Bio Lab shifted to a heavy emphasis on genetics. In 1962, the facilities of the Carnegie Institution were merged with those of the Bio Lab to form a new, single institution: Cold Spring Harbor Laboratory of Quantitative Biology (shortened in 1968 to Cold Spring Harbor Laboratory). When the two laboratories were joined, LIBA's operational role ended. LIBA then moved on to its current role as "Friends of Cold Spring Harbor Laboratory," taking on the job of explaining the Laboratory to the public and organizing community support for the Laboratory. So organized, LIBA mounted four major capital fund drives, culminating in the successful 1983 drive to build Grace Auditorium.

For more than 20 years, LIBA has continued in this role, although it retained its original name. The group's obsolete acronym, however, frequently misled newcomers as to its and the Laboratory's activities. Other biological centers have since developed on Long Island, and the Association no longer played an operational role for the Laboratory. In November, at a retreat at Arden House north of New York City, LIBA directors voted to remove this confusion by changing the group's name to the Cold Spring Harbor Laboratory Association. Confirmed at the January 1992 annual meeting, the new name removes all doubt as to the allegiance of this unique and valuable group. Hereafter known as the Association, this loyal band of Laboratory neighbors will continue to support and raise money for the Laboratory under the able presidency of George Cutting. The Laboratory welcomes and appreciates this support which is now channeled exclusively toward operating as opposed to capital funds. Last year, the Association members raised more than $350,000 which was used to provide stipends for postdoctoral fellows and new staff scientists.

Symposium: The Cell Cycle

This year's Symposium, organized by Laboratory scientists David Beach and Bruce Stillman, focused on the life cycle of the cell. This fundamental set of processes, which includes DNA synthesis and replication, cell division, and a "resting" phase, goes awry in cancer cells. The topic of this year's Dorcas Cummings Lecture for Laboratory neighbors, always held on the Sunday afternoon of the Symposium, was "Understanding Cancer." The speaker, Dr. J. Michael Bishop, a professor at the University of California at San Francisco Medical School, explained that a tumor is a cluster of cells descended from a single cell whose cell cycle ran amok due to some genetic aberration. When that cell divided, it passed along its genetic miscue to its two progeny, which then passed it along to their progeny, and so on in an exponentially increasing manner. Although cancer remains a frightening thing, research into cancer and the cell cycle is making it less of a terrible "black box" and is leading us closer to eventual understanding and cures.

Jan Witkowski

Banbury Meetings on the Cutting Edge

Banbury Center director Jan Witkowski continues to assemble leading experts to discuss important issues bridging biology, industry, and society. Typically three days in length, each meeting aims to be timely, rich in discussion, and on the

cutting edge of a field. This happens by bringing together the right people on the right topic and placing them in the idyllic setting of the Banbury estate generously donated to the Laboratory in 1976 by Charles Robertson. This year's meetings included conferences on the biology of Lyme disease; patents and molecular biology; the molecular genetics of breast cancer; and aging, from cells to health care. The conferences range from basic research, such as the meeting on the genome of *Escherichia coli*, to the impact of biology on the public sector, such as the meeting for science journalists on controversial issues in biology and society.

Important new support for the Banbury Center came from a $150,000 grant from the William Stamps Farish Fund that our trustee Martha Farish Gerry helped us obtain. It will go toward the holding of three Banbury conferences on complex genetic diseases like arthritis in which several genes are involved.

Baring Brothers Executive Conference

Baring Brothers & Co., Ltd., the celebrated English merchant bank, sponsors an annual meeting on new developments in biology which is held at the Banbury Center each October. The topic of this year's meeting was the molecular biology of signal transduction, the fundamental process by which cells communicate. The program featured presentations by leading researchers in genetics, cell biology, and neurobiology, including a special segment highlighting the research program at Cold Spring Harbor, during which attendees met in small groups with Laboratory scientists David Beach, Ron Davis, Mike Wigler, and others. Participants included Laboratory trustees, scientists, and executives from the biotechnology, pharmaceutical, and financial communities of the United States, Europe, and Japan. In addition to listening to a broad range of talks, the business leaders had a chance to "get their hands dirty" at the Neuroscience Center's new Hughes teaching laboratory, where, led by Nobel laureate Sidney Altman, they performed a simple experiment preparing DNA from bacterial cells.

Sadly, Trustee Terms End

Cold Spring Harbor Laboratory continues to benefit from the expertise and generosity of an outstanding board of trustees. Our board consists of 30 trustees, ten of whom are chosen because of their direct involvement with the world of science. The remaining 20 public members live primarily on Long Island or in New York City and join us because of their interest in promoting biomedical research and their willingness to contribute their own expertise in business, the law, or public service. This year, we have five new members, four of whom have never before served on our board and whose interests and knowledge span a broad range.

We lost the service of five valuable trustees this year. During the summer, Elinor Montgomery to our sorrow tendered her resignation because of illness. Then at our November board meeting, David Botstein, Jim Darnell, Fred Richards, and Sidney Strickland reached the ends of their second terms on our board. Each of these scientifically trained trustees brought us unique talents.

David Botstein, long a leading yeast molecular biologist, recently has become chairman of the genetics department at Stanford University. He is also well-known in human genetics circles for first proposing the use of distinctive segments of DNA as "flags" along the chromosomes for mapping disease-causing genes. Botstein taught our summer course in Advanced Bacterial Genetics be-

Wendy Hatch

tween 1976 and 1980, after spending a sabbatical year at the Laboratory in 1973–1974 studying yeast genetics. This was the second time Jim Darnell of The Rockefeller University completed two full terms. Earlier, he was on our board while serving as a professor at Columbia University. As a board member, Darnell served on the executive, nominating, and tenure committees. Jim first came to Cold Spring Harbor in 1958 to take the newly instituted course in Animal Viruses. Fred Richards first came here in 1971 as an organizer of that year's Symposium on the Structure and Function of Proteins at the Three-dimensional Level. Retiring trustee Sidney Strickland, who studies developmental biology in the pharmacology department at SUNY Stony Brook, served as a member of the Banbury committee.

Our single returning trustee is Wendy Hatch. Mrs. Hatch is a loyal neighbor whose enthusiasm for the Laboratory is unsurpassed. In her previous two terms on the board, she served on 7 of the board's 12 committees, including the Banbury, building, development, and executive committees, as well as serving as secretary of the Laboratory. This year she will chair the DNA Learning Center advisory committee and serve on three other committees.

New to the board are Mrs. Martha Farish Gerry and Mr. David H. Koch. A resident of Mill Neck, Mrs. Gerry is also a member of the board of Columbia Presbyterian Hospital as well as several other health and family services organizations. In addition, she is vice president and a trustee of the William Stamps Farish Fund, a fund with an emphasis on education and medical research. Her background will be a valuable asset to the Banbury, building, and DNA Learning Center committees.

David H. Koch of New York City is executive vice president for chemical technology for Koch Industries, Inc., of Wichita, Kansas. A graduate of MIT and captain of its basketball team, he continued his postgraduate training at MIT in chemical engineering. Early in his career, he worked at the Scientific Design Company, Inc., the petroleum-related engineering firm founded by our former trustee Ralph Landau. He is also a trustee or director of a variety of organiza-

tions, including the MIT Corporation, NYU Medical Center, Memorial Sloan-Kettering Hospital, the American Ballet Theater, and Earthwatch.

Three new scientific trustees were elected. Günther Blobel, a professor at The Rockefeller University and Howard Hughes Medical Investigator, studies the fundamental problem of how proteins are transported across cell membranes. Gerald Fink, director of the Whitehead Institute for Biomedical Research and a professor at MIT, although a newcomer to the board, is no stranger to the Laboratory. He helped initiate a Yeast Genetics course in 1970, continuing as an instructor with only one year's interruption until 1987, including one year in which he co-taught with David Botstein. He will serve on the Banbury and tenure and appointments committees. Joining us from SUNY Stony Brook is Dr. Eckard Wimmer, chairman of the department of microbiology. In prior years, he helped organize several important Banbury meetings and most appropriately will chair the Banbury committee.

Robertson Research Fund

In this year of recession, the Robertson Research Fund was more valuable than ever in maintaining the Laboratory's commitment to young scientists. The Fund supported the research of graduate students, postdoctoral fellows, visiting scientists, investigators in the plant genetics program, and key newly arrived scientists. The fund also supports the Outstanding Junior Fellow Award, which this year went to David Barford, who came here following his Ph.D. at Oxford and a postdoctoral year in Dundee. David works on the three-dimensional atomic structure of an important class of enzymes called protein phosphatases. In addition, the Fund contributes to meetings, courses, and travel and relocation expenses for Laboratory scientists and provides backup support for underfunded research projects and meetings.

Major Gifts

The Laboratory received a number of substantial gifts in 1991, making possible essential programs such as new construction, purchase of laboratory equipment, and funding for scientists. Among these were new pledges from the Upjohn, Ciba-Geigy, and Baxter International Corporations toward our infrastructure campaign, whose primary purpose is the refurbishment and expansion of Blackford Hall and the construction of the 11 guest cabins. In addition, Bristol-Myers Squibb, the Pall Corporation, and Westvaco increased their pledges toward the infrastructure campaign. A total of 13 corporations, primarily from the pharmaceutical industry, have now pledged contributions totaling over $2.8 million.

Unrestricted giving is one of the most important sources of money for the Laboratory. These funds are used at the discretion of the trustees wherever and whenever needed, whether it be to purchase equipment, fund a scientist, or renovate a building. Generous gifts and pledges to the Laboratory's unrestricted endowment were received from Dr. Bayard Clarkson, Mr. & Mrs. George Lindsay, David Luke, Dr. David Pall, Taggart Whipple, Martha Makanna, Mrs. Alexandra White Smith, Mrs. Bayard Walker, and Morris Williams.

Support for specific research programs at the Laboratory helps shape the direction of Cold Spring Harbor science. The Mathers Foundation donated

$1,028,000 to support Ron Davis' learning and memory research, and the Sara Chait Memorial Foundation pledged $125,000 to support research on Alzheimer's disease. The Donaldson Charitable Trust and Ira J. Hechler provided support for two Russian scientists here, Grisha Enikolopov and his wife Natasha. Toward equipment for the Neuroscience Center came a much appreciated gift of $300,000 from The Gould Foundation. We are also grateful to the Samuel Freeman Charitable Trust and the Mellan Family Foundation for their support of Robert Franza's laboratory and the Oxnard Foundation for its support of Michael Gilman. The Garfield Foundation supported Jacek Skowronsky's AIDS-related research with transgenic mice. Leonie T. Brigham, in addition to an unrestricted gift, provided endowment funds for the neuroscience program by means of a generous bequest.

Other gifts made possible a variety of programs. Mrs. Maxine Harrison had pledged a bequest of $1.5 million in honor of her mother, Alle Davis Harrison, to endow a neuroscience chair. Sadly, that bequest has materialized sooner than expected, and we offer sincere condolences to the Harrison family on the occasion of Maxine's passing last fall. Trustee Lita Hazen deserves special thanks. Adding to her past $1 million contributions to the Neuroscience Center and the Blackford Hall renovation, in June Mrs. Hazen donated $1 million toward the renovation of McClintock Laboratory. Former trustee Norris Darrell established the "William Shakespeare Fellowship" to support a student in the Undergraduate Research Program. Support for young scientists here at the Laboratory included a planned gift from Katya Davey and a donation from former trustee Elinor Montgomery, whose gift toward the Posy White Fund, named in honor of Mrs. Montgomery's mother, brought that endowment's total to $180,000 as of the end of the year.

Seligson Family Postdoctoral Fellowships for Cancer Research

Our neighbors Alan and Edith Seligson lived through the heroically long but finally losing battle of their son Andrew against neuroblastoma. When more conventional therapies had failed, Andrew underwent a then very experimental bone marrow transplant in Holland. Through this treatment, his life was prolonged for several more years, but finally the awful cancer reappeared and Andrew died at the age of 25. To encourage the new knowledge that we will need to fight such cancers successfully, the Seligson family has established a postdoctoral research program where they help choose several young scientists to do cancer research here. The Seligson Family Fellows of 1991 were Mart Ustav, who works on the cancer-causing papillomaviruses, and Jeffrey Gerst, who studies the *RAS* oncogene.

Honoring David Luke's Leadership of the Second Century Campaign

Initially, we considered a $25 million goal for the Second Century campaign. But by the time we started this fund drive, we had upped our sights to $44 million to be raised by the end of 1991. Happily, the expanded target was itself exceeded with more than $46 million either received or firmly pledged. This extraordinary success was achieved only through the help of a very effective group of

David Luke receives Steuben glass bowl

volunteers led by David Luke. To honor David, as well as more than 60 key volunteers who provided so much of their valuable time during the campaign's duration, a special dinner was held on April 11, 1992, in the new downstairs Blackford Hall dining room. Before the dinner, Barney Clarkson, David Helfman, and I made brief remarks in Grace Auditorium about the importance of the campaign for the Laboratory's future, with Barney presenting David with a suitably inscribed Steuben glass bowl to honor his extraordinary efforts. In response, David graciously told of his pleasure in so working for the good of the Laboratory.

Blackford Hall

A Much Expanded and Modernized Blackford Hall Is Now Nearing Completion

Through doubling the size of Blackford Hall, we now have two functioning dining rooms, which can seat a total of 400 people, enabling us to accommodate the ever-increasing numbers of scientists attending our meetings. Completely new is a 120-person dining room on the ground floor equipped with its own serving section. A brand new elevator connects it to the kitchen on the main floor. Renovation of the basement bar was completed by January 1992, allowing it to be the site of a Super Bowl party. Other renovations include enlarging the existing dining room through a major addition on the east (harbor) side, nearly quadrupling the kitchen area, adding an outside eating area more than twice the size of the old terrace, and adding much enlarged rest room facilities.

In other building news, a future athletic facility is in the planning stages. A committee has been formed to consider the facility's size, usage, and equipment.

Mike Wigler

David Helfman

Wigler Honored Once Again

Laboratory scientist Michael Wigler received the thirty-first Clowes Award from the American Association for Cancer Research for his work on the genetics of cancer. Wigler and his group share credit with Bob Weinberg at MIT for isolating the first human cancer gene, or oncogene. Wigler's laboratory continues to work on the *RAS* oncogene, gaining insight into this gene's function in healthy cells and its role in transforming healthy cells into cancerous ones.

Helfman Receives Award

David Helfman received an Established Investigator Award from the American Heart Association in recognition of his work on RNA splicing. Helfman's research focuses on the ability of multi-parted genes to be put together in different ways to make a variety of related proteins. The "splicing" of these neighboring stretches of RNA into different combinations could explain structural differences between heart muscle and skeletal muscle, as well as many other biological phenomena.

DNA Learning Center

The DNA Learning Center, under the able directorship of David Micklos, continues to push the envelope of science education to include ever-younger students. The new "Fun With DNA" workshop brings primary school students into the laboratory to learn about DNA fingerprinting, genetic engineering, and other aspects of DNA science. Perhaps nothing illustrates the rapid progress of biotechnology so well as the fact that here in Cold Spring Harbor, fourth graders are carrying out experiments that less than 15 years ago won Nobel prizes.

With support from the E.S. Webster Foundation, architectural plans were inititated for a 3500-square-foot addition at the rear of the DNA Learning Center's current facility. The Stone Foundation pledged $250,000 over three years toward

a new multimedia laboratory to be housed in the new addition, which will allow state-of-the-art computer technology to bring the sights and sounds of DNA to many young minds. The Weezie Foundation also pledged $100,000 toward the development of the DNA Learning Center's proposed new exhibit "Exploring the Human Genome."

CSHL Press

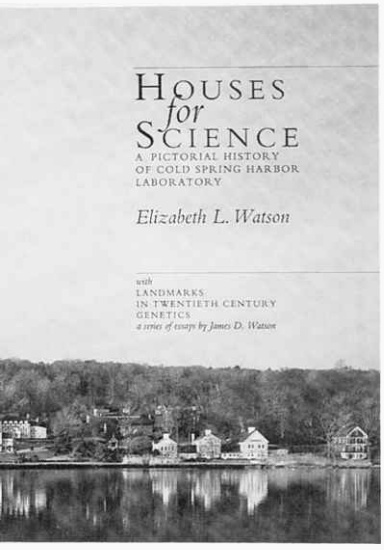
Cold Spring Harbor Laboratory Press published 15 new titles in 1991, up from 12 in 1990. The Press is also distributing a new line of children's books, published by Harper Collins Publishers in London. The first two books, entitled *Cells Are Us* and *Cell Wars*, have become two of our biggest sellers. This year saw the publication of our first audiotape, a recording of a concert given at the Laboratory this fall by geneticist/humorist/folksinger Ron Laskey, entitled *Songs for Cynical Scientists*.

The press launched a new journal this year, *PCR Methods and Applications*. Polymerase chain reaction is an increasingly important technique for scientists interested in cloning DNA, and this journal documents the latest uses and methods for researchers in genetics. *Genes & Development* continues to lead the field of professional journals in developmental and molecular biology. It was recognized for the second year in a row as the most-cited journal in its field. The Press's third journal, *Cancer Cells*, ended with the December 1991 issue. Although its quality was high, it suffered from an overcrowded field of competing publications and the effects of cuts in government research grants that has greatly reduced the monies available to purchase books and journals.

Three new series were begun in 1991. The first three volumes of a series on *Genome Analysis* were published, as were the first four volumes of *Current Communications in Cell and Molecular Biology* and the first two volumes of *Cancer Surveys*, published in collaboration with the Imperial Cancer Research Fund of the U.K. Finally, this year marked the release of the comprehensive review *Origins of Human Cancer*, the result of the centennial meeting of the same name held in September 1990, edited by Joan Brugge, Tom Curran, Frank McCormick, and former Laboratory scientist Ed Harlow.

Next year promises continued expansion for the Press, with 26 new titles, including a textbook on the *The Power of Bacterial Genetics*, laboratory manuals on nervous system probes and bacterial genetics, and a monograph on *Drosophila* development.

Finally, we bid goodbye this year to Charlie Apsel, business manager of CSHL Press. Charlie came to the Press in August 1980 from Human Sciences Press as fulfillment officer. She left at the end of January 1992 to pursue a career in hospice work near her home in Mt. Sinai.



Houses for Science Makes Its Appearance

Mid November marked the publication of *Houses for Science* by my wife Liz. Published by Cold Spring Harbor Laboratory Press, this book traces the unique architectural and scientific evolution of Cold Spring Harbor Laboratory from whaling village to world-famous village for DNA. Superbly illustrated with photos and drawings chronicling the nearly 200-year architectural heritage of our buildings, the 350-page *Houses for Science* is, as noted in a review in *Nature*, the first coffee-table book about science.

To mark its publication, LIBA sponsored a Sunday afternoon lecture by Michael Crosbie of *Architecture* magazine in which he compared new scientific laboratories elsewhere with our new Beckman Laboratory. Several days later, a small party at the Century Association introduced the book to friends in New York, and on our way to the Nobel Jubilee in Stockholm, we hosted a small gathering at the Travelers' Club in London.

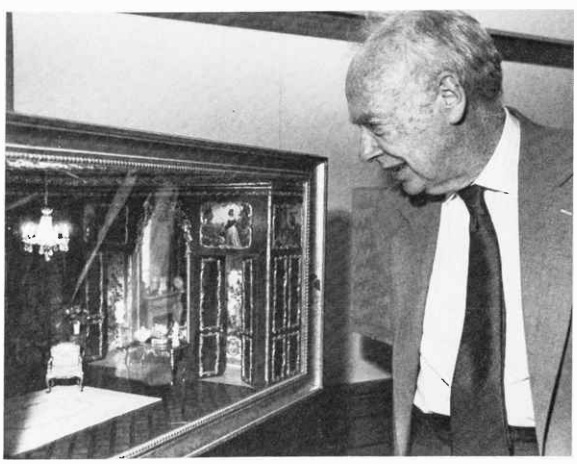
I have heard only great praise for my wife's book, and I am very proud of her achievement in bringing forth what should be the first of a new genre in books where intellectual history is seen through the form of the buildings that make it possible.

Liz Watson showing Jack Richards (*left*) and Jim Childress *Houses for Science*

Lloyd Harbor Seminar Series, Miniatures, McClintock's First 50, Orth Redux

The year was marked by a number of special events. The Lloyd Harbor seminar series at the Banbury Center was reinstated this year after a two-year hiatus. Organized by Laboratory Associate George Toumanoff, these public lectures present a broad range of topics for the enjoyment of Banbury's neighbors in the village of Lloyd Harbor. This fall, for example, Ann Gill of the Cold Spring Harbor Whaling Museum presented a talk and slide show on the village of Cold Spring Harbor during the whaling era. The talk was followed by a presentation by Liz Watson, who discussed the history of Cold Spring Harbor Laboratory as it developed out of the old whaling village here on the harbor's western shore. The evening also included a sneak preview of her book *Houses for Science*.

In September, the Laboratory hosted an exhibit of 32 miniature rooms designed by Mrs. Madeline Ridder. The miniatures, painstakingly detailed with period furniture, art, and functioning light fixtures, delighted 120 people and raised $7500 for the Laboratory. Very sadly, Mrs. Ridder suddenly passed away shortly after this exhibition. Happily, she was able to greet her many friends at the exhibit, and we will long remember her by the happy event of her miniatures show here.



Jim Watson enjoying one of the miniature rooms designed by Mrs. Ridder

Barbara McClintock

Near the close of the year, Barbara McClintock celebrated her 50th year at Cold Spring Harbor. Barbara has worked in her laboratory here nearly every day since December 1941. On December 19, Laboratory scientists and staff crowded the Grace Auditorium lobby to toast this milestone.

Piano virtuoso Peter Orth gave a second benefit concert in Grace Auditorium on January 25, 1992. Mr. Orth's Grace Auditorium debut was in 1989, during our centennial celebration. The concert, organized by Laboratory loyalist Mary Lenore Blair, was by all standards a great success. Mr. Orth's program of Chopin, Schumann, and Brahms lulled, aroused, and enthused his rapt audience and raised $8000 for the Laboratory.

Undergraduate Research Program

Each summer since 1959, up to 20 undergraduates from around the country and overseas have come to the Laboratory to carry out research projects with our scientists. This year, 19 students participated in the program, coming from as near as upstate New York and as far away as Paris. Each student worked for 10 weeks, learning techniques and developing and carrying out a project. This year's studies included gene cloning, protein analysis and characterization, isolation of cDNA, and X-ray crystallography and utilized molecules derived from yeast, plants, and humans. Each student in the program has a sponsor, with support coming from the National Science Foundation; Baring Brothers and Co., Ltd.; Philips Petroleum; Burroughs-Wellcome; the Olney Memorial Cancer Fund; and anonymous donors.

Partners for the Future

The second year of the Laboratory's *Partners for the Future* program began in October. Initiated in 1990 to involve high school students in research at the Laboratory, the program is highly competitive. From the 150 Long Island schools eligible, five students are selected to conduct research and hopefully to begin

Benefit concert given by piano virtuoso Peter Orth

building rewarding scientific careers. Sponsored by area businesses, students receive a stipend as well as hands-on experience unattainable in a classroom. The following is a list of 1991 *Partners*, their high schools, and their Laboratory mentors: Nicole Chasen, Oyster Bay High School (Dr. Jeff Kuret); Rose Cohen, Great Neck High School South (Dr. Jeff Kuret); Richard Lavi, Cold Spring Harbor High School (Dr. Hong Ma); Steve Xydas, Herricks High School (Dr. Richard Roberts); and Elizabeth Yong, Syosset High School (Dr. Venkatesen Sundaresan). The students work from October through March and commit at least 10 hours each week after their regular school day.

Changes in Scientific Staff

Senior Staff Investigator David Frendewey left this year to take a position at NYU Medical Center as an associate professor. Frendewey's work centers on the synthesis and splicing of RNA in yeast. The simplicity of this organism allows David to unravel details of the way genetic information is translated into the building blocks of cells. Edward Chang, an associate investigator in John Anderson's laboratory, left for a senior scientist position at the Development Center for Biotechnology in Taiwan.

Staff associates Jeffrey Field, Dallan Young, and Karl Riabowol also departed in 1991. Jeff took an associate professorship in Columbia's biology department, and Dallan and Karl have both migrated north to take assistant professorships at the University of Calgary in Alberta.

New Staff Members

The dedication and opening of Beckman Laboratory marked a record year for arriving staff. Ron Davis, senior staff scientist, and Tim Tully, senior staff investigator, came here from, respectively, Baylor College of Medicine, Houston, Texas, and Brandeis University, Waltham, Massachusetts. They study the process of learning and memory using the fruitfly *Drosophila* as a model organism.

We are also pleased to have Hiroyuki Nawa on board in Beckman as a senior staff investigator. Dr. Nawa came to Cold Spring Harbor Laboratory from Kyoto University in Japan. He studies the development of neurotransmitter phenotypes in the nervous system and its relationship to the learning and memory processes.

Two staff investigators are welcomed this year. Ryuji Kobayashi comes to us from Nagasaki University in Japan and is researching protein chemistry and enzymology in Beckman. Scott Patterson works with the QUEST group in Demerec Laboratory and utilizes his skills in computer science to identify proteins from two-dimensional gels. He worked previously at The University of Queensland in Brisbane, Australia.

James Cherry, a staff associate in Beckman, arrived in 1991 to research mammalian learning and memory.

New visiting scientists came to us from various corners of the globe. Nikolai Lisitsyn, associated with Michael Wigler's laboratory, comes to us from the Institute of Molecular Genetics in Moscow. Nikolai has a special interest in human genome mapping. Jean-Pierre Renaudin specializes in plant genetics and comes to us from the French National Institute for Agronomic Research to join Venkatesen Sundaresan's laboratory. Janos Posfai came from Hungary for the fourth time as a visiting scientist in Rich Roberts' laboratory and is continuing his work in mathematical analysis of biological sequences. Dr. Posfai has since

returned to The Academy of Sciences of Hungary, Department of Biophysics. Stefan Stamm, a German scientist currently researching alternative RNA splicing in David Helfman's laboratory, arrived in 1991 for a one-year sabbatical. Frederick Southwick, an M.D. from Boston City Hospital, spent the summer in David Helfman's laboratory researching the molecular biology of cytoskeletal proteins. Huanran Tan, from the People's Republic of China, researches c-*rel* in Robert Franza's laboratory. Magsood Ahmed, visiting scientist, has returned to Pakistan after researching restriction enzymes in Hershey Laboratory.

Staff Promotions

Many changes at the Laboratory occurred in 1991, including a number of staff promotions. David Helfman, former senior staff investigator, was promoted to senior staff scientist. David first came to Cold Spring Harbor Laboratory in 1981 to work with Steven Hughes and John Fiddes and developed an antibody screening–DNA cloning technique. He quickly became interested in cytoskeletal structure in normal and tumor cells. Helfman's laboratory continues to work on the molecular biology of the cytoskeleton.

Roymarie Ballester and James Lees-Miller were made staff associates. Roymarie continues to work in Demerec Laboratory, furthering her work in *RAS*-mediated signal transduction in yeast. James is studying the role of tropomyosins and actins in cell motility.

Eric Richards was promoted to staff investigator. Eric is conducting work on molecular chromosome structure in plants and works in Delbrück-Page Laboratory. Michael Laspia of Demerec Laboratory was also promoted to staff investigator. He researches the transcriptional regulation of HIV-1 gene expression by the viral *trans*-activator protein Tat. Susan Lobo of James Laboratory was promoted to research associate. She studies the transcription of small nuclear RNA genes.

Postdoctoral Fellows

The following scientists have departed after having completed their postdoctoral studies here: Ulrich Deuschule to the Basil Institute for Immunology as staff scientist; Ashok Dubey to the Indian Institute of Technology as associate professor; Jeffrey Gerst to Mount Sinai Medical School as assistant professor; Makoto Kawamukai to Shimane University (Japan) as associate professor; Daemyung Kim to Chonjin University; Tsfrira Pe'ery to Israel; and Ratneswaran Ratnasabapathy to Long Island University as assistant professor of pharmacology.

Also departed to continue their postdoctoral research are Gary Heisermann to the Marine Biological Laboratory; Ellen Katz to Albert Einstein Medical College; Li-Kuo Su to Johns Hopkins Oncology Center; Anne Vojtek to Fred Hutchinson Cancer Research Center; and Jong-Chang Wu returned to Taiwan.

We must also mention our feelings of deep regret regarding the tragic passing of the talented Australian M.D. William Clouston, then working with Winship Herr in James Laboratory, and express our condolences to his wife and family.

Several graduate students completed their doctorate work at the Laboratory and moved on: Tom Baxter left to pursue other activities in the Bronx, New York; Rui-Hong Chen moved to SUNY Stony Brook; Christian Dahmann to Universität Tübingen (Germany); Barbara Faha to MGH Cancer Center East; Qianjin Hu returned to Japan to pursue other activities; and Kenneth Mellits to ICRF (London).

Top row: Peter Stahl, Jim Watson, John Maroney; *Bottom row:* Guy Cozza, Roberta Salant, Bill Keen

Long-Term Service

During 1991, seven employees reached important milestones here at the Laboratory. William Keen, comptroller, and John Maroney, assistant administrative director, each completed 20 years of service. John began here as a research assistant in James Laboratory working with Joe Sambrook. From early 1973 to December 1982, he was head of Purchasing, and in early 1983, he became assistant administrative director. Bill Keen has been comptroller of the Laboratory since 1971 when he came here from KPMG Peat Marwick.

Five employees reached 15-year milestones: Guy Cozza, chief accountant; Joseph Ellis, groundskeeper; Roberta Salant, administrative assistant; Peter Stahl, maintenance manager; and Margaret Wallace, mediamaker.

My Resignation from the Directorship of the National Center for Human Genome Research

Bearing the responsibilities of two major administrative positions, one here at Cold Spring Harbor and the other at NIH in Bethesda, has been an ever-increasing burden during the past year to both me and this Laboratory, whose growing size demands a full-time director. At the January meeting of the Program Advisory Committee of the National Center for Human Genome Research, I told its members that the increasing scope of the Human Genome Project would soon necessitate the appointment of a full-time director who could take a more direct role in the management of an effort whose growing yearly budget already exceeded $100 million. While initially I could function effectively in getting the Human Genome Project off to an irreversible start, I was becoming frustrated over my inability to be the active manager the Project now needs. Also worrying me was a disagreement festering since last October between the new director of the NIH, Dr. Bernadine Healy, and me over the desirability of patenting expressed

DNA sequences whose functions have not yet been determined. By early April, it was clear that the time had arrived for me to resign, and on April 10, 1992, I submitted my resignation. Fortunately, there is every indication that Dr. Healy will continue to strongly back the Human Genome Project and she has indicated her desire to quickly appoint a scientist of major accomplishments to replace me. Naturally, I will continue to remain a strong proponent of genome programs and, if asked, will enthusiastically give the new director my assistance.

The Recession Begins to Affect Our Federal Funding

During the past 20 years, the NIH, in particular the National Cancer Institute component, has been the financial lifeblood of our scientists. Until recently, virtually none of our scientists failed in their initial attempt for funding. Now, however, we, like virtually every other major center for research, find ourselves frequently strapped for cash. This dilemma arises because the number of good scientists applying for grants is rising much faster than the NIH budget, which this past year has effectively just kept pace with inflation. To handle this crisis, NIH has been arbitrarily reducing by 10–20% the monies we thought had been firmly committed toward multi-year grants. At the same time, they have been delaying the starting dates for new grants and not funding grant proposals that in past years would have invariably been funded. So we have been scurrying everywhere trying to make up those funds that the government can no longer provide. The fact that so much of our science directly relates to the growing biotechnology industry obviously opens up the possibility that we may be able to replace a significant fraction of these lost federal funds with monies from industry. Such help, however, can only be a short-term palliative to most institutions like ours which need to continue doing science that does not seem to have commercial consequences. Indispensable for our long-term future must be a vigorous NIH growing in size commensurate with the extraordinary recombinant-DNA-generated advances in biology.

Real further increases in the NIH budget are likely to require not only our nation's recovery from the current recession, but also a reestablishment of the strong good will that previously existed between Congress and our biomedical research community. As academics, we had long been regarded as seekers more of knowledge than of financial rewards. Now the picture is not so clear with so many biotechnological implications arising from our science. At times over the past decade, many key scientists have seemed more interested in the companies they have formed than in the science that spawned them. The *greed is good* eighties philosophy, however, is gone, and we must again be perceived as asking what good we can do for our fellow citizens, not what more federal monies will do for us personally. Our future as a first-rate scientific institution demands that our thoughts continue to be dominated by ideas as opposed to stocks and their options.

We Must Constantly Strive to Retain Our Reputation of Working for the Good of All Science

With the completion of the Neuroscience Center, we have suddenly a 50% increase in our facilities for science. Likewise, the just being completed addition to Blackford Hall effectively doubles our dining facilities, finally giving us the capac-

ity to feed all of the visitors to our meetings in heated quarters. Gone forever are the leaky tents that so characterized our big meetings. Happily, both major building projects have been accomplished without a ruthless impersonalization of our character. Bungtown Road still retains a village feeling. We will not rest, however, on new architectural triumphs but continue to work hard to see that all of our visitors feel they are wanted. In a real sense, Cold Spring Harbor Laboratory belongs collectively to its staff and visitors, both of whom are indispensable to its present as well as to its future.

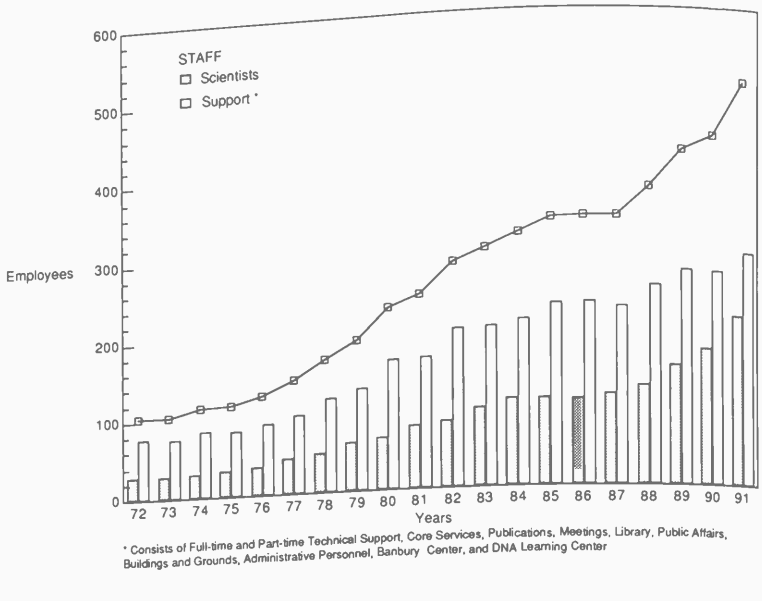
However, with 220 people already doing science here and some 600 total employees, we are just at the limits of what still can be called a village. Clearly, we must strive even harder to know each other and to make everyone feel that the Laboratory is as much a home as a place to work. Toward this end, we plan for Blackford Hall to return quickly to its former role as the social center that breaks down the walls between individuals who come here as competitors but leave as friends. We must also soon take steps to improve our athletic facilities, since such activities provide easy ways to start speaking to strangers. A decade ago, I thought we had the money to build near the tennis courts a building housing a squash court and an exercise room. But then suddenly the money was needed to build Grace Auditorium. Now our need for a complex is even greater, and a major goal of mine remains to ensure its construction over the next several years. We must also come to grips with how to provide the child care needed by many of our younger staff. A facility on the Laboratory grounds would immeasurably simplify the lives of those individuals who have very small children and yet want to work full time at the Laboratory.

I have much work cut out for me in the near future.

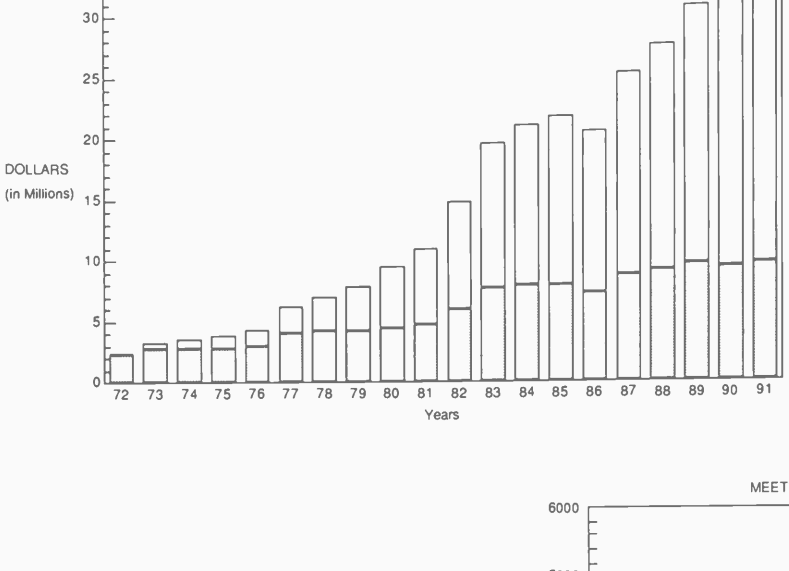
May 14, 1992

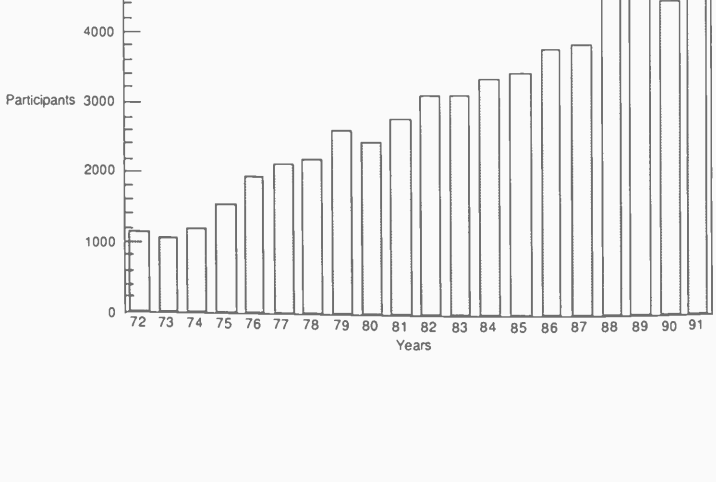
James D. Watson

DEPARTMENTAL REPORTS



* Consists of Full-time and Part-time Technical Support, Core Services, Publications, Meetings, Library, Public Affairs, Buildings and Grounds, Administrative Personnel, Banbury Center, and DNA Learning Center





ADMINISTRATION

The past year was difficult for most academic institutions engaged in basic research, and the Laboratory was no exception. Most of our federal research grants were funded at reduced levels due to federal budget pressures and because, nationwide, too many deserving projects are competing for the same funds. In addition, Congress began an investigation of reimbursement of research overhead costs. A few highly publicized abuses were found at some institutions, and thus a range of new regulations and rule interpretations have been proposed. As a result, the issue of how legitimate research overhead costs are to be recovered in the future has become clouded and makes forward planning difficult at best.

The Laboratory nevertheless completed 1991 with satisfactory operating results and a strong financial condition. The outstanding quality of our research and education programs enables them to compete successfully for a good share of available federal funding. In addition, foundations, corporations, and individuals support the Laboratory generously.

For the third successive year, there was a modest surplus from operations after full funding of depreciation, which amounted to $1.9 million. A reserve was established to provide for the present uncertainties of federal grants, and funds previously designated for start-up expenses of the Neuroscience Center were used only sparingly. The good financial results were helped importantly by a 6-month wage freeze instituted at mid-year and strongly supported by the great majority of staff. The normal schedule of salary reviews resumed, as planned, at the end of the year. Cost containment was emphasized by all departments, and new hiring of support personnel was curtailed despite rapid growth of the scientific staff associated with the opening of the Neuroscience Center. The cooperation and support of all employees were major factors in the successful financial results and are greatly appreciated.

The Laboratory's endowment, consisting of the Robertson and Cold Spring Harbor Funds, reached a new high level of over $57 million by the end of the year, a gain of more than $13 million over the previous year. This was the result of some new gifts to the Cold Spring Harbor Fund and another year of outstanding investment results achieved by our investment manager, Miller, Anderson & Sherrerd. Total return for all of the Funds ranged from 32% to 33% compared to a benchmark index return of 24.5%. The Laboratory continued its policy of reinvesting a substantial portion of endowment income to provide for inflation and the future growth of our research programs.

There were 53 meetings and courses held this year on the main Laboratory campus and at Banbury Center. As in the past, they dealt with a broad range of cutting-edge science and were attended by more than 5000 scientists from academia and industry. For the first time, our visitors enjoyed the new overnight accommodations available in our five new cabins and Dolan Hall. Blackford Hall, halfway through a major expansion of kitchen and dining space, was available for uninterrupted meal service despite the inconvenience of ongoing construction. Visitors and staff watched with growing anticipation the progress of what will surely be a spectacular facility when completed in April, 1992.

The DNA Learning Center extended its program for high school and first-year college students to include middle school children for the first time and moved

closer to its goal of modernizing the teaching of biology and genetics in the United States across a broad spectrum of ages. Increasingly, the Center is reaching out to minority and disadvantaged groups. An indication of its growing national impact can be seen from the rapid sales of Dave Micklos and Greg Freyer's laboratory text, *DNA Science*, already in its second printing. At the end of the year, plans were proceeding for acquiring the present DNA Learning Center building from the Cold Spring Harbor School District under terms of the purchase option included in the current lease. It is then intended to add a new addition to the rear of the building to include a second teaching laboratory, lunchroom, and multimedia computer facilities. A new exhibit dealing with the Human Genome Project and an auditorium are planned for the main building.

Following on the heels of two good years, the Cold Spring Harbor Laboratory Press had a very difficult year in 1991. Book sales declined substantially with the outbreak of war in the Persian Gulf and were adversely affected throughout the year by the limited funding available to scientists for such purchases. A number of important new titles scheduled for publication were delayed until 1992, further subtracting from expected sales. The journal *Genes and Development* for the second year in a row received "most cited" status in its field of genetics and developmental biology from the Institute for Scientific Information, and again earned a surplus. The newly launched journal *PCR* promises to be a resounding success in terms of both editorial and advertising content. *Cancer Cells*, however, after 2 years of competing bravely in an overcrowded market for cancer review journals, was unable to make sufficient progress toward break-even operation and was terminated with publication of a final issue in February, 1992.

As 1991 drew to a close, the Laboratory's first ever capital drive, the Second Century Campaign, surpassed its goal of $44 million. With more than $46 million in hand, including interest, the Campaign will now wind down as a last few pending, but important, solicitations are completed. Its successful conclusion represents a monumental achievement on the part of David Luke III, the Campaign Chairman, the Committee Chairs, Oliver Grace, Wendy Vander Poel Hatch, George Cutting, Taggart Whipple, William Miller, and Townsend Knight, and all the other volunteers. At a dinner this April, the Laboratory will express some small measure of gratitude to those who have devoted so much time and energy to this very large undertaking. Much credit is due to the Development Office and its Director, Konrad Matthaei. Konrad has left the Laboratory as planned following the successful completion of the 3-year long Campaign, and he will be greatly missed by his many good friends here and in the community. The Development Department is now headed by Gordon (Skip) Hargraves, who joined us in March of 1991 and was appointed Director of Development in January, 1992.

The task of raising capital for the Laboratory will continue on a more focused basis, as of course it must. The need for additional endowment and facilities will always be inherent at an institution as dynamic and forward looking as Cold Spring Harbor Laboratory. In the future, there will also be increased emphasis on raising unrestricted annual funds through annual giving. Such funds support our scientists by providing fellowships and start-up laboratory equipment and supplies, by bridging gaps between federal grants, and by maintaining many other research support functions around the Laboratory.

Each year while tending to the Laboratory's administrative affairs I find my task made immeasurably easier by the very capable and dedicated efforts of the administrative staff. The Laboratory functions so well because of Bill Keen, Jack Richards, Susan Cooper, Art Brings, Susan Schultz, Sande Chmelev, Cheryl Sin-

clair, and Barbara Ward. Thanks also should go to Roberta Salant, who cares so well for the needs of the Board of Trustees.

Special mention should be made of John Maroney who, in addition to administrative tasks, serves as in-house legal counsel and manages so very well our technology transfer activities. John acts as interface between the scientific staff, the Commercial Relations Committee, outside patent counsel, and the corporate world. John's efforts have been instrumental in a substantial and growing stream of royalty income which benefits both the Laboratory and its scientists.

Looking to the future, we must be concerned by the scarcity of funding for basic research. On the one hand, political and business leaders point to biotechnology as the answer to depressed economies at the federal, state, and local levels. Nations such as Japan and Germany target the industry as key to winning the competition for a higher standard of living in the next century. Last year, the American public invested some $3 billion in stock offerings of fledgling biotech companies. On the other hand, there remains the serious need for a dependable and growing source of support for the basic science that makes the industry possible.

G. Morgan Browne

BUILDINGS AND GROUNDS

The Buildings and Grounds Department had another busy year in 1991. With completion of some buildings, new construction in progress, and the relocation of many of the laboratory staff from one building to another, the workload on the department was tremendous.

Three major ongoing projects occupied Buildings and Grounds during the year: five new Log Cabins, the Neuroscience Building, and Blackford Hall. Two of these projects, the Log Cabins and the Neuroscience Building, were finally completed in 1991.

Log Cabins

The first project to be completed was the five new Log Cabins situated above the first complex of six cabins on the southwest edge of the Laboratory campus. Water, electricity, gas, and telephone systems were installed by the different Buildings and Grounds trades. Interconnecting boardwalks, including built-in benches and a small community area with a telephone, were built between the cabins. Finally, the area was landscaped and the roadway was finished, all in time for the spring meetings.

Neuroscience Building

Most of the construction of the laboratories and installation of the equipment was completed in March, and we immediately began to move the scientists into their new offices. At this point, however, much work still needed to be done to customize each laboratory area before their staffs could move in. This customization process included reworking many of the support rooms, wiring the rooms for emergency power, installing the pipes for the benchtop utilities, and setting up all of the sophisticated scientific equipment. Dan Marshak, John Anderson, and James Pflugraph from Demerec Laboratory were the first scientists to move into their new laboratories in the Neuroscience Building. While the relocation of equipment was in progress, the department was also engaged in installing the compressed air and vacuum systems, all of which were in place before the courses began. The building itself was also undergoing final touches by both the contractor and the department in order to be ready for the May Dedication Ceremonies.

The summer months continued to be just as hectic as the spring. In addition to the ongoing relocation of our own scientists, new incoming scientists had to be moved into Beckman Laboratory. The second floor of the Neuroscience Building had to be specialized for the use of fruit flies as a research tool, which meant adding environmental rooms; rewiring for emergency power; piping the carbon dioxide, compressed air, gas, and vacuum to the benchtops; and installing the new equipment.

Blackford Hall

The pace of the Blackford Hall project continued with little letup. The concrete structure was completed just in time for the spring meetings. The "Open Air" decks of the addition were used as temporary dining areas and were a pleasant change from the tents used in previous years. After the meetings were over, renovation of the lower level went into full swing. The bar was completed along with the new lavatory facilities, and the mechanical equipment was relocated to the new mechanical room. At the close of 1991, the lower dining room was complete, and so were the recreation room and many of the support facilities. The spring of 1992 should see the completion of the Blackford Hall project.

James, McClintock, and Demerec Play "Musical" Laboratories

James Laboratory: While work continued in the Neuroscience Building, we revamped the old P-3 laboratory in James Annex, renovated the darkroom, and added larger windows. Four of the postdoc offices were also renovated to accommodate more staff. In addition, the painting throughout James Annex and the second floor of Sambrook Laboratory was finished, the fume hood in Sambrook was relocated, and the existing cold room was enlarged. After completing these first steps, Bruce Stillman's laboratory was moved from Demereck Laboratory to Sambrook, Nicholas Tonk's laboratory was moved from Sambrook to the newly painted Demerec, and Mike Gilman's laboratory moved from Sambrook to the third floor of James.

McClintock Laboratory: A decision was made during the year to renovate and add to McClintock Laboratory. This meant that all of the scientific staff in the building had to be relocated to other areas. Bob Franza's laboratory, the first to

move out, was relocated to the second floor of Beckman Laboratory, and David Beach's laboratory, as well as his laboratory in Demerec, was relocated to the first floor of Beckman. As with the other areas in the new building, the Buildings and Grounds trades had to reinstall all of the existing equipment from the old locations, provide support utilities, complete the darkrooms, and make some alterations to the support rooms.

Demerec Laboratory: When the laboratories in Demerec were emptied, the Buildings and Grounds trades moved in to paint, add shelves, and complete minor alterations, which included reworking two rooms into a microscope room and a cell-culture room. Upon completion, Adrian Greider's laboratory was moved in from Page Laboratory, Scott Patterson moved into the lower level of Demerec, and Dave Helfman's and Dafna Bar-Sagi's laboratories were moved in from McClintock Laboratory, leaving that building quite empty and lonely, but ready for renovation in 1992.

Thanks for a Job Well Done

While all of the frantic activities described above were taking place, we were also working on many small projects, namely, converting from oil to gas heating in Nichols and Grace, altering the second floor of Wawepex from bedrooms to offices, and moving 200 phone extensions. Every department in Buildings and Grounds had a large part in completing all of these projects and bringing the year of 1991 to a successful end.

The Equipment Repair Service received and is maintaining more scientific equipment than ever before. It looks like more help will be needed for Cliff Sutkevich and Bob Borruso to keep up with the work load in 1992. The amount of mechanical equipment has also increased, and our staff of mechanics, plumbers, and electricians are doing an excellent job. Buck Trede and his grounds crew have done a great job with maintaining the Laboratory's grounds, cleaning up and putting in new plants in the spring, mowing and trimming in the summer, raking and pruning in the fall, and shoveling and sanding the roads and walkways through the winter months. In addition, they did a fine job of landscaping the Cabin and Neuroscience Building areas.

The year 1991 saw big changes in the Carpentry department, beginning with the hiring of Frank Russo in May. Three or four months later, we hired new men to work with Frank, and we now have a most competent crew of carpenters. Never before has the Receiving department delivered as much mail and material as it did this year. Jim Sabin, Randy Wilfong, and Christopher Oravitz did a great job in keeping up with the work load.

All of the Buildings and Grounds trades pitched in to help move the scientists, equipment, and material without disrupting the course and meeting schedules or the Dedication Ceremonies for the new Neuroscience Building. The entire staff did a great job. They are real professionals and I wish to thank them for a job well done.

Jack Richards

DEVELOPMENT

Second Century Campaign

The Cold Spring Harbor Laboratory Development Department came into being initially to coordinate the Laboratory's first *capital* drive, the Second Century Campaign. In addition, it was charged with expanding *annual* contributions and broadening its constituency. Despite the lackluster 1991 economy and the intense competition for the philanthropic dollar, I am pleased to report that during the year, more than $5,000,000 was added to the Campaign, surpassing our original goal of $44,000,000. Initiated in 1986, the Laboratory's first broad, public funding campaign, has now raised $45,965,194. Foundations, national and local, those new to the Laboratory as well as those familiar with it, have accounted for slightly more than 50% of this amount. Laboratory Trustees and other individuals have accounted for approximately 40%, and an ever widening group of corporations have contributed nearly 10%. Campaign Chairman David L. Luke III and his Steering Committee members, Oliver R. Grace, George W. Cutting, Jr., Mrs. Sinclair Hatch, Townsend J. Knight, and Taggart Whipple, provided enthusiastic and dynamic leadership for the Campaign. Fifty-five diligent and persistent volunteers ably manned the six different Campaign Committees (listed later in this report). Through their efforts, three foundation challenge grants were matched and countless new individuals and organizations were made aware of the Laboratory's existence and its mission.

The Second Century Campaign was designed to raise money for new and renovated facilities and for staff endowment. The Neuroscience Center, dedicated in May, is an unusually powerful and prompt testimony of the Campaign's success.

Several areas in the Neuroscience Center have been named as a result of significant campaign gifts: Arnold and Mabel Beckman Laboratory (Arnold and Mabel Beckman), Dolan Hall (The Dolan Family Foundation), The Gardner Neuroscience Library (Mr. and Mrs. Robert B. Gardner, Jr.), The Hatch Recreation Room (Mrs. Sinclair Hatch), Hazen Tower (Lita Annenberg Hazen), Hughes Teaching Laboratories (Howard Hughes Medical Institute), Keck Structural Biology Laboratory (The W.M. Keck Foundation), The Plimpton Seminar Room (Mrs. Pauline A. Plimpton and Mr. Amyas Ames), The Lucy and Edward Pulling Seminar Room (Mr. and Mrs. George W. Cutting, Jr.), and The Whipple Lounge (Taggart and Katharine Whipple). All donors of $5000 or more are being honored on a permanent plaque in Grace Lobby. A complete list of all Second Century Campaign participants may be found later in this report.

Annual Giving

Our Annual Giving program is an important source of unrestricted income. Under the very able direction of Chairman George W. Cutting, Jr., and its 28 directors, $354,750 was raised in Annual Giving (Jan. 1, 1991–Dec. 31, 1991), an increase of 28% from the previous high. This was a truly remarkable achievement as this program was run in direct competition with the capital campaign. Eighty percent of this goal was achieved by the newly formed Associates Committee, deftly chaired by John Reese. The Associates Committee is composed of David Banker, Charles Gay, Missy Geddes, Dawn Kisner, Gordon Lamb, Carol Large, Harry

Lee, Jordan Saunders, and Jim Spingarn. The Laboratory has the highest number of Associates it has ever had and is growing steadily.

In November, Chairman Cutting called his directors to a first-ever 2-day LIBA Retreat in Harriman, New York. It proved to be an exceedingly stimulating, thought-provoking meeting in which LIBA's strengths and weaknesses, threats, and opportunities were thoroughly discussed. As a result, several changes of structure and operation are being recommended to the Cold Spring Harbor Laboratory Board of Trustees. (A complete report of the Long Island Biological Association 1991 activities may be found in Financial Support of the Laboratory, later in this Annual Report.)

Planned Giving

In addition to making outright gifts to the Laboratory, to take effect immediately (cash, securities, insurance policies, real estate), gifts may also be made to take effect in the future. These can be made in trust or by will. These gifts are most often added to the Laboratory's endowment. Endowment means those funds of the Laboratory's that are held and invested in perpetuity, with only the annual investment income being applied to the current operating budget. The mechanism for two of these "planned gifts" is in place: the pooled income fund and the bequest. The Cold Spring Harbor Pooled Income Fund was established with the U.S. Trust Company as its administrator. This allows individuals to give cash or stock to the Laboratory that will be pooled with other such gifts. These funds are then invested, and the donors (or the persons they designate) will receive the net income from their share of the Fund for life. Afterward, the principal amount of the gift is paid to the Laboratory. There are several significant benefits of making such a gift: it provides long-term stability for Cold Spring Harbor Laboratory, it increases income for the donors or their relatives, it avoids capital gains tax, and it provides an immediate charitable deduction for income tax purposes. With a low-cost-based stock, for instance, it is now possible to make a gift to the Laboratory and increase your income! Of course, the most frequently used mechanism is that of the bequest, the basic building block of our budding Planned Giving effort. The excellence of an institution is almost directly proportional to the size of its endowment. Please consider and urge your friends to consider making gifts to Cold Spring Harbor Laboratory in their wills.

Staff

The considerable success of Development Department is due largely to its tiny, but excellent, staff. The Department has effectively handled a variety of demands from within and without the Laboratory *and* surpassed the Second Century Campaign goal! Although limited in numbers, George Cutting, Claire Fairman, Joan Pesek, and Debra Mullen were not in any way limited in their intelligence, their energy, their dedication, their creativity, their responsiveness, and their senses of humor.

We thank all who have supported the Laboratory in 1991. We urge those who have not yet participated in our various donor programs to consider doing so in 1992. (Methods of giving either outright or by bequest are outlined under Financial Support of the Laboratory.) Your generous support honors our distinguished reputation and ensures our vigorous future.

Konrad Matthaei

Neuroscience Center Library Opens

In conjunction with the opening and dedication of the Beckman Neuroscience Center at the Laboratory, a branch library was opened at the Center. Books and reference materials are housed in the Gardner Library and bound journals are housed in the Pulling Seminar Room, both of which have large windows overlooking the harbor. Just off the stairway from the main floor is a pleasant reading room where current journals and newspapers can be found.

The collection, comprised mainly of books and journals, was transferred to the Main Library in 1985 from the former neuroscience library housed in the McClintock building. Some new subscriptions, books, and reference tools were ordered in 1991, and, as the neurobiologists use the collection, it will be expanded to meet their needs.

The move to the Center was efficiently accomplished by the library staff with little or no disruption of library services at either building. A job well done!

Archives Continue to be Active

It will be another 100 years before the archives will be used as much as they were during preparations for the Centennial in 1990. There does, however, continue to be a great deal of activity that produces income for the Laboratory. The number of photographs invoiced has more than doubled since 1988. Archives showed a net profit of over $1500 in 1991. As the Laboratory gains more exposure, calls for information and literature are also increasing.

Archives provided a work haven and resource center for Elizabeth Watson during the writing of her book, *Houses for Science*. We prepared a photo essay of Edward Pulling that has been hung in the Pulling Room in the Neuroscience Center, and we provided the research for the Center's dedication booklet as well. The DNA Learning Center used the archives to research material for an exhibit on Nobel and Lasker prize winners which will open there in 1992.

The opening celebration of New York Archives Week, sponsored by the Long Island Archives Conference and coordinated by Laura Hyman, was held in Grace Auditorium in October. The theme for the exhibits focused on the early years of World War II. Lynn Kasso, who manages the archives, compiled an extensive booklet entitled "Wartime Research at Cold Spring Harbor...1942–45." She also borrowed a wonderful photograph called "Tea and Talk" from the Carnegie Institution for the Conference. It features 15 Carnegie Institution investigators at an informal talk, including Al Hershey and Milislav Demerec. A copy of this photograph is being considered for permanent display in Bush Auditorium, where it was taken in the 1940s.

Interlibrary Loans from Around the World

Wanda Stolen, who handles interlibrary loans, is now able to procure copies of patents, theses, and foreign publications, as well as journal articles and books. Searches go as far afield as the British Lending Library, and we are presently negotiating an agreement with the St. Petersburg Lending Library in Russia.

Reference Services

INTERNET access played an important role in literature searching this year. Scientists were able to do online searching from their personal computers by connecting directly to the National Library of Medicine. The GENINFO databases available include a subset of Medline designed for molecular biologists by the National Biotechnology Center at NLM. GEN BANK, PIR, EMBL, Swiss Prot, HIV Databases, Mendelian Inheritance in Man, Cloning Vector Database, and the Brookhaven Protein Crystallographic Database are also available. The library has been a beta test site for a subset of the Medline database on CD-ROM called ENTREZ, which enables scientists to find related citations and associated sequences in protein and/or nucleotide databases.

Genemary Falvey, Head of Library Services, taught several GENINFO system workshops to the scientific and administrative staff. She also serves as the beta test coordinator between the Laboratory and National Library of Medicine and is a member of the Laboratory's computer committee.

The Library's journal holdings were computerized in 1991 and will go online next year. This will contribute to a more efficient system for both the user and the library staff. Leigh Johnson efficiently but manually processes over 600 claims for missing issues each year. This new system should greatly reduce the workload. Several current awareness services were provided, and more than 3600 reference questions were answered by the library staff.

Space Needs

The Library and its staff continue to endure a critical lack of space. By 1995, there will be no more room for the physical collection. Use of library services has increased in proportion to the growth of the Laboratory. Additional staffing and equipment will be required to handle this increase, and there is no room to put people or machinery.

The time has come to address the need for a dedicated library to complement its dedicated staff.

Susan Cooper

PUBLIC AFFAIRS

Neuroscience Center Opens

The final observance of the Laboratory's three-year Centennial celebration concluded with the dedication of the Neuroscience Center in a two-part ceremony that began on April 27, 1991. Dr. Arnold Beckman, who accepted our invitation to join the Laboratory's Trustees at the ribbon-cutting ceremony, attended with his daughter Pat and cut the ribbon to officially open the Arnold and Mabel Beckman Neuroscience Center. A specially engraved pair of ceremonial scissors was presented to Dr. Beckman. Afterward, his portrait was unveiled in the Center's main

floor lobby, as Dr. Watson offered a champagne toast. This ceremony was followed by a luncheon in the new Plimpton seminar room with the Trustees and guests.

Just six days later, on May 3, 1991, 500 friends and contributors were present for the second part of the dedication of the Neuroscience Center. Among the speakers were Dr. Susan Hockfield, who described the history of neurobiology at the Laboratory, Dr. Max Cohen, who discussed the future of neuroscience, and Dr. Stephen Heinemann, who related his experience as one of the students in the Laboratory's first neurobiology course. Keynote speaker U.S. Congressman Robert Mrazek spoke in support of government funding for science. On a lighter note, he shared with the audience his experience of mowing lawns at the Laboratory as a teenager.

The Public Affairs staff produced the dedication booklet for the event, including three other pieces that detailed the individual components of the Neuroscience complex. In record time, Margot Bennett designed each of the four pieces: *Beckman Neuroscience Center*, *Dolan Hall*, *Hazen Tower*, and *The Neuroscience Center Dedication*.

Staff Moves On and Moves In

Following the dedication of the Neuroscience Center, Emily Eryou, true to her conviction that she would join her husband to run their specialized engineering consulting firm, completed her successful career at the Laboratory on May 24. She began working in the Laboratory's library in 1987 and joined Public Affairs in 1988 where she projected the patience of Job, the wit of Lucille Ball, and her own brand of grace that mobilized the staff to produce the Centennial. Emily's last task was to train Lisa Gentry, who began working as the assistant in Public Affairs on April 29. Before coming to the Laboratory, Lisa worked for ABC Sports in New York, where she was an assistant director. As a freelance public relations person, Lisa designed advertising campaigns for New York retailers; prior to coming to New York, she was chairman of broadcasting at Fugazzi College in Lexington, Kentucky, where she taught courses in radio and television. Her background at ABC matched our need for the difficult multi-tasking done by our department. Lisa handles the requests from both television and radio, and her teaching background lends a helping hand to the Partners for the Future program for which she has complete responsibility.

David Siegel, science writer from September to May, left to pursue a degree in law. Nathaniel Comfort came to us from Cornell University, where he earned his Master's degree in neurobiology, to take on the responsibility of communicating the Laboratory's science to our lay audience. Nathan was associate editor of *The Living Bird Quarterly* at Cornell, where he wrote extensively; this coupled with his neuroscience background and a substantive body of freelance writing makes him eminently qualified to expand the Laboratory's reading public.

Because of these staff changes, we relied heavily on Clare Bunce, who shares her time between the Library and Public Affairs. Clare capably handled the details of public information and kept work moving while the new people learned their jobs. Help was also received from four wonderful volunteers. Jim Kurfess, who has been working for the department during winters since the beginning of our Centennial planning, now proofreads, stuffs envelopes, answers phones, and generally supports the work of Public Affairs. Katya Davey, who has her own full-time job at Robertson House, still finds time to affix labels for the *Har-*

bor Transcript; Mia Hao-Peng, a high school student, volunteered during the summer and did anything we asked. She was solely responsible for the inventory of our postcards and note cards. James Elkus, a prep school student and talented photographer, had the opportunity to take pictures during meetings and work in the Laboratory's bookstore.

New Pieces Produced

The third edition of *F.A.C.E.S.* will be distributed in January 1992; more than 130 new photographs were taken to complete this printing. Since the staff moved from a variety of places to the new Neuroscience Center and to Wawepex, it was difficult to place the faces with the right buildings.

Several brochures and flyers were updated in 1991. An especially nice brochure detailing the planned renovation of McClintock Laboratory was a combined effort between Bill Grover of Centerbrook Architects, who provided the beautiful poster that became the cover art, and Margot Bennett and Nathaniel Comfort, who designed and wrote the piece. Margot also created the 1992 meetings and courses posters, due out in December and January. This year, we have a new poster that combines the information from neurobiology and the molecular biology posters of the past. This "all courses poster" includes the new spring and fall listings as well. Three *Harbor Transcript* issues were generated in 1991; the third and fourth issues were combined because of staff changes and delayed hirings. Mailing of the *Transcript* has been simplified through Lisa Gentry's persistence and sound investigation. We have found an exceptional mailing house that handles all of our bulk mailings at an affordable cost, saving countless hours of staff time.

Media Mentions

Without the Centennial, it would seem that there might be less all-around coverage, but this does not appear to be the case. The Laboratory was featured in more than a dozen articles and mentioned in more than 100 others. Our senior scientific and administrative staffs cooperate regularly with the Public Affairs staff, fielding a wide variety of queries by the press. Dr. Watson had another active press year, producing an additional 40 mentions in 40 newspapers and magazines. Key articles appeared in *Forbes, Nature, Science, Newsday, The New York Times,* and *The Wall Street Journal,* as well as many in local Long Island newspapers and magazines.

Our newest celebrities are *Houses for Science* and its author Elizabeth Watson. A half dozen reviewers and reporters interviewed Mrs. Watson, and as of this writing, 11 articles and reviews have appeared. We anticipate an extensive article in *Newsday* on the growth of the Laboratory as seen through this new book. The literary press, book buyers, and the interested public were exposed to our "best book" of the season at several parties organized by the Publications and Public Affairs Departments. In Grace Auditorium, members of the Long Island Biological Association heard Michael Crosbie (Senior Editor of *Architecture* magazine) address "Science and Architecture." At a Lloyd Harbor seminar featuring Mrs. Ann Gill, Executive Director of the Whaling Museum, Mrs. Watson unveiled freshly printed copies of her book and talked about some of the interesting historical findings she discovered. At the Century Club in Manhattan and the

Travellers' Club in London, Dr. and Mrs. Watson hosted friends and publishing colleagues at inaugural parties for *Houses for Science.*

Other Events

Art Brings, the Laboratory's director of Environmental Health and Safety, worked with the Occupational Safety and Health Administration (OSHA) to co-sponsor a meeting entitled "Safety and Health and the Working Woman." Public Affairs also assisted the Development department in planning the exhibit of miniature rooms designed by Mrs. Eric Ridder.

At each event, every meeting, and all of the courses, indoors or out, Herb Parsons and Ed Campodonico, our audiovisual team, are there to manage the special sound, lights, and equipment needs. This year, during some very difficult times for Herb Parsons and his family, Ed Campodonico often worked 7 days a week to keep up with the demands in the AV department. His loyalty and good common sense kept everything on schedule.

Special Tours

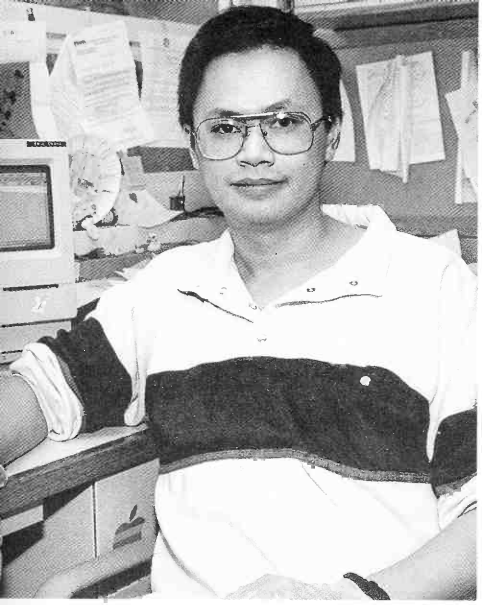
In addition to our regularly scheduled tours held four times each year, Public Affairs provides tours for various individuals and special groups. This year, among the more than 20 tours scheduled, we hosted the USA-Soviet Running Cultural Exchange, providing a tour led by visiting scientist Nikolai Lisitsyn. The Friends of the UN were treated to a very special tour by Elizabeth Watson, and a class of young women from Georgian Court College, studying maize genetics and Barbara McClintock, were given a talk by Rob Martienssen from the Laboratory's plant group.

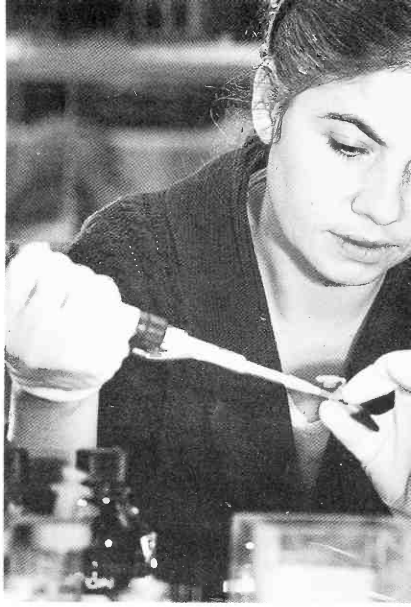
Plans for New Materials

In 1992, we will be creating a new press kit for use by both the Development and Public Affairs Departments. The kit will contain two new four-color brochures: One will detail the many facets of Cold Spring Harbor Laboratory and illustrate its accomplishments and its beauty and the other will be a brief introductory flyer. Supplementing these pieces will be detailed flyers on each of our scientifc programs. Rounding off the package will be a revision of the walking tour brochure.

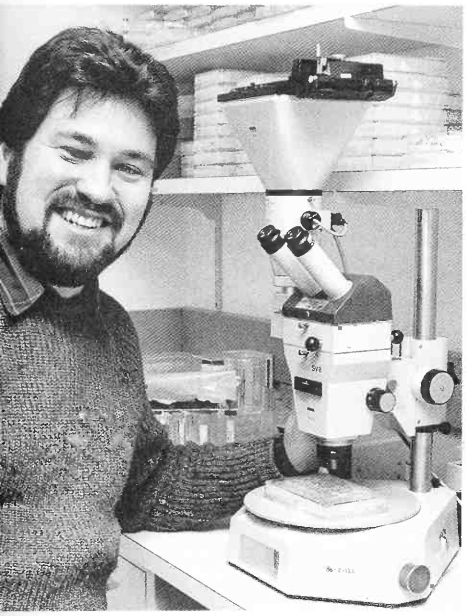
We look forward to a busy year of events planning, writing, and design.

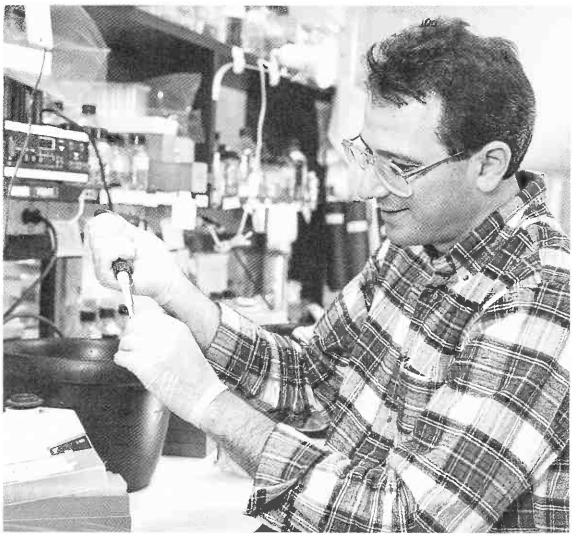
Susan Cooper

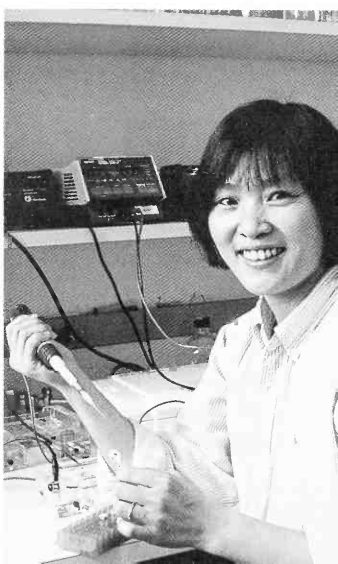




RESEARCH







Top row: Vincent Jung, Carol Greider, Nouria Hernandez
Middle row: John Horton, David Helfman, Stephen Bell
Bottom row: Jeff Kuret, Nancy Walworth, Hong Sun

TUMOR VIRUSES

Research in the Tumor Virus Section covers a broad sweep of topics in eukaryotic molecular biology. Originally, this work was tightly focused on the investigation of cellular transformation and gene expression in mammalian cells, studied principally through the medium of two DNA tumor viruses, adenovirus and SV40, and funded by a single large program project grant. Transformation remains a central theme, but the work has now expanded in its scope and diversified to embrace additional viruses (human immunodeficiency virus type 1 and bovine papillomavirus) and simpler model systems such as yeast. Eight laboratories participate in this section, and their reports follow in the order listed.

- Bruce Stillman's laboratory (DNA Synthesis) is dissecting the mechanism of DNA replication and chromosome assembly in mammalian and yeast cells with a view to understanding how the genetic blueprint is duplicated from generation to generation.

- Arne Stenlund's group (Molecular Biology of Bovine Papillomaviruses) is characterizing the viral elements that control gene expression and DNA replication in an animal virus that is closely related to the human papillomaviruses associated with warts and carcinomata.

- Work in Elizabeth Moran's group (Adenovirus Transforming Functions) is defining the pathway whereby the protein encoded by an adenovirus oncogene interacts with cellular proteins and modulates the activity of cellular transcription factors, thereby contributing to cell transformation.

- Together with their co-workers, Michael Mathews, Michael Laspia, and Gilbert Morris (Protein Synthesis) are investigating the transcriptional and translational alterations of cellular metabolism produced by adenovirus and HIV-1 gene products.

- In Adrian Krainer's group (RNA Splicing), attention is devoted to messenger RNA splicing, whereby the information present in the primary transcript of a gene is edited, with a view to understanding the mechanism and control of this process.

- Winship Herr and Masafumi Tanaka (Transcriptional Regulation) and their fellow workers are exploring the regulation of gene expression at the transcriptional level, focusing on the structure and function of viral and cellular transcription factors.

- Daniel Marshak and Ryuji Kobayashi with their colleagues (Protein Chemistry) are applying sophisticated protein chemistry techniques to illuminate issues of signal transduction and cell growth control.

DNA SYNTHESIS

B. Stillman G. Bauer, S.P. Bell, S. Brill, S.-U. Din, A. Dutta, T. Melendy, J.M. Ruppert, S. Waga, F. Bunz, K. Fien, Y. Marahrens, H. Rao, L. Borzillo, N. Kessler

We continue to seek a better understanding of the mechanism and regulation of DNA replication. As in past years, our studies have focused heavily on the replication of DNA tumor virus chromosomes, with the ultimate goal of transferring this knowledge to understand the replication of cellular chromosomes. Toward this end, we have increasingly studied DNA replication in the yeast *Saccharomyces cerevisiae* because it offers a wonderful mix of biochemical and genetic approaches. Information obtained with this experimental system can then be used to better understand DNA replication in human cells. This year has produced new results that should be of great import in the future.

SV40 DNA Replication

F. Bunz, L. Borzillo, T. Melendy, M. Ruppert, S. Waga, B. Stillman

The small double-stranded DNA chromosome containing the genetic information for simian virus 40 (SV40) replication contains a single origin of DNA replication (*ori*) where initiation of DNA synthesis begins. The virus encodes a single protein that is required for DNA replication, the SV40 large tumor antigen (TAg). This *initiator* protein recognizes the *ori* and initiates a series of complex events that ultimately lead to the unwinding of *ori* and the bidirectional replication of DNA.

During the past 6 years, we have concentrated on identifying cellular DNA replication proteins from human cells that combine with TAg to replicate DNA containing the SV40 *ori*. Seven proteins or activities have been described in detail in previous Annual Reports. When combined, these replication factors will support initiation and elongation of DNA replication. The products, however, are not the same as the products obtained when the crude cellular extract is combined with TAg, and we continue to search for cellular DNA replication proteins that will yield completely replicated DNA.

A new replication activity called MF1 has been purified to apparent homogeneity. This DNA replication factor is required, together with the previously identified replication factors and other partially purified proteins, for complete replication of template DNAs containing the SV40 *ori*. The DNA products of this highly fractionated system contain covalently closed, circular DNA that is identical to the products obtained with the crude cell extract in vitro or following DNA replication in vivo. MF1 has a 5′ to 3′ exonuclease activity and is similar to a DNA replication exonuclease identified in J. Hurwitz's laboratory.

Detailed studies on the functions of the cellular proteins required for SV40 DNA replication in vitro continue. DNA-binding studies with the purified proteins replication factor C (RFC), proliferating cell nuclear antigen (PCNA), and RPA (formerly called RFA) and DNA polymerases α and δ have revealed an ordered assembly of these proteins onto the replication template DNA. Other studies have shown that polymerase α binds to SV40 T antigen, probably reflecting the mechanism of assembly of this DNA polymerase onto the template DNA when TAg and RPA are bound to *ori*. Following the assembly of this initiation complex, other replication factors are assembled onto the DNA. We have demonstrated that RFC binds to the primers made by DNA polymerase α–primase complex and that PCNA can subsequently bind RFC. The assembled PCNA/RFC primer recognition complex is then recognized by DNA polymerase δ. Together with RPA coating the single-strand template DNA, the RFC/PCNA/polymerase δ multiprotein complex functions as a processive leading-strand DNA polymerase complex (Fig. 1). These results, coupled with our studies over the past 3 years, have clearly demonstrated that DNA polymerase δ functions as the leading strand polymerase. Recently, Akio Sugino and his colleagues have found that a third DNA polymerase, polymerase ε, is essential for growth of the yeast *S. cerevisiae*. However, addition of polymerase ε purified from human cells (obtained from S. Linn) has failed to produce any ef-

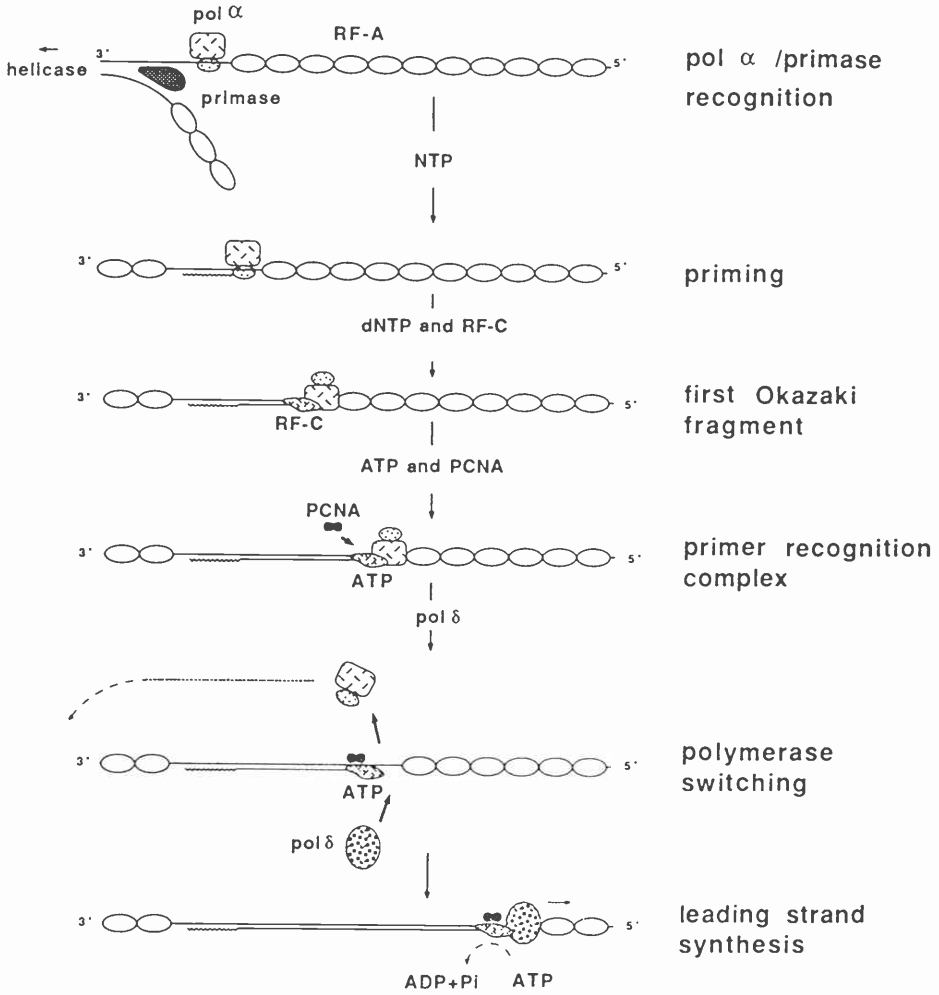


FIGURE 1 Model for the function of RFC and PCNA in loading DNA polymerase δ onto a DNA replication fork. (From Tsurimoto and Stillman, *J. Biol. Chem. 266:* 1967 [1991].)

fect on SV40 DNA replication in vitro. Thus, the role of polymerase ε in cellular DNA metabolism remains to be determined.

Cellular DNA Replication

S. Bell, S. Brill, K. Fien, Y. Marahrens, B. Stillman

Substantial progress has been made in the past year in our attempts to understand the mechanism and regulation of cellular DNA replication. We have taken a number of paths to approach this complex problem, and all have yielded interesting new results.

The first of these approaches was to identify and characterize functional homologs of the cellular DNA replication factors that are required for SV40 *ori*-dependent DNA replication. Because the yeast *S. cerevisiae* offers an excellent combination of biochemical and genetic approaches, we have sought to purify these replication factors from *S. cerevisiae*, clone the genes encoding these proteins, and study the role of these proteins in the S phase of growing cells. RPA from *S. cerevisiae* was purified previously, and the genes encoding the three subunits have been cloned. A genetic analysis is currently under way. Most recently, we have purified the *S. cerevisiae* homolog of RFC and have shown that the multi-protein complex has all of the functions found with the human RFC, including a DNA-dependent ATPase activity, a primer-template DNA-binding function, and the ability to stimulate DNA polymerase δ activity in cooperation with *S. cerevisiae* PCNA and RPA. Except for the 5′ to 3′ exonuclease

activity (MF1), all of the known cellular DNA replication proteins required for SV40 *ori*-dependent replication have been purified from *S. cerevisiae* by our laboratory or others, and we are currently concentrating on cloning the genes encoding these proteins so that their function in the cell can be studied genetically.

A second approach to understanding cellular DNA replication, and particularly initiation, has been to characterize fully a cellular origin of DNA replication. In last year's Annual Report, we described the identification of multiple *cis*-acting elements that contribute to the function of the cellular DNA replication origin *ARS1*. Four functional elements were identified, one of which was essential for *ori* function and was designated element A (Fig. 2). This element contained the previously recognized ARS consensus sequence that is found in all origins of DNA replication in *S. cerevisiae*. The other three elements, designated B1, B2, and B3, were individually not essential, but when each one was mutated, *ori* function was reduced. Interestingly, mutation of any combination of two of the B elements rendered the cellular *ori* nonfunctional. We have now determined probable functions for each of the four *ori* elements.

Element B3 was shown to bind the cellular transcription factor, *ori*-binding protein called ABF1. ABF1 was initially identified in our search for cellular origin-binding proteins and by numerous other laboratories as a transcription factor that activates cellular genes. We have now been able to replace the ABF1 DNA-binding site within element B3 with the binding sites for other *S. cerevisiae* transcription factors, including RAP1, GAL4, and a hybrid LexA-GAL4 fusion protein. The observation that all of these proteins stimulated *ori* function led to the suggestion that transcription activation domains present on these transcription factors activate DNA replication from cellular origins. A similar activation of DNA replication by transcription factors has been observed with several eukaryotic virus genomes.

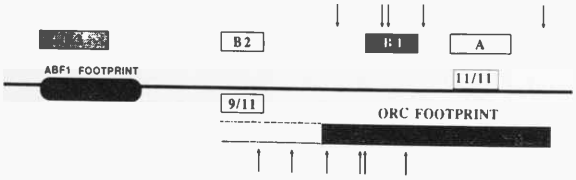


FIGURE 2 Summary of the structure of the *ARS1* chromosomal origin of DNA replication. The four functional elements A, B1, B2, and B3 are indicated by boxes. Elements A and B2 contain 11/11 and 9/11 matches to a sequence found in all *S. cerevisiae* origins of DNA replication. The binding sites for two origin-binding proteins, ABF1 and ORC, are shown. Under some circumstances, ORC binds to the B2 element in addition to binding the A and B1 elements. Arrows represent protein induced DNase I hypersensitive sites.

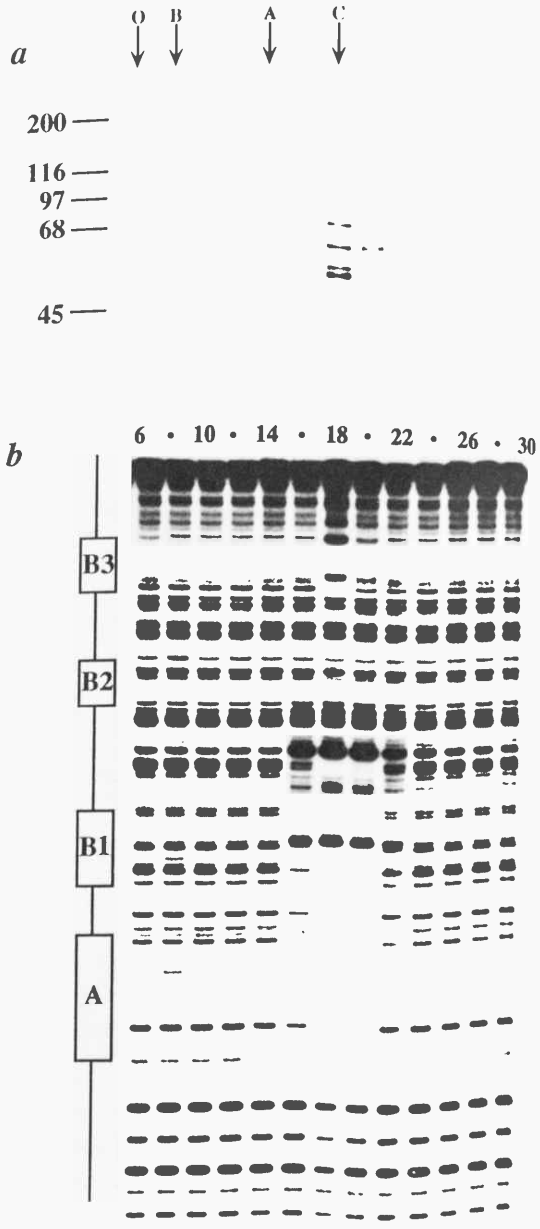


FIGURE 3 Glycerol gradient sedimentation of purified ORC protein. (*a*) SDS-polyacrylamide gel of the proteins in each fraction; (*b*) DNase I footprint.

Perhaps the most interesting observation was the recent discovery of a multiprotein complex that recognizes the essential A domain and interacts with both the B1 and B2 elements (Fig. 3). This multi-

protein, *o*rigin *r*ecognition *c*omplex (ORC) binds in a sequence-specific manner to the A element. Mutations in this element that reduce or eliminate origin function also reduce or eliminate ORC binding to *ori*, respectively. Thus, there is a precise correlation between origin function and ORC binding. Furthermore, we have found that ORC binds to all origins of DNA replication (ARS sequences) that have been tested, including *ARS1*, *ARS307*, *H4ARS*, *ARS121*, *HMR E ARS*, and the 2 μM plasmid *ARS*.

In the studies of the DNA-binding properties of ORC to the origins of DNA replication, an unusual DNA-binding property was revealed. ORC requires ATP for its sequence-specific DNA binding to the origins of DNA replication. This mode of DNA binding is unusual and perhaps unique, placing ORC in a new class of nucleotide-dependent, sequence-specific DNA-binding proteins.

These data strongly implicate ORC as the initiator protein for eukaryotic chromosomes, analogous to other well-characterized initiator proteins such as SV40 TAg, *Escherichia coli* DnaA protein, and bacteriophage λ O protein. The identification of homologs of *S. cerevisiae* ORC in metazoan species should facilitate the identification of replicator sequences that have heretofore remained elusive.

Regulation of DNA Replication

S.-U. Din, A. Dutta, B. Stillman

One of the cellular DNA replication proteins, RPA, is involved in the early stages of unwinding the SV40 *ori* with TAg. We had demonstrated previously that one of the three subunits of RPA, the RPA 34K protein, is phosphorylated in a cell-cycle-dependent manner. The protein was not phosphorylated during the G_1 phase, but it was phosphorylated in the S and G_2 phases of the cell cycle in proliferating cells. Both the yeast and human RPA 34K subunits were phosphorylated in this manner.

Experiments during the past year have continued to explore the functional significance of this phosphorylation. Detailed timing studies with synchronized human cells revealed that RPA first becomes phosphorylated at the G_1/S-phase transition, and the increase in RPA phosphorylation parallels the onset of S phase. Phosphopeptide analysis demonstrated that RPA was phosphorylated on multiple serine residues, and this pattern of phosphorylation was constant throughout the S and G_2 phases of the cell cycle.

To identify the protein kinases that phosphorylate RPA, large amounts of the human RPA protein were purified and used as a substrate to search for RPA kinases that are in human cell extracts. Three separate activities have been extensively purified, one of which has been identified as the human cdc2/ cyclin protein kinase. This kinase, which associates with different cyclin subunits in a cell-cycle-dependent manner, phosphorylates a subset of the phosphoserines present in the human RPA 34K subunit. These cdc2 kinase sites have been mapped, and mutation of these residues, using the human cDNA clone encoding the RPA 34K subunit (obtained from L. Erdile and T. Kelly), blocked all phosphorylation of RPA when the cDNA was transfected into mouse cells. This suggests that phosphorylation of the 34K subunit by cdc2 kinase might be critical for all subsequent phosphorylation and for the function of the protein. A full characterization of these mutants is under investigation.

As previously demonstrated by J. Roberts and his colleagues, we have shown that addition of the human cdc2/cyclin kinase to extracts prepared from human cells that were in the G_1 phase of the cell cycle stimulated SV40 DNA replication. The G_1 extracts otherwise have a low specific activity for DNA replication compared to similar extracts made from S-phase cells. Recently, we have carried this further and demonstrated that the cdc2 kinase stimulated the *ori* unwinding reaction that is dependent on TAg and RPA. Indeed, the cdc2 kinase was required for *ori* unwinding if an extract from G_1-phase human cells was included in *ori* unwinding reactions containing RPA and TAg. We are currently attempting to identify the component(s) in this extract that causes initiation to be absolutely dependent on cdc2 kinase.

Chromatin Assembly

G. Bauer, N. Kessler, B. Stillman

In the presence of a purified protein called chromatin assembly factor 1 (CAF1), plasmids containing the SV40 *ori* replicate and assemble into a chromatin structure that is identical to the structure of cellular chromatin. Chromatin assembly is dependent on concomitant DNA replication, and we have demonstrated that CAF1 functions to assemble histones H3 and H4

onto the DNA. Histones H2A and H2B are then assembled to complete the nucleosome in a reaction that is independent of ongoing DNA replication.

Purified CAF1 contains multiple protein subunits of 150K, a triplet at 62K, 60K, and 58K, and another subunit of 50K molecular weight. To study the function of the CAF1 protein further, we have prepared a series of monoclonal antibodies that independently recognize either the 150K or the 62K–58K proteins. Immunoprecipitation studies demonstrate that all of the CAF1 subunits form a complex. Interestingly, however, the 150K subunit appears to be in excess amounts that are not associated with the 62K–58K proteins, suggesting that in addition to being a CAF1 subunit, it may be bound to other proteins. In support of this, we have found that the monoclonal antibodies that recognize the 62K–58K proteins will immunoprecipitate all of the CAF1 activity present in nuclear extracts, but they will not deplete all of the 150K protein present in these extracts. These monoclonal antibodies have also been useful reagents to begin cloning cDNAs encoding the multiple CAF1 proteins. To date, we have obtained a partial cDNA encoding the 150K subunit.

In addition to CAF1, other proteins are required for the replication-dependent assembly of chromatin in addition to the DNA replication factors themselves. We have extensively purified a second such activity called CAF2 and have demonstrated that CAF2 interacts with CAF1. We are in the process of trying to identify the proteins that constitute CAF2 and determine the biochemical function of these proteins.

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MOLECULAR BIOLOGY OF BOVINE PAPILLOMAVIRUS

A. Stenlund M. Ustav E. Ustav
P. Szymanski L. Gandhi

The papillomaviruses are a large family of closely related, small DNA viruses that infect a variety of mammalian hosts. These viruses all carry transforming genes that are expressed in the natural course of infection and result in the production of (usually) benign tumors. In recent years, the human papillomaviruses have been recognized as a major cause of disease and have therefore attracted considerable attention. Bovine papillomavirus (BPV) has come to serve as the prototype virus for the papillomavirus group because a part of the viral life cycle for this virus can be reproduced in tissue-culture cells. Our interest in this virus is specifically related to the ability of the viral DNA to persist as a stably replicating plasmid in BPV-transformed cells. This capacity for regulated replication appears to be intrinsic to BPV and has been suggested to take place in two stages. In a hypothetical stage I, the viral DNA enters the cell and is replicated at a rate more rapid than that of the cellular DNA (more than once per cell cycle), resulting in an amplification of the viral DNA and an increase in the viral copy number to approximately 100 copies per cell. In stage II, which corresponds to the state of the viral DNA in BPV-transformed mouse cells, the viral DNA persists over many generations without apparent loss or change in copy number. We refer to stages I and II in our terminology as amplification and maintenance, respectively.

In last year's Annual Report, we described the development of a short-term replication assay that we designed and used to define the replication apparatus of BPV. This assay has allowed us to initiate a detailed study of the viral *trans*-acting factors and *cis*-acting elements that are required for replication of BPV. The assay was developed expressly to study the early events (stage I, amplification) and measures primarily the requirements for initiation of replication and DNA synthesis. With the use of this assay, we have identified the two viral polypeptides, E1 and E2, that are required for replication and also defined the necessary *cis*-acting elements. During the past year, we have concentrated our work on the functional role of these two polypeptides and their interaction with the *ori* sequence.

Maintenance replication (stage II) has always been assumed to involve a number of functions in addition to DNA synthesis. These include copy number control, segregation control, and mitotic stability, which are not readily measured in the short-term replication assay. However, our description of amplification replication has allowed us to create an assay where we provide the stage I functions and then ask specifically what additional functions are required for maintenance replication. This year, we have therefore initiated studies to determine what viral components, if any, are required specifically for maintenance replication.

In addition, we have expanded our scope, and in collaboration with the laboratory of L. Chow and T. Broker (University of Rochester Medical Center), we have addressed the issue of how representative BPV is for the large group of human papillomaviruses in terms of viral replication. These studies indicate remarkable similarities between these viruses and clearly validate BPV as a model for papillomavirus DNA replication.

Role of E1 and E2 Polypeptides in DNA Replication

M. Ustav, P. Szymanski, E. Ustav, A. Stenlund

Our initial in vivo studies demonstrated that the two viral polypeptides, E1 and E2, were necessary and sufficient for replication of BPV in tissue-culture cells. A commonly encountered role of viral replication factors is to recognize the *cis*-acting sequence in the DNA where replication will be initiated. Due to the large number of binding sites for the E2 polypeptide in the viral genome, it seemed unlikely that the E2 polypeptide could serve as an *ori* recognition factor in spite of the presence of an E2-binding site in the minimal *ori* sequence. The other required polypeptide, E1, had previously been shown to bind nonspecifically to DNA. We therefore decided to ask directly if the E1 polypeptide could bind to the origin

region. To generate a convenient source of E1 polypeptide, we used an *Escherichia coli* expression system. The E1 polypeptide, tagged with an epitope from the influenza hemagglutinin (HA), was used for DNA precipitation assays, with a monoclonal antibody directed against the epitope tag. Using this procedure, we showed that the E1 protein in addition to its nonspecific DNA-binding activity specifically recognized a single sequence in the viral genome, located in the origin region. By using DNase footprint analysis and methylation and ethylation interference analyses, we found that the recognition sequence was located in the center of the minimal *ori* fragment, overlapping an interrupted palindrome (Fig. 1). Mutations in this palindrome that affected binding of E1 also affected DNA replication. On the basis of these results, we concluded that E1 functions as the viral *ori* recognition factor.

The role of the E2 polypeptide was investigated initially by generation of deletion mutants in the E2-coding sequence. These mutants were subsequently tested for their ability to support replication and to *trans*-activate transcription when expressed from a heterologous expression vector. The deletion mutants were designed to affect the amino-terminal part of the protein that has been shown to be required for *trans*-activation, as well as the carboxy-terminal part of the protein that contains sequences required for DNA binding and dimerization. All deletions were, as expected, defective for *trans*-activation. Interestingly, all mutants were also defective in their ability to support replication, indicating that sequences both from the amino-terminal *trans*-activation domain and from the carboxy-terminal DNA-binding/dimerization domain were important for replication. To determine if the *trans*-activation function per se was of importance, we tested a chimeric protein consisting of the *trans*-activation domain of the herpesvirus protein VP16 fused to the DNA-binding domain of E2. This construct was capable of activating transcription but

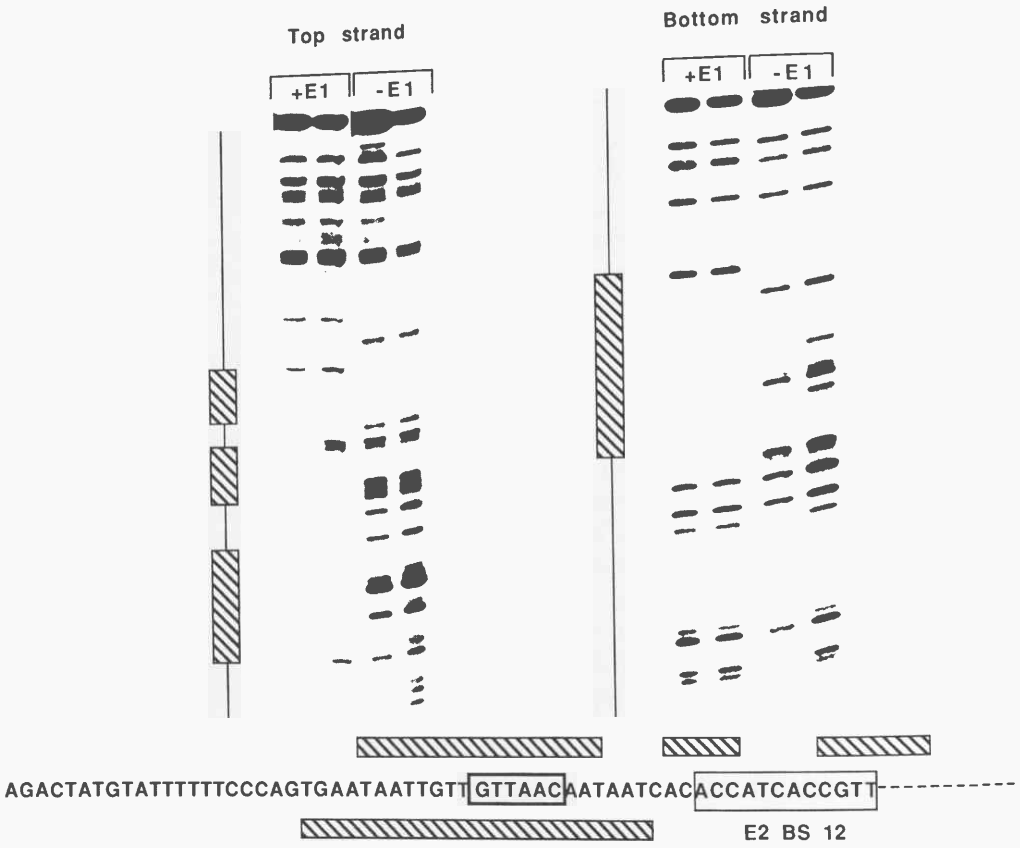

FIGURE 1 E1 binds specifically to the minimal origin region. DNase footprint assays were used to determine the specific binding site for the E1 polypeptide. The protected regions on each strand are indicated by a hatched bar, and the actual sequence protected is shown below.

was not able to support replication, indicating that the *trans*-activation function was not sufficient for replication but that, more likely, a specific domain of the amino-terminal portion of E2 was required. To determine if the lack of function of the carboxy-terminal deletion resulted from the loss of DNA-binding ability or was caused by the inability of the protein to form dimers, we wanted to generate mutations in this region that would affect DNA binding but not the ability to dimerize. Therefore, point mutants were generated in conserved motifs in the carboxy-terminal part of E2, and the resulting mutants were tested for their ability to bind to DNA and subsequently tested also for their ability to dimerize in an in vivo dimerization assay, since dimerization is a prerequisite for DNA binding. Mutants that were capable of dimerization but were defective in their ability to bind DNA were chosen and tested for their ability to support replication. A perfect correlation was observed between the DNA-binding ability and the ability to support replication. These results indicate strongly that the DNA-binding capacity of E2 is required for DNA replication and that both the E1 and E2 polypeptides are required to bind to the *ori* region.

Definition of the *cis*-acting Elements Required for Replication

E. Ustav, M. Ustav, A. Stenlund

Our previous studies had demonstrated that the *cis*-acting elements required for replication were confined to an approximately 100-nucleotide sequence located between the E2-dependent enhancer of BPV and the early coding sequences. We have continued the mutational analysis of this region by generating deletions and point mutations. Deletion analysis from either end of the fragment resulted in a minimal *ori* fragment of approximately 60 nucleotides (Fig. 2). This sequence contained three recognizable elements: an A/T-rich region, a palindromic element, and a low-affinity E2-binding site. The palindromic sequence was identified as a binding site for the E1 polypeptide (see above), and point mutations within this element that affected binding of E1 in vitro also affected DNA replication. Our initial mutational analysis indicated that mutations in the E2-binding site that rendered E2 binding to this site undetectable still allowed replication of the origin fragment, which led us to the conclusion that binding of E2 to the *ori* was not required for replication. However, on the basis of the results obtained with mutations that rendered the E2 polypeptide defective for DNA binding (see above), we suspected that this conclusion was incorrect. Further mutational analysis of the E2-binding site, with mutations that either increased or decreased the affinity of E2 for the site, showed good correlation between how well E2 supported replication and the strength of the E2-binding site. These results indicated strongly that binding of E2 to the *ori* sequence was necessary for a functional *ori*. This conclusion was further supported by experiments showing that a deletion of the entire E2 BS12, which was inactive for replication, could be restored to a functional *ori* by insertion of an unrelated E2-binding site at a different position in the plasmid. This apparent paradox—that the E2-binding site, even when mutated so that binding of E2 in vitro could no longer be detected, still appeared to be functional—indicated a stabilizing interaction with another DNA-binding component in vivo. To address this question, we generated a series of *ori* constructs in which we inserted a spacer sequence between E2 BS12 and the palindrome that serves as a recognition sequence for E1 binding. Insertion of a 10-bp spacer resulted in loss of *ori* function with the low-affinity E2 BS12, whereas high-affinity E2-binding sites functioned at

FIGURE 2 DNA sequence of the minimal *ori*. The 60-bp minimal *ori* region contains three recognizable elements: an A/T-rich region of unknown function, an inverted repeat that serves as a recognition sequence for binding of the E1 polypeptide (E1 BS), and a low-affinity binding site for the E2 polypeptide (E2 BS 12).

considerably greater distances. These results were consistent with an interaction between the E2 polypeptide and another component of the replication machinery, resulting in a stabilization of the E2 interaction with a low-affinity binding site. A potential partner in this interaction is the E1 polypeptide, which has been reported to interact with E2 in vitro by M. Botchan and colleagues (University of California, Berkeley).

Maintenance Replication

M. Ustav, A. Stenlund

A unique and interesting aspect of BPV replication is the ability of the viral genome to persist in BPV-transformed cells by replicating as a nuclear plasmid with a constant copy number under cell cycle control. To study this particular aspect of BPV replication that we call maintenance, we have started to design experiments addressing the requirements for maintenance replication. The following are the initial questions that we asked: (1) Are viral gene products other than E1 and E2 required? (2) Are *cis*-acting sequences other than the minimal *ori* required? (3) Can maintenance be achieved without the transcriptional control that is normally provided when E1 and E2 are expressed from the viral genome? Our strategy to address these questions has been to establish stable cell lines that constitutively express the E1 and E2 polypeptides transcribed from heterologous promoters. We chose Chinese hamster ovary (CHO) cells for this purpose since these cells support short-term replication of BPV efficiently and also can be transfected with virtually 100% efficiency. The cell lines were verified to express both E1 and E2 polypeptides both by detection of the polypeptides by immunoprecipitation and by functional assays where short-term replication of a minimal *ori* fragment was assayed. To assay for maintenance replication, we generated a vector containing a *neo* resistance marker into which we could insert various fragments from BPV. A 2.5-kb fragment from BPV containing the origin of replication was initially inserted and transfected into the E1+E2 cell line. After selection for G418-resistant colonies and further passage for 6–8 weeks, DNA was prepared and analyzed for the presence of episomal BPV sequences. In all cases, the origin construct was maintained at high copy number exclusively in episomal form.

These results indicated clearly that the E1 and E2 gene products were sufficient for maintenance and also that transcriptional control of the levels of these two proteins was not required. The next question that we addressed was whether the *cis*-acting sequences that were sufficient for short-term replication (i.e., the minimal *ori*) were also sufficient for maintenance replication. These experiments were carried out by inserting small fragments containing the minimal *ori* into the *neo* plasmid. The *ori* constructs were tested initially in a short-term replication assay in which all constructs replicated to similar levels. After G418 selection and passaging, however, the minimal *ori* constructs could not be recovered as episomal DNA. These results indicated that the minimal *ori* was not sufficient and that additional sequences were required for stable maintenance of BPV. The inability to function in maintenance was not purely a consequence of a lower replication efficiency, since the minimal *ori* functioned in short-term replication with an efficiency similar to that of the larger fragment. Further mapping of the sequences important for maintenance indicates that these are located in the E2-dependent transcriptional enhancer.

Viral Gene Regulation

P. Szymanski, A. Stenlund

Last year, we reported that the E2 *trans*-activator through its action on the E2-dependent enhancer appeared to function as a master regulator of viral transcription. During this year, we have continued our work on E2 by studying its role in negative regulation. The E2 open reading frame encodes, in addition to the full-length polypeptide that functions as a *trans*-activator, two shorter polypeptides that contain the same carboxy-terminal DNA-binding/dimerization domain as E2. These two polypeptides have been shown to act as competitive repressors of transcription from E2 responsive promoters; however, the mechanism of action has not been determined. Two obvious possibilities are either competition for DNA binding, since these three proteins have the same DNA-binding specificity, or formation of inactive heterodimers, since the dimerization domain is also

shared. To distinguish between these possibilities, we utilized the point mutants in the carboxyl terminus of E2 that are deficient for DNA binding but still are capable of dimerization (see above). In functional assays where an E2 responsive reporter construct is cotransfected with an expression vector for the E2 activator, the reporter is activated. If either of the short forms of E2 is included, repression of this activation is observed. If instead the DNA-binding-deficient mutant version of the short E2 is used, no repression is observed. However, the DNA-binding-deficient form of full-length E2 in the same assay can efficiently repress E2-dependent *trans*-activation. The conclusion from these experiments is that the three forms of E2 despite their common dimerization domain cannot heterodimerize efficiently. In addition, hybrid dimers, formed between the wild-type activator and the DNA-binding deficient mutant, are incapable of activating transcription due to a lack of DNA-binding activity of the hybrid. Taken together, these results indicate that the naturally occurring repressor forms of E2 act largely through competition for DNA binding but that mutant forms of the full-length E2 can function as repressors through formation of inactive hybrid dimers.

Replication of Human Papillomaviruses

M. Ustav, A. Stenlund [in collaboration with C.-M. Chiang, T. Broker, and L. Chow, University of Rochester Medical Center]

To determine whether the information that we have gathered about replication of BPV also applies to the very large group of clinically important human papillomaviruses, we have initiated a collaboration with the laboratory of L. Chow and T. Broker. Consistent with the similar genomic organization of all papillomaviruses, expression vectors containing the E1 and E2 open reading frames from HPV-11 supported efficient replication of the HPV-11 genome in a variety of cell lines in a short-term replication assay. A surprising finding in these experiments was that the E1 and E2 polypeptides from BPV were also quite efficient in supporting replication of the HPV-11 genome. Furthermore, both HPV-11 and BPV E1 and E2 polypeptides could also replicate a variety of other animal and human papillomaviruses. In addition, the combination of BPV E1 and HPV-11 E2 (or the converse) also functioned well for replication. These results taken together clearly demonstrate the universality of BPV as a prototype for the papillomavirus group. Another important point is the apparent lack of cell type or species specificity, indicating that the high degree of tissue and species tropism that is observed in vivo for these viruses is not a result of restrictions in DNA replication but is more likely due to restrictions in expression of viral proteins.

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ADENOVIRUS TRANSFORMING FUNCTIONS

E. Moran S. Abraham, Y. Rikitake, H.-G.H. Wang, P. Yaciuk, M. Carter, A. Pepe

Our goal continues to be to understand the genetic and biological mechanisms that are the basis of adenovirus E1A transforming functions. In previous years, we have identified two independent active sites that are the basis of E1A cell-growth-regulating functions. These sites can be inactivated selectively by mutations confined to either the extreme amino terminus or the region designated conserved region 2 (Fig. 1). Although the immortalization function of E1A requires that both of these sites be functional, we have shown through a variety of biological and genetic analyses that either site alone is sufficient to induce a transcriptional program sufficient to bring cells out of a resting state, through G_1, and into S phase. Our most recent work has focused on determining how these transcriptional signals are communicated from the E1A products to the various cellular promoters. This process must involve intermediate products because E1A is not itself a DNA-binding protein.

Within the last several years, it has become apparent that the two E1A active sites involved in cell growth control function as the binding sites for specific cellular proteins. Since the identification by Ed Harlow, then at Cold Spring Harbor Laboratory, of one of the region-2-associated proteins as the retinoblastoma gene product (pRB), E1A became an important tool for probing the function of this tumor susceptibility gene. Last year, we reported genetic evidence, from a study done in collaboration with Joseph Nevins at Duke University, that indicated a link between E1A region 2 and activation of the cellular E2F transcription factor. This suggested that pRB, as a region-2-associated protein, might constitute a physical link between E1A and the E2F transcription factor. Subsequent work in the Nevins laboratory has shown that pRB is indeed a component of E2F complexes and, moreover, that other region-2-associated proteins participate in these complexes as well. It is thus very likely that activation of E2F is one pathway by which E1A can regulate cell-cycle-specific gene expression.

But it is not the only pathway. In this laboratory during the past year, we have focused on an alternate pathway: that signaled by the E1A amino-terminal active site. To date, the only cellular protein known

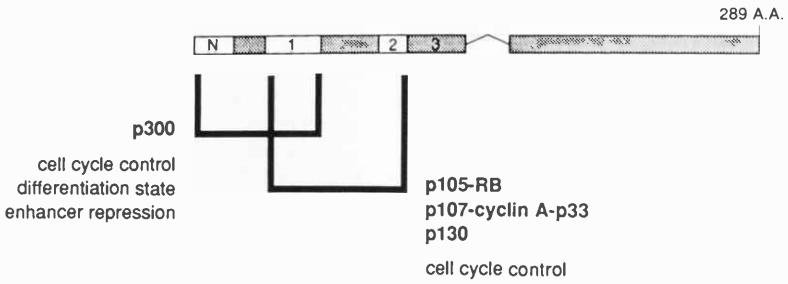

FIGURE 1 Schematic diagram of E1A active sites and associated proteins. The E1A regions required for cell-growth-regulating functions (shown as unshaded regions in this diagram) are the binding sites for several cellular proteins. These regions constitute two independent active sites that can be inactivated selectively by mutations confined to either the extreme amino terminus or region 2. The activity of either site is sufficient to induce G_1 and S phase in resting cells, although both sites are required for the E1A immortalization function. Although both sites can induce cell cycle functions, the ability of E1A to block cellular differentiation and repress enhancers controlling tissue-specific gene expression is linked with the amino-terminal active site and the binding of a cellular protein designated p300.

to bind to E1A via the amino terminus is a 300-kD protein, designated p300, that has become a special focus of our studies. The biochemical basis of the amino-terminal active site has a special interest because this site is linked not only with cell cycle activation, but with the abilities of E1A to repress enhancer activity required for tissue-specific gene expression and to block cellular differentiation.

p300 Binding Is Dependent on Specific, Conserved, Positively Charged Residues at Positions 2 and 3 of E1A

H.-G.H. Wang, P. Yaciuk, S. Abraham, Y. Rikitake, M. Carter, E. Moran

p300 binding is dependent on residues included within the amino-terminal 25 amino acids of E1A. Because the extreme amino-terminal sequence is not strongly conserved among the E1A products of the various adenovirus serotypes, there are few clues to the nature of the essential residues. However, these sequences are implicated in the E1A functions targeting the control of cell growth and differentiation. Therefore, it is of significant interest to determine which E1A residues are essential for amino-terminal function, especially as these sequences could conceivably contain motifs homologous to important cellular control proteins.

To determine whether there are specific residues required for p300 binding in the E1A amino terminus, we first constructed a chimera in which E1A residues 1 to 24 were replaced with the first 24 residues from SV40 large T antigen, a transforming protein encoded by a different class of DNA tumor virus. The chimeric protein showed no evidence of binding p300, even when highly overexpressed. (The chimera did, however, continue to bind the region-2-associated proteins.) These results suggest that there are indeed specific residues in the E1A amino terminus required for p300 binding. To explore this possibility further, we considered the significance of the small degree of homology that occurs in the E1A amino-terminal sequences. Two conserved features have been noted (Fig. 2). All serotypes sequenced show a leucine residue at approximately position 20, which is invariably followed by a negatively charged residue, either glutamic or aspartic acid, in the next position. However, changing either of these residues

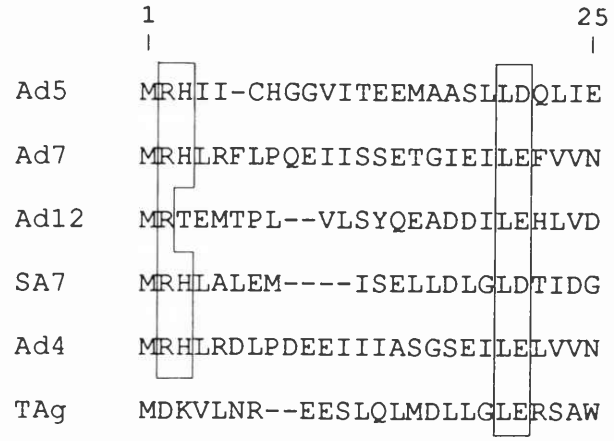

FIGURE 2 Comparison of amino-terminal E1A protein sequences from various primate adenoviruses. The first 25 amino acid residues of Ad5 E1A are compared with the amino-terminal sequences of E1A from other serotypes. Conserved features are boxed. The first 24 residues of SV40 large T antigen are also shown for comparison.

(Leu to Ser or Asp to Ala) had only a minimally disruptive affect on p300 binding. The other conserved feature is the occurrence of positively charged residues in positions 2 and 3. The E1A gene of almost every primate serotype sequenced encodes an arginine at position 2 and a histidine at position 3. The conservation of these positively charged residues is intriguing, especially as the occurrence of positively charged residues in the highly acidic E1A products is rare. We have found that mutation of either of these residues (Arg to Gly or His to Asn) severely disrupts the p300 interaction, indicating that the integrity of this small region of positive charge is essential for stable p300 binding. These results combine with results reported last year, from which we concluded that p300 and the region-2-associated proteins bind to distinct subregions in conserved region 1, to give a clearer picture of the requirements for p300 association in each of its binding regions.

Amino-terminal Point Mutations Support the Link between p300 Binding and the E1A-mediated Enhancer Repression Function

Y. Rikitake, S. Abraham, P. Yaciuk, H.-G.H. Wang, E. Moran

It has been known for some years that E1A can repress the activity of viral and cellular enhancers.

Examination of this function with the newly constructed point mutants strongly supports previous evidence indicating that the enhancer repression function is primarily a property of the amino-terminal active site.

Enhancer repression assayed on the long terminal repeat (LTR) of the human immunodeficiency virus (HIV) is shown in Figure 3. Enhancer activity of the HIV.LTR is dependent on the presence of two NFκB-related enhancer elements and can be detected by expression of a heterologous reporter gene product, an enzyme designated CAT, whose presence is detected by the conversion of chloramphenicol (Cm) to its acetylated forms (Ac-Cm). Coexpression of the E1A 12S wild-type products with the HIV.LTR.CAT construct strongly represses CAT activity. Mutation of either of the two amino-terminal positively charged residues essential for p300 binding (12S.RG2 and 12S.HN3) severely impairs this enhancer repression activity. Nearby mutations with little disruptive effect on p300 binding (12S.LS20 and 12S.DA21) have, likewise, little effect on the enhancer repression function. In contrast to the extreme amino-terminal mutations, a combination of the two point mutations (12S.928/YH47), which together abrogate the region-2-specific associations without impairing p300 binding, has no effect on enhancer repression. These results indicate that enhancer repression via the NFκB-related elements is not linked with any region-2-associated products, but is tightly linked with p300 binding, and is indeed dependent on the same individual E1A residues.

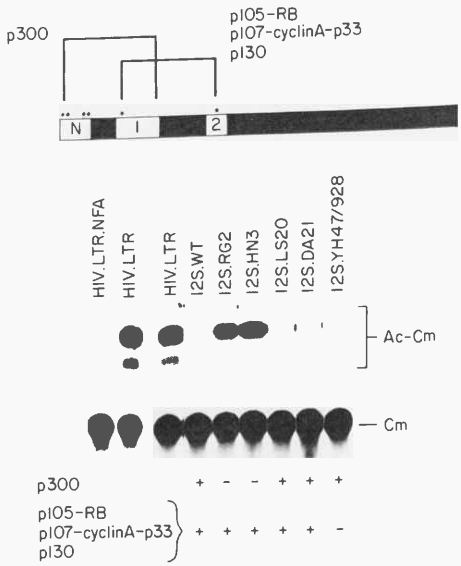

FIGURE 3 The enhancer repression function is linked with the p300-binding activity of E1A. In this experiment, the activity of the HIV.LTR enhancer was visualized by the ability of a reporter gene product to convert chloramphenicol (Cm) to acetylated forms (Ac-Cm) with distinguishable chromatographic properties. The activity of the HIV.LTR is dependent on the presence of two NFκB-related elements in the enhancer; in their absence (HIV.LTR.NFA), activity is very low. Coexpression of the E1A 12S wild-type products (12S.WT) with the HIV.LTR construct strongly represses the enhancer. Mutation of either of the two amino-terminal positively charged residues essential for p300 binding (12S.RG2 and 12S.HN3) impairs the repression activity severely. Nearby mutations with little disruptive effect on p300 binding (12S.LS20 and 12S.DA21) have, likewise, little effect on the enhancer repression function. In contrast to the extreme amino-terminal mutations, a combination of two point mutations (12S.928/YH47), which together abrogate the region-2-specific associations without impairing p300 binding, has no effect on enhancer repression function. The approximate locations of the single point mutations are indicated by dots in the upper bar diagram.

p300 Is a Sequence-specific DNA-binding Protein with Affinity for Known Enhancer Motifs

Y. Rikitake, S. Abraham, P. Yaciuk, H.-G.H. Wang, E. Moran

Last year, we reported preliminary biochemical evidence indicating that p300 is a DNA-binding protein. During the past year, we have confirmed this observation and determined that the interaction of p300 with DNA shows a preference for specific sequences. Moreover, the consensus sequence derived for p300 binding shows a provocative degree of similarity to known enhancer motifs, raising the exciting possibility that p300 is a direct intermediate between E1A and upstream DNA elements that control cellular gene expression.

To determine whether p300 has preferred DNA-binding sequences, we asked whether immobilized p300 can select sequence-specific oligonucleotides out of a random pool. (This technique was described in detail in last year's Annual Report.) The proportion of retained oligonucleotides increased through each of several rounds of selection, indicating that p300 is indeed capable of distinguishing preferred sequences. Cloning and sequencing of the selected oligonucleotides indicated that the selected fragments were in fact related and not random. From the sequencing of 16 cloned fragments, we derived a 7-bp consensus sequence: 5′-GGGAGTG-3′, (5′-

CACTCCC-3′). This sequence bears an intriguing degree of similarity to several enhancer elements, including the NFκB element, that are the best-characterized targets of E1A repression. This observation prompted us to test the ability of p300 to bind to specific enhancer motifs. NFκB-related motifs include an element found in major histocompatibility complex (MHC) class I genes and bound by a cellular factor designated H2TF1. The ability of p300 to bind to an oligonucleotide containing the H2TF1 motif (5′-TGGGGATTCCCCA-3′) is shown in the UV cross-linking experiment illustrated in Figure 4. A positive autoradiography signal in this assay depends on the ability of the protein to form a direct physical bond with the DNA molecule and indicates that the DNA-binding activity of the protein is direct and intrinsic. The ability of p300 to bind to preferred sequences was tested in competition assays. The amount of radioisotope signal transferred from the oligonucleotide to the protein can be competed by the presence of unlabeled oligonucleotide; the efficiency of the competition is a measure of the affinity of the protein for the probe sequence. In Figure 4, the affinity of p300 binding to the H2TF1-binding sequence was tested against competition from each of three different unlabeled oligonucleotides: a random sequence oligonucleotide (ran), the H2TF1 motif itself (H2TF1), and a double point mutation variant of the H2TF1 motif (H2TF1.DM: 5′TGCGGATTCCCGA-3′), which reduces the extent of homology with the p300 consensus sequence, and is known to impair binding of the H2TF1 protein. The H2TF1 motif itself competed very strongly (lanes 9–12). The double point mutant variant competed less efficiently (lanes 13–16), showing both that p300 is capable of distinguishing a very subtle level of difference among preferred binding sequences and that p300 can make the same qualitative distinctions as known enhancer-binding proteins such as H2TF1. The random sequence oligonucleotide competed very poorly; a 125-fold excess of this oligomer was required to compete the H2TF1 signal (lanes 5–8), showing that p300 has

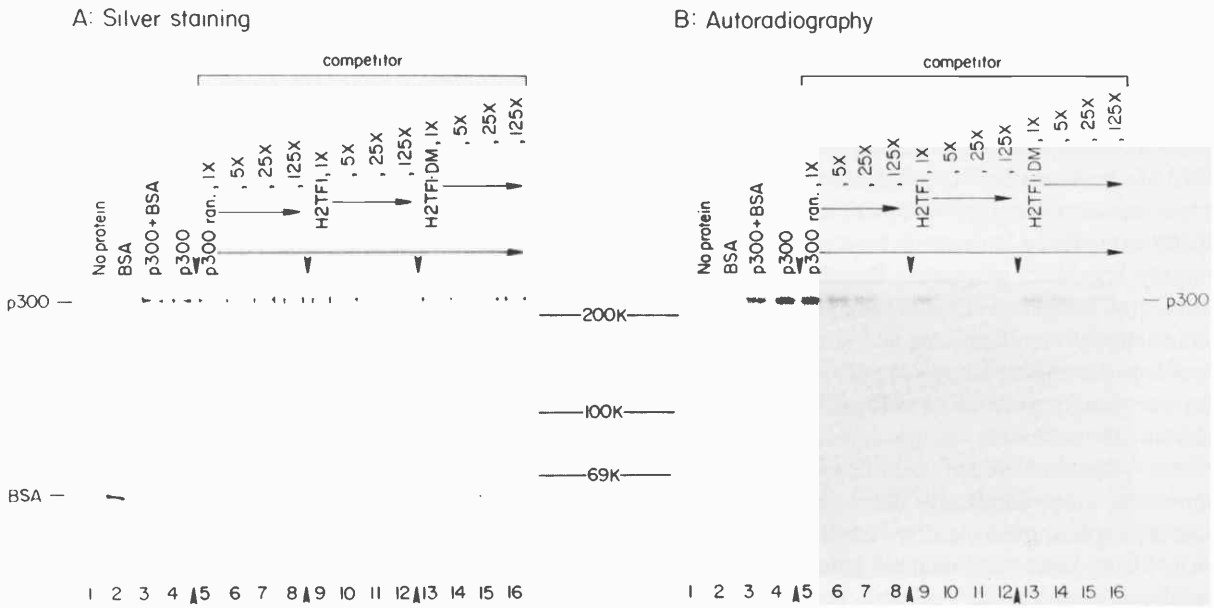

FIGURE 4 UV cross-linking of p300 to a specific DNA enhancer motif is resistant to competition from mutant or random DNA sequences. Protein products on the same gel were visualized either by silver staining (panel *A*), which reveals all detectable proteins, or autoradiography (panel *B*), which reveals only those proteins that successfully cross-linked to a radioisotope-tagged DNA probe containing the H2TF1 enhancer-binding motif. The silver-stained gel shows the presence of equal levels of p300 in each reaction; the samples in lanes *2* and *3* contained a control protein, BSA. Only p300 shows a radioactive signal on the autoradiogram; BSA neither binds to the H2TF1 probe (lane *2*) nor interferes with p300 binding (lane *3*). The ability of various (nontagged) DNA probes to compete with the tested enhancer motif for p300 binding is indicated by the decrease in p300 signal in each of the three competition experiments shown. The H2TF1 enhancer motif competes strongly with the identical labeled sequence (lanes *9–12*). A double point mutant variant of the H2TF1 motif does not compete as well (lanes *13–16*), and random sequences compete very poorly (lanes *5–8*).

a relatively high affinity for the enhancer-specific motif over random DNA sequence.

Basis of p300 Function

P. Yaciuk, S. Abraham, A. Pepe, Y. Rikitake, H.-G.H. Wang, E. Moran

The progress in the past year raises the exciting possibility that p300 is an enhancer-binding protein and that its targeting by E1A results in the repression of a variety of tissue-specific genes. The implication that p300 plays a basic role in tissue-specific gene expression suggests that further characterization of p300 will give us novel and important insights into this fundamental process. To understand why p300, of all the enhancer-binding proteins in the cell, is targeted by E1A, it will be important to understand both the structure of p300 and the nature of p300 interactions in the cell in the absence of E1A. Work on both of these fronts is in progress. We are currently screening expression libraries for the purpose of obtaining a cDNA clone corresponding to the p300 product. We are also exploring the protein-protein interactions of p300 and have begun to characterize a number of protein species that interact specifically with p300. We are particularly interested in associated proteins that may modulate p300 function. We expect that p300 is regulated because its function is implicated in highly regulated processes. Regulation may be effected at various levels, including expression, post-translational modifications, and protein-protein interactions. As reported last year, p300 does not appear to be strongly growth- or cell-cycle-regulated at the levels of expression or posttranslational modifications. (Posttranslational modifications of p300 do, however, vary specifically during the cell cycle, and we are exploring the significance of this observation.) It is likely, however, that p300 is regulated via its associations with other cellular products. Important preliminary results obtained this year suggest that the p300-associated products include those whose expression or association is strongly growth-regulated. Characterization of the p300 associations will be greatly facilitated by the availability of monoclonal antibody lines specific for p300, and the development of these reagents is a high priority for the coming year. Indeed, recent preliminary evidence suggests that we may have already succeeded in developing these important reagents.

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PROTEIN SYNTHESIS

M.B. Mathews P. Clarke, R. Galasso, S. Green, S. Gunnery, B. Hoffman, C. Kannabiran, M. Kessler, C. Labrie, M.F. Laspia, Y. Ma, L. Manche, K.H. Mellits, G.F. Morris, R. Packer, T. Pe'ery, D. Taylor, P. Wendel

Work in this laboratory continues to investigate the regulation of gene expression, focusing on three systems. The first of these is concerned with the regulation of protein synthesis by a cellular protein kinase. The kinase forms an important part of the interferon-induced antiviral response, and it is the target of viral countermeasures designed to neutralize this cellular defense mechanism. Our studies seek to understand the enzyme's role in translational control and its interactions with viral components, specifically RNAs, that modulate its activity. The second project deals with the mechanism whereby an oncogene activates cellular genes that are required for cell division, an issue that is central to cellular transformation and carcinogenesis. In particular, we are investigating the transcriptional activation of the gene encoding a DNA replication factor, PCNA (proliferating cell nuclear antigen), by the adenovirus E1A oncogene product. Finally, in the third project, we are examining an unusual form of transcriptional regulation adopted by the AIDS virus, human immunodeficiency virus type 1 (HIV-1). The virus encodes a protein, Tat, which binds to the nascent transcript generated from the viral promoter and then increases the ability of subsequent RNA polymerase molecules to transcribe the template. During the year, Paul Clarke and Debbie Taylor joined our laboratory, and Tsafi Pe'ery and Ken Mellits, with his newly awarded and well-earned Ph.D. degree, left Cold Spring Harbor Laboratory.

Adenovirus VA RNA and Translational Control

S. Green, P. Clarke, F. Ma, T. Pe'ery, K.H. Mellits, D. Taylor, L. Manche, M.B. Mathews

Interferon induces a cellular response that serves to limit viral infection and protect the host. One component of the response involves the induction of a protein kinase known as DAI, the double-stranded (ds) RNA-activated inhibitor of protein synthesis. This enzyme phosphorylates a translation factor, eukaryotic initiation factor 2 (eIF-2), interfering with its function in protein synthesis. DAI is present in most cells in an inactive state, but during viral infection, the presence of low levels of dsRNA (probably generated by symmetrical transcription of the viral genome) leads to the autophosphorylation and activation of the kinase. Once activated, DAI blocks translational initiation thereby preventing the virus from usurping this process for its own proliferation. For their part, a number of viruses have evolved strategies to circumvent this cellular defense mechanism: The best understood of these involve small highly structured yet single-stranded (ss) RNAs that inhibit the activation of DAI. This laboratory has for many years studied the inhibitor VA RNA produced by adenovirus, but a similar effect is also seen with RNAs derived from Epstein-Barr virus and HIV-1.

The recent cloning of a cDNA encoding DAI afforded a new approach to studying this system, using mutagenic methods to determine which regions of DAI are involved in interacting with dsRNA activators and ssRNA inhibitors. Our procedure is to generate functional DAI protein in vitro through the use of T7 RNA polymerase transcription and wheat germ translation systems. Wild-type and mutant forms of DAI are then tested to determine whether they are still capable of binding RNA. From the analysis of mutants carrying large internal deletions or extensive carboxy-terminal truncations, we conclude that the RNA-binding domain lies within the first 170 amino acids of the protein. This region is devoid of obvious RNA-binding domains, so we examined the role of basic amino acids with the idea that the positively charged side chains of these amino acids might interact with the negatively charged RNA backbone. A number of basic amino acids were replaced by uncharged amino acids using site-directed mutagenesis, and the RNA-binding properties of the resultant proteins were studied. Most of the basic residues appear to function in a synergistic manner, which is

also dependent on their location within the protein molecule, but two regions were identified in which mutations completely abrogate the ability of DAI to bind to RNA. The binding of VA RNA is considerably more susceptible to mutation of the enzyme, with most substitutions of basic residues resulting in loss of function. Studies are now in progress to determine which individual amino acids are involved and how they interact spatially.

Even in the most favorable situations, DAI is a scarce protein and difficult to obtain in sufficient quantities for biochemical work. To remedy this deficiency, we attempted to use the cDNA encoding DAI to overexpress the kinase in *Escherichia coli*. Surprisingly, despite the use of a strongly regulated promoter, once the protein is produced, it is highly toxic, killing the bacteria before significant levels of DAI accumulate. To overcome this problem, we made a mutant protein containing a single-amino-acid substitution that renders the protein nonfunctional. This mutant produces a significant amount of protein during overexpression. After the development of a suitable purification scheme, the bacterially expressed protein was used as antigen for the successful production of polyclonal antibodies that should facilitate large-scale protein purification. We now hope to follow a similar method for the production of monoclonal antibodies.

As mentioned above, several viruses have evolved measures to evade the DAI-mediated antiviral defense mechanism. One of these exploits a characteristic of DAI that high concentrations of dsRNA or of specific ssRNA effectors block its activation. At late times in adenovirus infection, large quantities of two small RNAs, VA RNA_I and VA RNA_{II}, are synthesized by RNA polymerase III. Previous studies have shown that the VA RNA_I encoded by adenovirus-2 (Ad2), and that encoded by the closely related virus Ad5, is a highly structured molecule. It contains two short duplexes, the apical and terminal stems, and a central domain that is largely single-stranded. Structural and mutagenic studies suggested two possible conformations for the central domain but so far have not allowed us to decide between the two alternatives. Taking a fresh approach to this problem, we have begun a phylogenetic comparison of the RNA from several sources. Six other VA RNA species from human, simian, and avian adenoviruses have been analyzed at the DNA and RNA levels. Alignment of their primary sequences revealed regions of homology corresponding to transcription signals, notably the A and B boxes of the promoter and the initiation and termination sites at the two ends of the RNAs. The region to the 3′ side of the B box is heterogeneous in sequence, but in all cases, it can pair with the B box region to form a stem-loop structure equivalent to the apical stem of Ad2 VA RNA_I. Similarly, the sequences at the 5′ and 3′ ends of the molecules can pair, forming a terminal stem. The regions corresponding to the central domain of Ad2 VA RNA_I are heterogeneous in sequence (except in two cases that display ~65% homology), but they all form a structure corresponding to the central domain of Ad2 VA RNA_I. Although this region is largely open, there are some bases that are inaccessible to nucleases, suggesting that they are base-paired or protected because of tertiary folding. Strikingly, there is also a short conserved sequence that occupies a similar position in all six VA RNA species. The significance of this sequence remains to be ascertained.

Earlier studies using deletion and linker scanner mutants indicated that the apical stem of VA RNA is important for DAI binding. Mutants that disrupt the central domain retain the ability to bind DAI efficiently but are unable to block DAI activation by dsRNA, suggesting that binding and functional domains of Ad2 VA RNA_I can be distinguished. These studies have now been extended using further mutational analysis to investigate the roles of the apical stem, the central domain, and the potential base pairing within the central domain. Extensive disruptions of base pairing within the apical stem were observed to reduce binding, whereas small mutations that maintain significant base pairing still allow efficient binding to DAI. A mutation that brings the apical stem closer to the central domain abolishes DAI binding, implying that spatial separation of the binding domain and central domain is crucial for efficient binding. As predicted, mutations in the apical stem have no effect on the function of the molecule provided the ability to bind DAI is retained. On the other hand, mutations in the central domain disrupt the function of VA RNA but in many cases do not affect its ability to bind to DAI. Other mutations within the central domain were observed to decrease binding efficiency, however, suggesting that DAI binding is influenced by sequences within the central domain as well as by the apical stem. One possibility is that both parts of the molecule are involved in forming a tertiary structure that binds to DAI. We are now developing chemical methods to examine the interaction of DAI with RNA directly: These studies will complement studies of the enzyme itself.

Regulation of PCNA

G.F. Morris, C. Kannabiran, C. Labrie,
R. Packer, M.B. Mathews

The transforming region of adenovirus (E1) stimulates the expression of the gene encoding a DNA replication factor, the human proliferating cell nuclear antigen (PCNA). In a cotransfection assay, either the 12S or 13S product of the E1A gene can induce CAT expression from a PCNA promoter-CAT reporter gene fusion. The induction of this cellular gene by the E1A 12S product, more familiar as a transcriptional repressor, is clearly distinct from *trans*-activation by the E1A 13S product and appears to be related to the transforming properties of the E1A protein. Our objective is to understand how the 12S product activates transcription of this growth-regulated gene.

Last year, we reported that a site for the activating transcription factor (ATF), located approximately 50 nucleotides upstream of the transcription initiation site in the PCNA promoter, mediates a positive response to the E1A 12S and 13S products. Homology with an ATF site cannot be sufficient for an inducible response to 12S, however, because such sequences are quite common. The adenovirus E3 promoter, for example, also possesses a sequence that has homology with an ATF site about 55 nucleotides upstream of the transcription initiation site, and this promoter is repressed by the E1A 12S product. The conservation between humans and mice of the ATF site and of sequences further downstream in the PCNA promoter suggests that more extensive stretches of sequence may play a part in the response to E1A 12S. To address this possibility systematically, we constructed a series of substitution mutations throughout the minimal E1A-responsive PCNA promoter by linker scanning mutagenesis. Eight-base-pair linkers were substituted for wild-type sequences in the PCNA-CAT reporter gene fusion following bidirectional digestion with BAL-31. On average, each mutant contains six altered bases out of a maximum of eight. The resulting contiguous and overlapping mutant constructs were assayed for their response to E1A 12S. Transfection experiments in HeLa cells showed that the mutation of flanking sequences upstream of the ATF site decreases basal expression of the PCNA promoter and abolishes its response to E1A 12S. These results suggest that PCNA promoter sequences in the proximity of the ATF site contribute to the specificity of transcription factor binding to this site. Alteration of downstream sequences has little effect on basal or 12S-stimulated transcription in HeLa cells. We are presently determining if the same holds true in other cell types.

It has been reported that an ATF site may require a TATA element downstream for activity and that bound ATF may alter TFIID binding to the TATA motif. No correctly positioned A/T-rich segment is evident in the PCNA promoter, but sequence homology suggests that the transcription initiation site may be specified by an initiator motif located between nucleotides −10 and +10 (relative to the transcription start site at +1). Consistent with this idea, deletion of sequences from −1 to +60 results in ill-defined transcription initiation sites in vitro. This deletion causes no appreciable change in the response of the PCNA promoter to 12S or 13S, however, and several other mutations in the vicinity of the transcription initiation site also have no effect on the response to 12S or 13S. These observations argue that sequences near the initiation site may select the precise location of the transcriptional start but are not absolutely required for the E1A response in vivo, although the possibility that cryptic elements subserve the same function in the initiation site mutants has not yet been excluded.

To examine the ATF site requirement in the response of PCNA-CAT to E1A 12S, we replaced it with five copies of the Gal4 recognition motif. The resulting construct, G5PCNA-CAT, contains PCNA promoter sequences from −47 to +60 but lacks all upstream PCNA promoter sequences. Like promoters that contain a correctly positioned TATA motif, G5PCNA-CAT can be activated by a variety of transcriptional activation domains (Sp1, E1A 13S, and VP16) supplied as Gal4-fusion proteins. However, G5PCNA-CAT differs from TATA-containing promoters (such as G5E1B-CAT) in its response to these chimeric activators when E1A 12S is coexpressed. In the absence of fusion proteins that recognize the Gal4-binding sites, G5PCNA-CAT is unresponsive to E1A 12S. Coexpression of Gal4-Sp1, a fusion protein that can bind the Gal4 sites and weakly activates transcription via the glutamine-rich transcriptional activation domain of Sp1, produces a positive response to E1A 12S by G5PCNA-CAT. Thus, it seems that bound Gal4-Sp1 can serve the same function in the response of G5PCNA-CAT to E1A 12S as the ATF motif binding protein serves in the wild-type PCNA-CAT promoter. Coexpression of E1A 12S with either of the two strong transcriptional activators Gal4-VP16 or Gal4-E1A, however, re-

duces expression from G5PCNA-CAT. In contrast, with the TATA-containing construct G5E1B-CAT, E1A 12S produces similar effects upon transcriptional activation by these three chimeric transcriptional activators, but the magnitude of the effect is greatly reduced.

These experiments indicate that the PCNA basal promoter is configured in a manner that permits the response of some transcriptional activators to be modulated by E1A 12S, whereas the TATA-containing E1B promoter remains less responsive to the effects of E1A 12S. Supporting this conclusion are experiments with a Gal4-Rb fusion protein that can bind either the E1A 12S or 13S proteins to DNA containing the Gal4 recognition site. Gal4-Rb can cooperate effectively with either E1A 12S or 13S to activate G5PCNA-CAT, whereas only E1A 13S can cooperate with Gal4-Rb to activate G5E1B-CAT. Thus, E1A 12S can activate transcription when positioned at the PCNA promoter but does so only weakly at the E1B promoter. Our current hypothesis is that the E1A 12S response of the PCNA promoter relates to the recruitment of TFIID to this TATA-minus promoter.

Many of the activities of the E1A 12S gene product are due to its interactions with cellular proteins, some of which are known to be concerned with cell cycle regulation. The regions of the 12S product that bind to these cellular proteins have been defined, and in several cases, the cellular proteins can be distinguished by their E1A-binding specificity. To shed further light on the mechanism of PCNA regulation, we are mapping the regions of the 12S protein that are required for induction of PCNA-CAT expression in HeLa cells. A variety of E1A deletion and point mutants were assayed for their ability to stimulate expression of PCNA-CAT in HeLa cells. *Trans*-activation was most severely affected by deletion of amino acids 2–60, including the amino-terminal nonconserved region of E1A as well as conserved region 1 (CR1). A point mutant in CR2 (pm928) that is defective for binding the retinoblastoma susceptibility gene product (Rb) stimulated expression of the PCNA promoter as efficiently as wild-type 12S E1A. These results suggest that PCNA induction in HeLa cells may correlate with p300 binding and probably is independent of Rb, despite the fact that the PCNA promoter contains a potential Rb control element and is repressed by Rb expression. Additional mutants are being tested to confirm and refine these conclusions.

We went on to study the regulation of PCNA promoter activity in human and rodent cells that display a greater degree of growth regulation than HeLa cells do. Surprisingly, in these cells, the full-length PCNA promoter is repressed by cotransfection of CMV 12S. The repression activity maps to the amino-terminal 60 amino acids of the E1A protein, again correlating with the binding of p300 and colocalizing with enhancer repression mediated by 12S. The significance of the repression is uncertain and is the subject of active investigation. As a first step, we have determined that the promoter element responsible for repression is located between nucleotides −234 and −213 relative to the transcriptional start site of PCNA mRNA transcription.

Regulation of HIV Gene Expression

M. Kessler, S. Gunnery, B. Hofmann, M.B. Mathews

The HIV-1 Tat protein is a potent *trans*-activator of HIV gene expression, capable of greatly stimulating transcription and also implicated in regulating translation. Tat acts through the *trans*-activator response element (TAR) situated in the R region of the long terminal repeat (LTR) between residues +1 and +59 relative to the transcription start site. (Residues +14 to +44 are required for minimal activity.) TAR functions at the RNA level in a position- and orientation-dependent manner. RNA encompassing the TAR region folds into a stem-and-loop structure of which three parts are required for *trans*-activation: an intact stem, a three-nucleotide pyrimidine-rich bulge at +23 to +25 important for Tat binding, and loop sequences (+30 to +35) that can bind cellular proteins (e.g., p68, TRP185). TAR is dispensable if Tat is brought to the promoter by alternative means, suggesting that TAR functions, at least in part, to provide a suitable Tat binding site.

As described in previous Annual Reports, by comparing transcription complex distributions on HIV-promoted templates, we found that Tat increases both the initiation rate and the efficiency of elongation. The degree to which Tat stimulates initiation is determined by the basal rate of LTR-directed transcription: Tat stimulates initiation severalfold when the basal rate of transcription is low but has little or no effect on initiation when the basal transcription

rate is high. Interestingly, when plasmids containing an SV40 replication origin are transfected into COS cells (monkey cells that express SV40 T antigen and permit plasmid amplification as a result of DNA replication), the basal transcription rate from the HIV promoter varies depending on the position of the origin region relative to the promoter. As a consequence, the degree of stimulation by Tat of the initiation rate also varies. When the origin region is situated immediately upstream of the HIV promoter, the basal transcription rate is relatively low and Tat acts predominantly to stimulate initiation. Alternatively, when the origin is situated approximately 2 kb downstream from the promoter, basal transcription is severalfold higher and Tat primarily stimulates elongation.

The influence of the origin on HIV-directed transcription does not appear to be due to differences in plasmid copy number related to the position of the origin. Furthermore, in CV-1 cells (monkey cells lacking T antigen), the promoter's basal transcription rate is unaffected by the SV40 origin. However, the position effect returns when T antigen is transiently expressed, indicating the involvement of T antigen in its induction. Moreover, the effect is not observed in COS cells treated with hydroxyurea, an inhibitor of DNA replication, or when the origin is rendered nonfunctional by mutating T-antigen-binding site II. Taken together, these results suggest that the position effect is due to T antigen functioning as a DNA replication factor and not as a viral *trans*-activator. On the basis of the comparison of reporter gene expression in the presence and absence of DNA replication, it appears that the origin region is acting to enhance basal expression when situated distal to the HIV promoter, rather than to suppress basal expression when situated adjacent to the promoter. Since virus replication is dependent on the proliferation of the host T cell, this mechanism of activation may play an important role in the transition from viral latency to active infection when resting T cells are induced to proliferate.

Tat enhances transcriptional elongation by increasing the processivity of complexes initiated at the HIV promoter. In COS cells, the majority of the complexes on HIV-promoted templates accumulate within the 83-nucleotide promoter-proximal region when transcription is carried out in the absence of Tat. Last year, we reported that when the distribution of these complexes is mapped directly by sizing the RNA that is pulse-labeled in nuclear run-on reactions, several transcripts of 60–80 nucleotides are detected. The accumulation of these transcripts indicates that the complexes are stopping in the promoter-proximal regions as the result of an inherent inability to elongate efficiently and not because of a specific elongation block. In the presence of Tat, the same short transcripts are detected, as well as long RNA, suggesting that Tat acts at least in part to overcome this inability to elongate efficiently.

When pulse-labeling run-on transcription is followed by 30 minutes of "chase" transcription in the presence of a high concentration of the limiting nucleotide, the short transcripts elongate, implying that their accumulation in vitro is due to pausing rather than termination events. During chase transcription, a new short transcript accumulates. This RNA is observed when α-amanitin is added at the beginning of the chase reaction but not at the beginning of the pulse-labeling, suggesting that it is produced from longer RNAs that have undergone processing. Direct analysis of RNA labeled in vivo with ^{32}P-phosphate revealed that short RNAs of 57–66 nucleotides accumulate. In contrast, RNase protection analysis indicates that the short RNAs that accumulate in vivo are predominantly smaller and less heterogeneous (55–60 nucleotides). On the basis of these and other results, we propose that short transcript formation is a multistep process. Transcription complexes tend to pause at several sites and (perhaps because they are stalled) to terminate before reaching the end of the transcription unit. Such attenuated transcripts, generated by the premature termination of transcription at heterogeneous sites, undergo processing to generate RNAs terminating just beyond the 3′ base of the TAR stem-and-loop structure. These RNAs are then processed further to generate the 50–55-nucleotide species that are detected in RNase protection assays.

We reported previously that TAR RNA (nucleotides 1–83) can inhibit the activation of DAI in vitro. As described above, DAI is a protein kinase that forms part of the host's antiviral defenses, and several viruses have devised countermeasures to defeat this cellular defense mechanism. The TAR sequence is found at the 5′ end of all full-length HIV mRNAs as well as in the form of short RNAs, raising the possibility that it plays a role in translational control and viral pathogenesis. The inhibition of DAI by TAR RNA was initially observed in kinase assays conducted in vitro using TAR RNA made by transcribing the HIV-LTR with T7 RNA polymerase. The transcript was purified through two gels to rid it

of any dsRNA contaminants. This form of TAR RNA contains a short stem of 4 bp and a loop of eight nucleotides, in addition to the main 29-bp-long stem and loop.

We have now confirmed the ability of TAR RNA to inhibit ribosome-associated DAI in a translation system from rabbit reticulocytes. Activation of the enzyme by incubation with dsRNA leads to an inhibition of in vitro translation which is reversed by both TAR RNA and VA RNA, presumably by blocking the activation of DAI. To extend this work, we have tested several TAR RNA mutants for their ability to reverse protein synthesis inhibition. The mutations were made at different points in the main stem-and-loop structure, and nuclease sensitivity analysis was performed to determine the structure of each mutant RNA. In general, mutants in which the stem structure is disrupted lost their translation rescue ability, consistent with observations that the stem of the TAR RNA structure is crucial for DAI binding. Mutants in which the loop is shortened or deleted exhibit reduced activity, possibly because these mutations created structures with shorter stems. Unexpectedly, TAR RNA truncated at +68 is inactive even though nuclease sensitivity analysis indicated that the RNA has the same first stem-and-loop structure as the wild-type TAR RNA. This finding suggests that although the stem is essential, it may not be sufficient for the DAI inhibition activity. To study the action of TAR RNA in vivo, we have placed it under the control of heterologous promoters and are currently examining its expression in transfected cells.

Mechanism of *Trans*-activation by the HIV-1 Tat Protein

M.F. Laspia, P. Wendel

Human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS), exhibits a novel genomic organization. In addition to the structural genes *gag*, *pol*, and *env* that are common to all retroviruses, HIV also has at least two essential regulatory genes. One of these genes, *tat*, encodes a *trans*-activator protein that greatly increases the expression of genes linked to the long terminal repeat (LTR) of the virus. *Trans*-activation is mediated by the binding of Tat to an RNA secondary structure, called TAR, that is present in the 5′ end of all HIV mRNAs. The molecular mechanism of *trans*-activation of HIV gene expression by Tat has been controversial as regulation has been proposed to occur at several different levels. The principal mode of regulation, however, appears to be transcriptional. During the past several years, our research has focused on elucidation of the mechanism of stimulation of HIV-1 transcription by Tat. To do this, we have analyzed *trans*-activation in vivo, in a recombinant adenovirus model system and, more recently in vitro, in a cell-free transcription system with purified Tat protein.

On the basis of findings in vivo, which are summarized in previous Annual Reports, we have proposed a model for *trans*-activation by Tat. In the absence of Tat, the level of HIV-directed transcriptional initiation is low, and transcriptional elongation is unstable such that the number of transcriptional complexes declines with increasing distance from the promoter (polarity). Tat interacts with TAR to stimulate HIV transcription bimodally by increasing transcriptional initiation and stabilizing transcriptional elongation. The combination of these two effects of Tat leads to large stimulation in HIV LTR-directed transcription rates. We have also studied the basis of the synergistic stimulation of HIV gene expression produced by Tat and general *trans*-activators. Such a synergistic interaction could be important in the transition from low levels of gene expression, early in infection or during viral latency, to high levels of gene expression in the productive stages of infection. Interestingly, synergy does not result from increased rates of transcription; rather, synergy is due principally to increased rates of transcriptional elongation.

At the time of last year's Annual Report, we had begun to study *trans*-activation by Tat in a cell-free system. A cell-free approach should permit analysis of the mechanism of *trans*-activation in detail and should enable us to dissect the interaction between Tat and TAR and the cellular transcriptional apparatus. As discussed previously, Tat protein was overexpressed in bacteria and purified to greater than 90% homogeneity by C18 reversed-phase high-pressure liquid chromatography. Purified Tat was biologically active in vivo since it stimulated LTR-directed transcription levels when introduced into HeLa cells by the technique of scrape-loading. In a cell-free transcription system consisting of an HIV promoter DNA template and a nuclear extract prepared from HeLa cells, Tat stimulated HIV-directed transcription of a 745-nucleotide runoff

RNA 12-fold. In addition, there was a class of short Tat-responsive HIV RNAs approximately 83 nucleotides in length. Synthesis of the short RNA was decreased about threefold in the presence of Tat and is presumed to represent prematurely terminated, possibly processed RNA generated in the in vitro transcription system. *Trans*-activation by Tat in vitro was specific since Tat was unable to stimulate transcription from either an HIV TAR mutant promoter or the adenovirus major late promoter. In addition, *trans*-activation required TAR RNA since it was inhibited by synthetic competitor TAR RNA.

To characterize *trans*-activation in the cell-free system, we have investigated the basis of the stimulation in runoff transcription produced by Tat. To do this, HIV transcription was analyzed by RNase protection assay with antisense riboprobes that distinguish between promoter proximal and promoter distal effects of Tat. Tat differentially increased promoter distal RNA levels, suggesting that it acts to stimulate the efficiency of transcriptional elongation. To further examine *trans*-activation by Tat in vitro, HIV transcription rates were analyzed directly. In these experiments, short single-stranded DNA probes were used to measure the distribution of elongating transcriptional complexes along the HIV-directed DNA template. In the absence of Tat, polarity caused less than 2% of the initiating RNA polymerases to transcribe beyond +630 nucleotides. Thus, HIV transcription in vitro is also subject to polarity. Tat did not increase the number of promoter proximal RNA polymerases significantly, but it did suppress polarity, resulting in a 15-fold increase in the number of promoter distal transcription complexes. These data suggest that Tat does not increase transcriptional initiation in vitro; rather, *trans*-activation is due principally to increased transcriptional elongation. Among the possible explanations for the lack of an initiation effect is that high basal levels of HIV promoter activity in vitro might reduce the stimulation of initiation by Tat. Alternatively, low levels of reinitiation in the in vitro transcription system might bias against

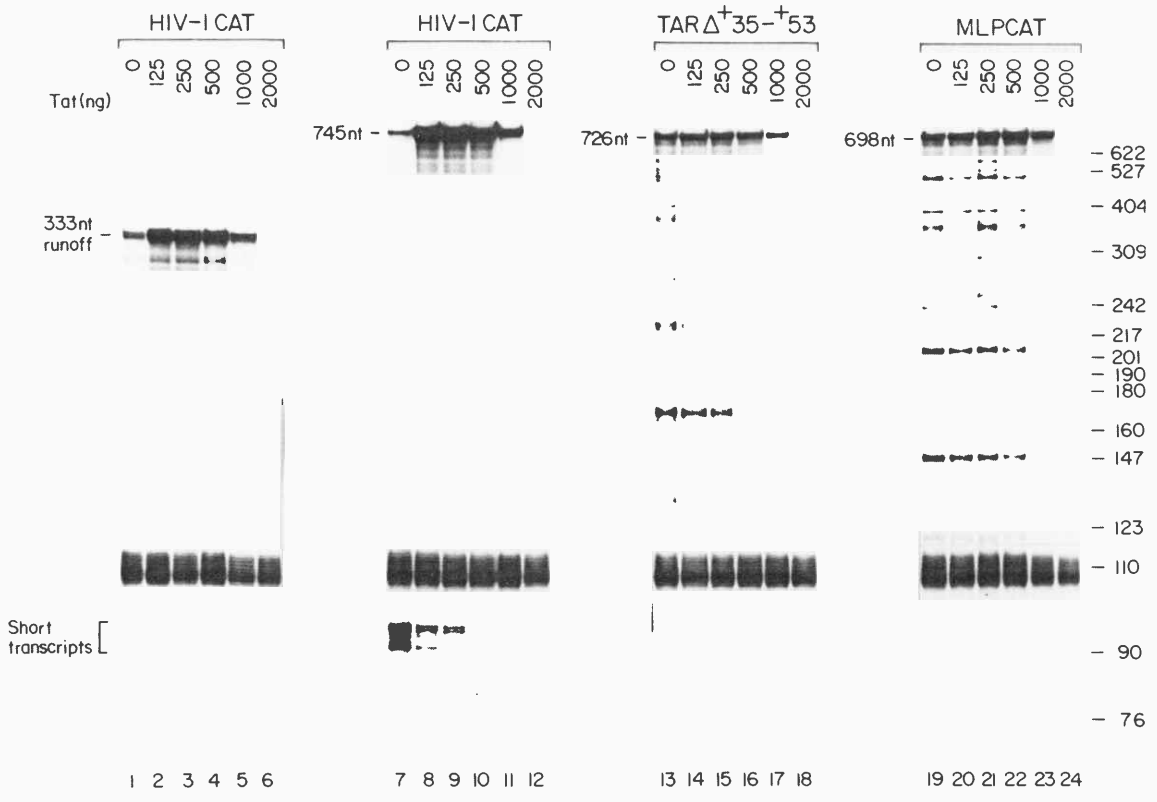

FIGURE 1 Tat stimulates HIV-directed transcription in a cell-free system. Transcription reactions contained a wild-type HIV DNA template (lanes *1–12*), a TAR mutant template (lanes *13–18*), or an adenovirus major late promoter template (lanes *19–24*), and a HeLa cell nuclear extract with the indicated amounts of Tat. On the left are the positions of the correctly initiated runoff RNA transcripts as well as the short, prematurely terminated HIV RNA.

detecting an effect on initiation. We are examining both of these possibilities and are attempting optimize conditions that favor stimulation of initiation in vitro.

To explore the basis of the inefficient elongation by HIV-directed complexes, the effects of the detergent sarkosyl on HIV transcription in vitro have been analyzed. Interestingly, the addition of sarkosyl to transcription reactions produced a 15-fold increase in LTR-directed transcription in the absence of Tat. This indicates that sarkosyl is able to suppress the requirement for Tat in order to achieve high levels of transcription. Furthermore, it may imply that low levels of HIV transcription are due to a repressor of transcription present in the HeLa cell nuclear extracts. Interestingly, low basal levels of HIV transcription are primarily due to repression of elongation, since sarkosyl increased the efficiency of elongation without similarly increasing initiation. In addition, repression caused pausing and termination of transcription by RNA polymerase complexes. We believe that these data suggest that Tat stabilizes transcriptional elongation by acting as an antirepressor, which when bound to TAR RNA enables transcriptional complexes to overcome repression of elongation.

Preliminary results suggest that repression is also exhibited by other eukaryotic genes. We have also recently observed that, in vitro, Tat is capable of stimulating expression from a heterologous RNA polymerase II transcription unit containing the TAR element cloned downstream from the promoter. These data suggest that repression of elongation may be a general phenomenon and intimate that Tat operates to stimulate transcription by a general mechanism. Elucidation of the basis of repression and the mechanism by which Tat increases efficient elongation is likely to provide important insights into regulation at the level of transcriptional elongation. Our efforts in the future will be directed toward identification of the activity(s) in HeLa cell nuclear extracts responsible for repression of elongation. In addition, we will attempt to characterize HIV-1 transcription in vitro using fractionated nuclear extracts and to reconstitute transcriptional *trans*-activation with purified components.

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RNA SPLICING

A.R. Krainer E. Birney, J. Cáceres, B. Dong, G. Joshi-Topé, D. Kozak, A. Mayeda, M. Wallace, K. Otto

BIOCHEMISTRY OF MAMMALIAN PRE-mRNA SPLICING AND SPLICE SITE SELECTION

RNA splicing is a required step in the expression of most eukaryotic protein-coding genes. In addition, alternative pre-mRNA splicing is a widespread mechanism for generating structurally and functionally distinct protein isoforms from single genes, often in a tissue-specific, developmentally regulated, or physiologically controlled manner. Our laboratory is studying the mechanisms of splice site selection and RNA cleavage/ligation during mammalian pre-mRNA splicing. To this end, we are purifying and characterizing several of the nucleoprotein and protein factors that are necessary for the catalysis of splicing in vitro. In addition, we are purifying and characterizing protein factors that modulate the selection of alternative splice sites in vitro. These experiments should help elucidate the contribution of RNA and protein catalysis to pre-mRNA cleavage/ligation and provide insights into the molecular basis of the specificity of splice site selection.

STRUCTURE AND FUNCTION OF HUMAN SPLICING FACTOR SF2

We previously purified from HeLa cells a protein factor required for pre-mRNA splicing in vitro and showed that the same factor, called SF2, could modulate alternative 5′ splice site selection in vitro in a concentration-dependent manner. This effect is not substrate-specific but occurs with a conserved polarity, i.e., proximal sites are activated at high SF2 concentrations. We isolated full-length HeLa SF2 cDNAs and were able to show that a single 28-kD polypeptide expressed in *Escherichia coli* was active and sufficient for both general splicing and splice site selection activities. We have also shown that SF2 is required for the initial steps of spliceosome assembly and that it can accelerate the annealing of complementary RNAs. SF2 binds RNA in a sequence-independent manner, but sequence preferences can be observed, which are currently under detailed investigation by E. Birney and A. Mayeda. SF2 is identical to ASF, a factor isolated independently in J. Manley's laboratory (Columbia University), which can modulate alternative splicing of the SV40 tumor antigens.

The carboxyl terminus of SF2 (Fig. 1) contains clusters of alternating arginine and serine residues (RS domain) similar to regions in the *Drosophila* proteins *tra*, *tra2*, and Suppressor of white apricot, all of which are involved in the regulation of specific alternative splicing pathways. A similar region is present in the U1 small nuclear ribonucleoprotein (snRNP)-specific 70K protein from several species. P. Bingham's laboratory (SUNY, Stony Brook) has shown that one the functions of RS domains is to serve as a signal for localization of proteins within the speckled region of the nucleoplasm, which is thought to be a site of RNA processing and/or storage of splicing factors (see D. Spector in Molecular Genetics of Eukaryotic Cells). The amino terminus of SF2 comprises a region that has strong homology with an 80-amino-acid RNA recognition motif (RRM), including the two internal RNP1 and RNP2 consensus elements, which is common to a large family of RNA-binding proteins. These include several heterogeneous nuclear ribonucleoprotein (hnRNP) and snRNP proteins, the *Drosophila* alternative splicing regulators *sxl* and *tra2*, and many other prokaryotic and eukaryotic proteins. These two domains of SF2 are separated by a putative hinge region consisting of multiple consecutive glycines. An additional octapeptide motif of unknown function, EFEDPRDA, is shared by SF2 and U1 70K polypeptides.

J. Cáceres is improving the expression of SF2 in *E. coli* and has generated a number of mutants in order to examine the roles of conserved SF2 structural domains in splicing, splice site selection, RNA binding, and RNA annealing by biochemical analysis of the respective recombinant proteins. In addition, wild-type and mutant SF2 are being tested in co-transfection experiments with several alternatively spliced genes to determine whether transient overexpression of SF2 can modulate alternative splicing in mammalian cells and to allow an in vivo analysis

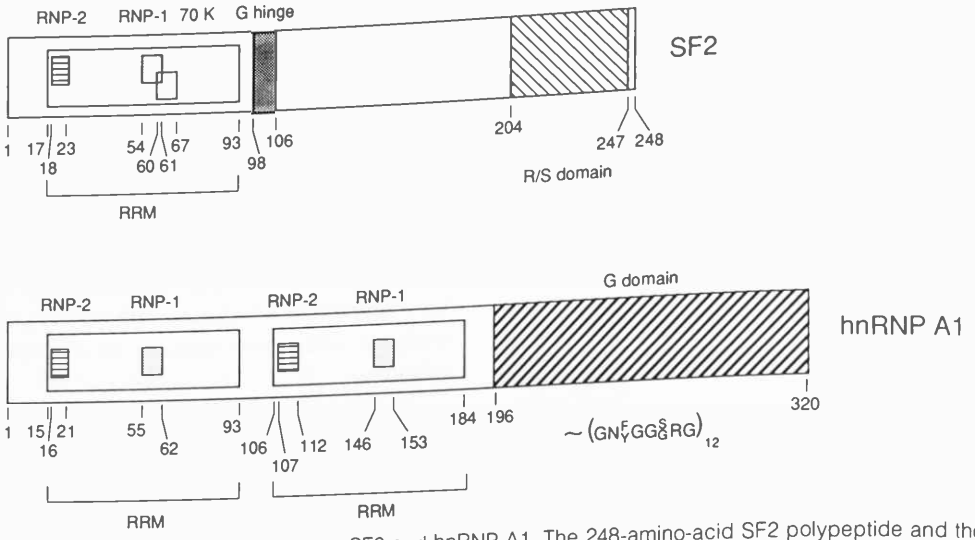

FIGURE 1 Domain structure of human SF2 and hnRNP A1. The 248-amino-acid SF2 polypeptide and the 320-amino-acid hnRNP A1 polypeptide are drawn to scale. The RNA recognition motifs (RRM), glycine hinge, glycine-rich domain, and arginine plus serine-rich domain are indicated. Within the RRMs, the smaller boxes denote the RNP1 and RNP2 consensus elements, and an eight-amino-acid identity to a region in the U1 snRNP specific 70K polypeptide.

of SF2 structure and function. To facilitate the analysis of SF2 function and in vivo expression, D. Kozak is in the process of generating monoclonal antibodies to this protein, in collaboration with C. Bautista and M. Falkowski, of the Monoclonal Antibody Facility.

A phylogenetic analysis of SF2 structure and function was undertaken by A. Mayeda in collaboration with A. Zahler and M. Roth (Fred Hutchinson Cancer Research Center, Seattle). This was made possible by the previous structural and immunological characterization in the Roth laboratory of a conserved family of nuclear phosphoproteins associated with active sites of RNA polymerase II transcription and with amphibian B-snurposomes. This family

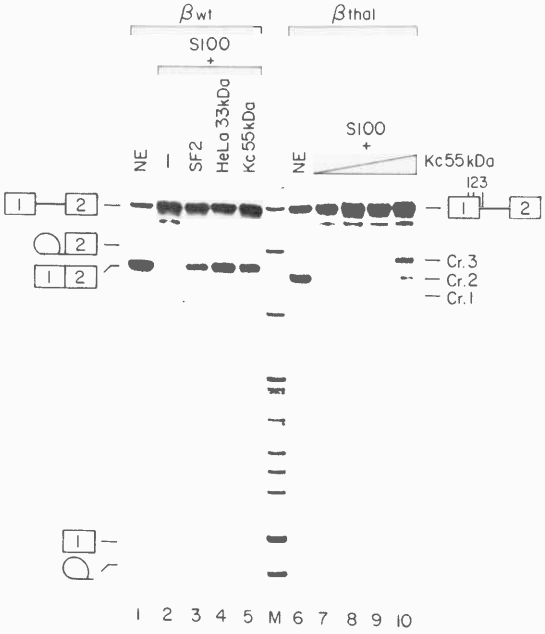

FIGURE 2 *Drosophila* SRp55 can replace human SF2 in a human cell-free system. Wild-type (lanes *1–5*) or β-thalassemic (lanes *6–10*) pre-mRNAs containing the first two exons and first intron of human β-globin were spliced in vitro with the indicated extracts and fractions in 25-μl reactions containing 15 μl of extract and splicing extract buffer. HeLa SF2 was obtained by our previously published chromatographic procedures. HeLa or Kc extracts were fractionated by ammonium sulfate and magnesium chloride precipitation, and 33-kD (human SRp33) and 55-kD (*Drosophila* SRp55) polypeptides were isolated from the respective extracts by preparative SDS-PAGE. The purified proteins were denatured in guanidinium hydrochloride and renatured. (Lane *1*) 8 μl HeLa nuclear extract; (lane *2*) 7 μl S100; (lane *3*) 7 μl S100 plus 8 μl chromatographically purified HeLa SF2; (lane *4*) 7 μl S100 plus 8 μl gel-purified human SRp33; (lane *5*) 7 μl S100 plus 8 μl gel-purified *Drosophila* SRp55; (lane M) *Hpa*II-digested pBR322 molecular weight markers; (lane *6*) HeLa nuclear extract; (lanes *7–10*) 7 μl S100 plus 0, 2, 4, or 8 μl of gel-purified *Drosophila* SRp55, respectively.

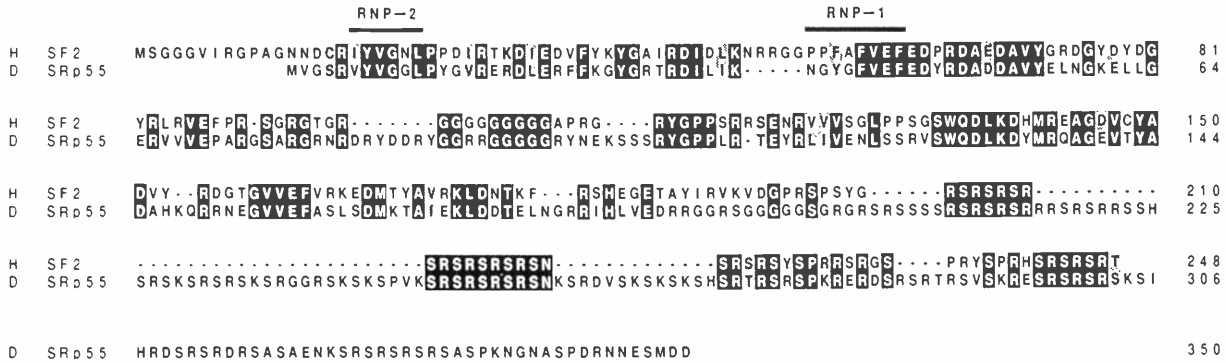

FIGURE 3 Primary sequence homology between human SF2 and *Drosophila* SRp55. The complete amino acid sequences of human SF2 (H. SF2) and *Drosophila* SRp55 (D. SRp55) are shown in the single-letter code. Amino acid identities are indicated in reverse type, amino acid similarities are shown in stippled boxes, and dashes denote gaps introduced to maximize homology. Amino acid positions in each protein are shown at the right. The conserved RNP-1 and RNP-2 elements of the RNA recognition motif (RRM) are indicated by solid bars. The alignment shown preserves the RRM homology and minimizes the number of gaps (nine in H. SF2, two in D. SRp55).

comprises proteins with approximate molecular masses of 20, 30, 40, 55, and 75 kD in the somatic tissues of a variety of animal cells. Remarkably, as shown in the Roth laboratory, the entire family of proteins can be purified from an ammonium sulfate cut by precipitation with millimolar concentrations of magnesium. Our recent work in collaboration with the Roth laboratory demonstrated that SF2 is a member of the same phosphoprotein family and that a different member of this family from *Drosophila*, SRp55, can replace human SF2 in the human in vitro system (Fig. 2). *Drosophila* SRp55, cloned in the Roth laboratory, is a 39-kD protein that is 50% homologous to human SF2 (Fig. 3).

It appears that many other, perhaps all, members of the family have several conserved structural features and similar functional properties (M. Roth, pers. comm.). Another example is the human splicing factor SC-35, which shares 25% homology with SF2 and has similar functional properties in vitro (in collaboration with X.-D. Fu and T. Maniatis, Harvard University; M. Roth, pers. comm.). The family members so far cloned, which include human SF2 (ASF), human SC-35, *Drosophila* SRp55 (as well as a closely related variant, B52), mouse X16 (an 18-kD tissue-specifically expressed protein of unknown function), and *Drosophila* rbp1 (a 15-kD protein of unknown function; B. Baker, pers. comm.), share all of the conserved motifs described above. Further work will be necessary to understand the significance of this apparent functional redundancy, as well as to establish whether each member of the family has unique functional properties in vivo.

hnRNP A1 AS A REGULATOR OF ALTERNATIVE SPLICING

A. Mayeda recently identified an activity in HeLa cell nuclear extracts, termed SF5, that specifically counteracts the effects of SF2 on splice site selection. SF5 is not a general inhibitor of SF2, because the latter activity is essential for splicing, and a large excess of SF5 still results in efficient splicing. However, the ratios of these two activities precisely determine which splice site is used in vitro. Thus, high levels of SF5 result in utilization of distal 5′ splice sites, whereas high levels of SF2 favor use of proximal 5′ splice sites. Like SF2, this activity does not operate in a substrate-specific manner, but rather, it has a general effect on the polarity of 5′ splice site selection.

A functional assay was designed for the purification of this activity, based on the switch toward distal 5′ splice sites. Purification of SF5 (Fig. 4) led to the demonstration that this factor is identical to hnRNP A1, one of the abundant core hnRNP proteins that are thought to package hnRNA into ribonucleosomes. This was confirmed by overproducing cloned hnRNP A1 in *E. coli* and by demonstrating that the soluble recombinant product was fully functional in stimulating distal 5′ splice sites with a variety of alternatively spliced pre-mRNAs. For example, increasing the concentration of hnRNP A1 causes a partial switch in

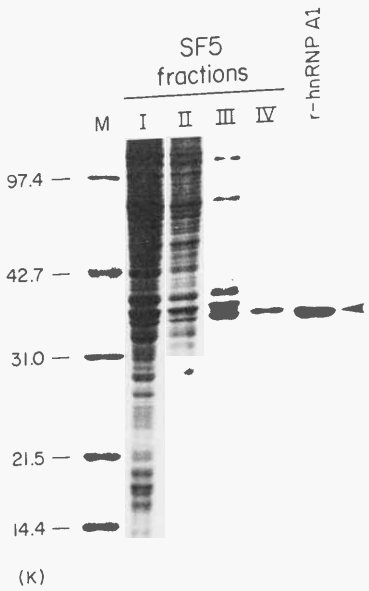

FIGURE 4 Purification of HeLa cell SF5. SF5 active fractions were analyzed by SDS-PAGE and Coomassie blue staining. (M) Molecular weight markers (M_r shown in kilodaltons); (I) crude HeLa cell nuclear extract (67 μg); (II) ammonium sulfate fraction (22 μg); (III) CsCl density gradient fraction (12 μg); (IV) phenyl-Superose fraction (1.7 μg). Recombinant rat hnRNP A1 (4 μg; a gift from S. Wilson) is also shown. The arrowhead indicates the M_r of the active p34 polypeptide.

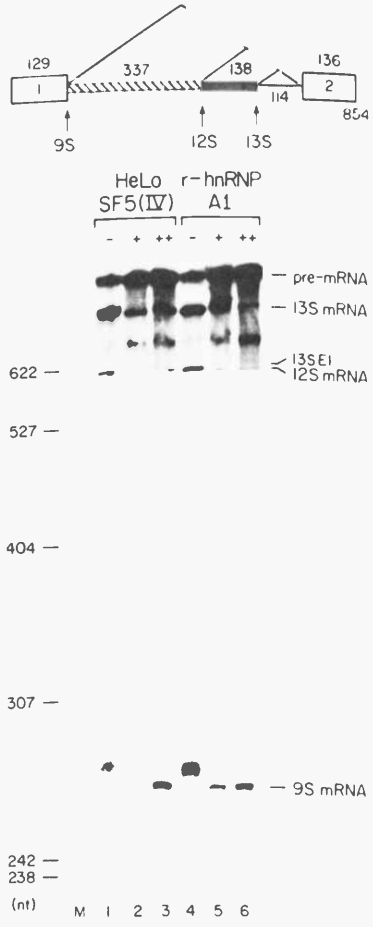

FIGURE 5 Effect of HeLa cell and recombinant human hnRNP A1 on alternative splicing of adenovirus E1A pre-mRNA. The structure of the adenovirus pre-mRNA is shown schematically (sizes in nucleotides). Constant amounts of S100 extract (6 μl) and partially purified SF2 (3 μl) and variable amounts ([-] none; [+] 3 μl; [++] 6 μl) of purified HeLa cell hnRNP A1 (SF5 fraction IV, concentrated to 474 μg/ml by ultrafiltration) or recombinant human hnRNP A1 (431 μg/ml) were used. (M) ^{32}P-labeled pBR322/*Hpa*II DNA markers (sizes shown in nucleotides). Spliced products derived from use of 13S, 12S, and 9S 5′ splice sites are indicated. A longer exposure of the bottom of the gel is shown to facilitate simultaneous visualization of the short 9S mRNA and the longer 13S and 12S mRNAs.

the splicing of adenovirus E1A pre-mRNA in vitro, such that there is a decrease in use of the proximal 12S and 13S sites and an increase in use of the 9S site (Fig. 5). A comparable switch occurs in the early to late transition of an adenovirus infection.

We have proposed that the intracellular ratios of SF2 and hnRNP A1 may control the specificity of splice site selection. Furthermore, in vivo regulation of one or both of these activities may play an important role in the tissue-specific or developmental regulation of alternative splicing. Interestingly, the levels of hnRNP A1 were previously reported to be increased in proliferating cells, and on the basis of our recent findings, such changes may explain the alterations in the alternative splicing patterns of many pre-mRNAs that have been observed in transformed cells. Current experiments are aimed at determining whether the in vivo ratio of the levels or activities of SF2 and hnRNP A1 varies among different cell lines, e.g., in response to transformation. These studies of SF2 and hnRNP A1 must take into account the existence of complex gene families, as well as isoforms generated by alternative splicing and posttranslational modification. Interestingly, a previously documented but unknown posttranslational modification of hnRNP A1 appears to eliminate activity in our assay. We have shown that SF2 is multiply phosphorylated but do not know at present the consequence of this modification on activity. In collaboration with R. Kobayashi and D. Marshak (see this Section), we are attempting to map the phosphorylation sites on SF2.

Previous work on hnRNP A1 has shown that it binds single-stranded nucleic acids in a sequence-independent fashion and in a cooperative manner due

to protein-protein interactions between carboxy-terminal domains. However, in crude extracts, distinct binding preferences for 3′ splice sites, as well as changes in the ability to cross-link this protein to pre-mRNA depending on the integrity of snRNAs, have been shown in other laboratories. To understand the mechanisms by which hnRNP A1 antagonizes SF2, such that the ratio of the two proteins can determine the outcome of alternative 5′ splice site selection in vitro, E. Birney and A. Mayeda are studying the RNA-binding properties of hnRNP A1 and SF2 as purified proteins and in the presence of other factors.

ALTERNATIVE 3′ SPLICE SITE SELECTION AND EXON SKIPPING

Interestingly, even high levels of SF2 result in proper discrimination between authentic and cryptic 5′ splice sites. These properties are consistent with a role for SF2 in ensuring the specificity of splice site selection during constitutive splicing in vivo by helping to avoid aberrant exon skipping. Furthermore, SF2 may play a role in the tissue-specific or developmental regulation of certain alternatively spliced genes. In collaboration with D. Helfman (Molecular Genetics of Eukaryotic Cells Section), SF2 was shown to be able to modulate the pattern of splicing of a natural alternatively spliced pre-mRNA in vitro. High levels of SF2 led to the preferential utilization of the proximal skeletal-muscle-specific tropomyosin 5′ splice site, at the expense of the fibroblast-specific 5′ splice site. Thus, high levels of SF2 can overcome tissue-specific differences between 5′ splice sites, at least in some cases. In addition, these studies showed that SF2 can prevent aberrant exon skipping in vitro.

Using model substrates containing alternative 3′ splice sites, A. Mayeda has identified and partially purified activities that stimulate proximal (SF6) or distal (SF7) 3′ splice sites. Further purification and characterization of these activities is in progress. These factors will be used together with SF2 and hnRNP A1 to investigate the molecular mechanisms of splice site selection in vitro. So far, A. Mayeda has shown that the ratio of SF2 to hnRNP A1 is sufficient to determine whether exon skipping or exon inclusion takes place in vitro. In collaboration with D. Helfman, we are investigating this phenomenon further as it relates to natural alternative splicing and to the specificity of splice site selection in constitutively spliced pre-mRNAs.

OTHER ESSENTIAL PROTEIN SPLICING FACTORS

We are continuing to purify and characterize additional protein factors that are essential for one or both RNA cleavage-ligation reactions. G. Joshi-Topé is pursuing the general strategy of selective depletion or inactivation to isolate a novel activity required for cleavage at the 5′ splice site and lariat formation. B. Dong is using immunological and genetic approaches to identify and attempt to isolate cDNA homologs of the *Saccharomyces cerevisiae* PRP18 splicing factor, which is required for 3′ splice site cleavage and exon ligation (in collaboration with D. Horowitz and J. Abelson, California Institute of Technology).

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TRANSCRIPTIONAL REGULATION

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The regulation of transcription in multicellular organisms is more complex than anticipated from paradigms first established in bacteria. First, *cis*-regulatory sequences are rarely confined to the sequences neighboring the transcriptional start site; instead, distal enhancers and silencers can profoundly affect the levels of transcription. Second, enhancers represent complex arrangements of subelements or enhansons, which cooperate with one another to regulate transcription. Third, there exist large numbers of sequence-specific and basal transcription factors. With the advent of cloned cDNAs encoding many of these regulatory factors, the field is now in a position to uncover how these proteins, assembled into promoter complexes, interact with one another to regulate transcription.

We use three primate viruses to probe the cellular transcriptional machinery: the simian DNA tumor virus SV40 and the human pathogens herpes simplex virus (HSV) and human immunodeficiency virus (HIV). Our earlier studies focused on the structure of the SV40 enhancer. SV40 relies entirely on the cellular transcriptional apparatus to activate transcription from its early promoter, and perhaps not surprisingly, its enhancer is an accurate reflection of the structure of cellular enhancers. We now study the structure and function of the cellular transcription factors Oct-1 and Oct-2, which bind to the SV40 enhancer. In contrast to SV40, HSV encodes its own immediate early promoter *trans*-activator, called VP16 or Vmw65, which it brings into the cell upon infection. Therefore, as our studies have progressed toward an understanding of the structure and function of the proteins that regulate transcription, we have increasingly used HSV as a model. Our current studies on *cis*-acting regulatory sequences involve an analysis of HIV promoter sequences in infected individuals. HIV provides an excellent model to study variation in promoter sequences in vivo, because, like other retroviruses, it mutates rapidly and because, unfortunately, there exist a large number of infected individuals.

* deceased

DIFFERENTIAL TRANSCRIPTIONAL REGULATION BY PROTEINS THAT BIND TO THE SAME DNA SEQUENCE

The promoter specificity of transcriptional activators is generally thought to be conferred by the sequence specificity of the DNA binding domain, which brings the activator to the appropriate promoter sequence. Many of our studies over the past several years have focused on the question of how the two activators Oct-1 and Oct-2, which recognize the same 8-bp "octamer" sequence, ATGCAAAT, can selectively activate different promoters. These studies have elucidated two mechanisms: First, promoter-selective activation domains can selectively activate transcription from different promoters even when tethered to the same DNA binding domain. Second, selective interactions with a second *trans*-activator, in this case the HSV activator VP16, can result in recruitment of one activator (Oct-1) but not the other (Oct-2) to a new *cis*-regulatory element.

These studies arose from a convergence several years ago of two separate lines of investigation in our laboratory. On the one hand, we were characterizing the structure and activity of subelements of the SV40 enhancer. These studies, done in collaboration with N. Hernandez and colleagues (Cold Spring Harbor Laboratory), showed that multimerized copies of the octamer motif can activate an unusual type of RNA polymerase II transcription unit exemplified by the U2 small nuclear RNA (snRNA) gene in both B cells and non-B cells, whereas a more typical mRNA transcription unit was active in B cells but not in HeLa cells, a non-B cell line. These different patterns of activity corresponded to the known ubiquitous and B-cell expression patterns of the two octamer motif-binding proteins Oct-1 and Oct-2, respectively, suggesting that Oct-1 preferentially activated RNA polymerase II snRNA promoters and Oct-2 activated mRNA promoters. These studies ultimately led to the discovery of promoter-selective activation domains.

On the other hand, we serendipitously purified and subsequently cloned cDNAs encoding Oct-1. The

deduced amino acid sequence of Oct-1 led to the discovery of a new class of homeodomain-containing DNA binding domains called the POU domain. The 160-amino-acid POU domain is bipartite, containing an unusual amino-terminal POU-specific region and a carboxy-terminal homeodomain that are tethered by a short nonconserved linker sequence. We were surprised to find in our early studies of Oct-1 that it could bind to two very different sites within the SV40 enhancer even though only one octamer consensus sequence had been recognized within the SV40 enhancer sequence. The second atypical Oct-1 binding site instead displayed similarity to the target of VP16 activation in the HSV immediate early (IE) promoters, called TAATGARAT. We subsequently showed that the TAATGARAT element, which is sometimes associated with an overlapping octamer motif, is by itself a bona fide, albeit low affinity, binding site for Oct-1.

After HSV infects a cell, the virion-associated VP16 enters the nucleus and forms a multiprotein-DNA complex on the TAATGARAT target site with two cellular factors, Oct-1 and a less well characterized factor we refer to as HCF. VP16 itself does not bind DNA effectively, but, by virtue of a very potent carboxy-terminal acidic transcriptional activation domain, once tethered to the HSV IE promoters by Oct-1 and HCF, VP16 is able to activate HSV IE transcription. R.G. Roeder and colleagues (Rockefeller University) showed that VP16 does not associate effectively with Oct-2, and our mapping studies revealed that VP16 selectively associates with Oct-1 by distinguishing among the seven amino acids that differ between the Oct-1 and Oct-2 homeodomains. This selective association between Oct-1 and VP16 can lead to differential transcriptional regulation by Oct-1 and Oct-2, because only Oct-1 is recruited by VP16 to the TAATGARAT element. As described below, many of our current studies continue to focus on how Oct-1 and Oct-2 activate transcription from different promoters.

The POU Domain Is an Integral DNA Binding Unit

R. Aurora

Since discovery of the POU domain, a central question has been how this unusual type of DNA binding domain recognizes DNA. Early studies showed that both the POU-specific region and the POU homeodomain are involved in DNA binding, but given the novelty of the POU-specific segment, it was not initially evident whether it might be involved in making sequence-specific contacts with DNA. Last year, we described studies with protein chimeras carrying different subsegments of the POU domain from either Oct-1 or the pituitary-specific POU protein Pit-1 (GHF-1), which differs in binding specificity. These studies showed that the POU-specific region as well as the nonconserved linker region can influence DNA binding specificity. Their influence on DNA sequence recognition, however, was dependent on the origin, either Oct-1 or Pit-1, of the other POU-domain segments.

This year we showed, using a DNA cross-linking assay and an engineered POU domain containing a protease cleavage site between the POU-specific and homeodomain segments, that the POU-specific region contacts DNA in the DNA-bound POU domain. To elucidate the three-dimensional structure of the POU domain, we have developed a protocol for high-level expression and purification of the Oct-1 POU domain from *Escherichia coli* and are using this material for NMR spectroscopy in collaboration with N. Assa-Munt and P. Wright (Scripps Research Clinic) and for X-ray crystallography in collaboration with J. Klemm and C. Pabo (Massachusetts Institute of Technology). Although these approaches have yet to bear fruit, we have already obtained structural information about the POU domain free in solution and bound to DNA by tryptophan fluorescence in collaboration with P. Hensley (SmithKline Beecham Pharmaceuticals) and J. Knutson (National Institutes of Health).

Fortuitously, the POU domain contains two tryptophan residues, one within the POU-specific region and the other within the POU homeodomain. Polarized fluorescence can be used to measure the rotational motion of macromolecules in solution, which is a reflection of their size, shape, and flexibility. If a fluorophore is excited by a polarized beam of light, only properly aligned molecules will be excited and their initial fluorescent emission will also be polarized. But if the fluorophore rotates between the time of excitation and fluorescent emission, then the amount of decay over time (in nanoseconds) of the fluorescence polarization (or fluorescence anisotropy) is a measure of the rotation of the particular fluorophore.

The two tryptophans within the POU domain represent two fluorophores whose respective fluores-

cent anisotropy can be measured. If the POU-specific region and POU homeodomain are not fixed with respect to one another in solution, then the anisotropy decay of the two tryptophans will probably differ. But if the POU domain segments are fixed with respect to one another in solution as an integral structure, then the anisotropy decay pattern of the two tryptophans should be identical. Analysis of the fluorescence anisotropy decay of the Oct-1 POU domain reveals a single exponential decay pattern, suggesting that the POU-specific and POU-homeodomain regions are indeed fixed with respect to one another in solution as a single structural unit. These results suggest that the POU domain is an integral DNA binding unit, which may explain why the POU-specific region and the POU-type of homeodomain have always been found associated with one another in nature.

Regulation of RNA Polymerase II and III Transcription from snRNA Promoters by Oct-1 and Oct-2

G. Das, M. Tanaka, K. Visvanathan

When we first obtained cDNAs encoding Oct-1 and a cDNA encoding Oct-2 from P. Sharp (Massachusetts Institute of Technology), we set out to determine how these two proteins might differentially activate mRNA and snRNA promoters. Determining how Oct-2 can preferentially activate an mRNA promoter was relatively easy because this protein is cell-specific, and therefore we only had to assay activation in cells lacking Oct-2. These results showed that Oct-2 is a more potent activator of mRNA promoters than Oct-1 because it contains two interdependent mRNA promoter activation domains, whereas Oct-1 only contains one corresponding domain. Assaying Oct-1 activation of snRNA promoters, however, was complicated by the fact that Oct-1 is expressed ubiquitously in mammalian cells and transient overexpression of Oct-1 had little effect on activation of the U2 snRNA promoter.

To circumvent the endogenous Oct-1 protein, we altered the DNA binding specificity of Oct-1 without grossly altering its structure. We took advantage of the different DNA binding specificities of the Pit-1 POU domain and created Oct-1 and Oct-2 derivatives in which the majority of the Oct POU domain had been replaced by the corresponding Pit-1 POU-domain sequences. We then used a Pit-1 *cis*-regulatory target from the prolactin promoter in place of the octamer motif in the model β-globin mRNA and U2 snRNA promoters. Figure 1 shows the activity of these Oct/Pit-1 POU domain chimeras, called Oct-1.P.1 and Oct-2.P.2, in a transient expression assay in HeLa cells. As expected from our previous results, Oct-2.P.2, but not Oct-1.P.1, activated the β-globin promoter (compare the bands labeled β in lanes 9 and 12). On the U2 promoter, however, these two activators display the opposite activities: Oct-1.P.1, but not Oct-2.P.2, activates transcription from this promoter (compare the bands labeled U2 in lanes 3 and 6). Thus, the Oct-1.P.1 and Oct-2.P.2 proteins can activate different promoters containing the same DNA binding sites while possessing the same DNA binding domain. Therefore, here the specificity of promoter activation is conferred by qualitatively distinct promoter-selective activation domains that lie outside the DNA binding domain.

These studies have progressed in two different directions. First, we are mapping the regions of Oct-1 responsible for activation of the U2 promoter to identify minimal snRNA promoter-selective activation domains. Our hypothesis is that mRNA and snRNA promoter-selective activation domains will interact with different targets within the transcriptional machinery to stimulate transcription; we plan to identify these different targets by using well-defined activation domains as probes. Second, we are analyzing the ability of Oct-1 and Oct-2 to activate RNA polymerase III transcription from the U6 snRNA promoter. The U6 promoter differs from the U2 snRNA promoter by containing a TATA box, which serves as a binding site for the general RNA polymerase II transcription factor TFIID. Paradoxically, the TATA box and the TATA box-binding TFIID core protein TBP, which are commonly associated with RNA polymerase II transcription, are responsible for ensuring that the U6 gene is transcribed by RNA polymerase III and not RNA polymerase II. Thus, although transcribed by RNA polymerase III, the U6 gene represents a composite of elements found within the very different mRNA and snRNA promoters transcribed by RNA polymerase II. Consistent with such a composite structure, the U6 promoter can be effectively activated by the snRNA promoter activator Oct-1.P.1 or the mRNA promoter activator Oct-2.P.2, suggesting that the U6 promoter can respond to both mRNA and snRNA promoter-selective activation domains.

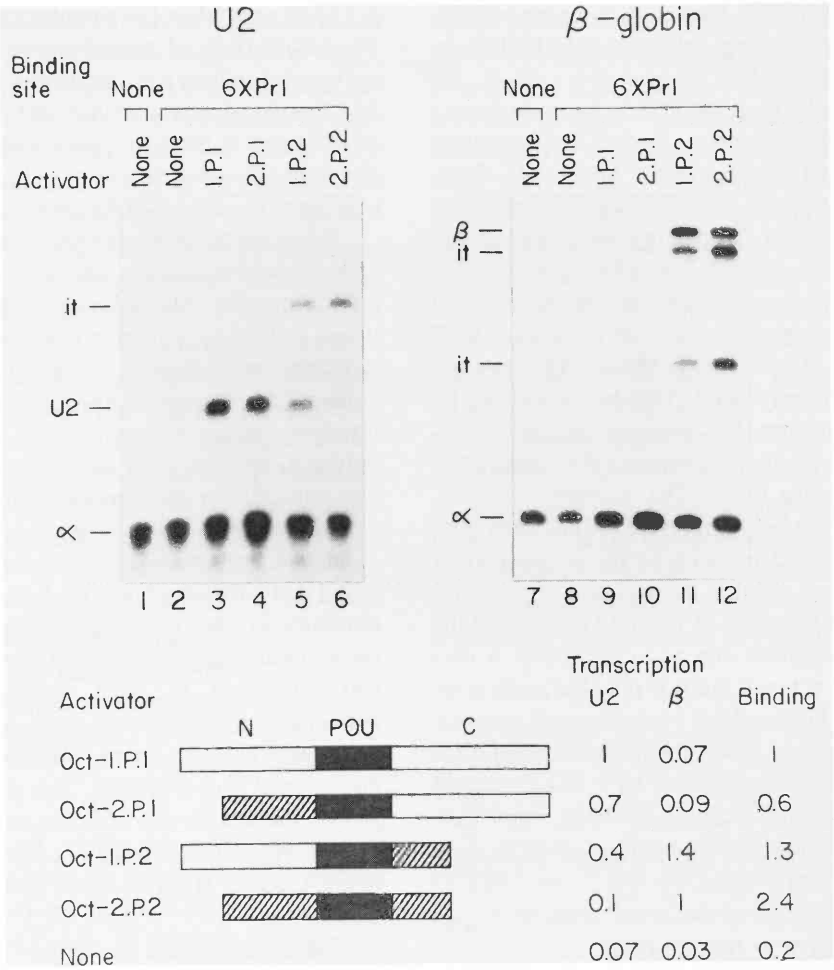

FIGURE 1 Oct-1 and Oct-2 possess distinct promoter-selective activation domains. An assay of transcriptional activation by Oct/Pit-1 POU-domain chimeras is shown. The structure of the chimeras is illustrated at the bottom of the figure with sequences derived from Oct-1 (*open rectangle*), Oct-2 (*hatched rectangle*), and Pit-1 (*closed rectangle*) indicated alongside the quantitative results of activation of the U2 and β-globin promoters by each protein. The relative expression levels (Binding) of the various activators were measured in a DNA-binding assay. The position of the α-globin (α) internal reference signal and the fragments corresponding to correctly initiated U2 (U2) and β-globin (β) transcripts are indicated as well as incorrectly initiated transcripts (it) that arise in each assay. Activation of the U2 and β-globin promoters containing six Pit-1 *cis*-regulatory sites (6XPrl) are shown to the left and right, respectively.

Activation of mRNA Transcription in Nonlymphoid Cells by the Octamer Motif: The Histone H2B Promoter

W. Thomann

As multimerized copies, the octamer motif can activate mRNA-type transcription from the β-globin promoter when positioned either close or far from the start site of transcription in cells expressing Oct-2, but not in cells only expressing Oct-1. Our studies indicate that Oct-1 fails to activate mRNA transcription under these conditions because it possesses only one of two interdependent mRNA promoter activation domains found in Oct-2. As shown by Schaffner and colleagues (Zurich), when assayed in an mRNA promoter context in which Oct-2 does not require both activation domains, Oct-1 is able to activate mRNA transcription, albeit less effectively than Oct-2. To understand the promoter context required for effec-

tive activation of mRNA transcription by an octamer motif in Oct-1 expressing cells, we use the histone H2B promoter as a model.

The histone H2B promoter is unique among octamer-containing promoters because the octamer motif is positioned adjacent to the TATA box. Point mutational analysis of the human histone H2B promoter showed that it contains three critical upstream elements: the TATA box, which positions the start site as well as activates transcription, and two upstream elements, the octamer motif and a CCAAT box, which can activate transcription independently of one another. When the CCAAT box is deleted by truncation, the histone H2B promoter displays complete dependence on the octamer motif for activity in cells only expressing Oct-1. The position of the octamer motif is apparently important for this activation because insertion of 5 bp between the octamer motif and the TATA box reduces transcription significantly. But the exact structure of the TATA box and the transcriptional initiation site associated with a correctly positioned octamer motif is not absolutely critical, because the histone H2B octamer motif can still activate a promoter in which the histone H2B TATA box and initiation site sequences have been replaced with the corresponding β-globin promoter sequences. To uncover the mechanism of position-dependent mRNA promoter activation by Oct-1, we plan to study interactions between Oct-1 and other transcription factors involved in histone H2B gene transcription.

Transcriptional Activation by Reiterated 18-amino-acid Glutamine-rich Sequences In Vivo and In Vitro

M. Tanaka

Oct-2 effectively activates a β-globin mRNA promoter through two interdependent activation domains, one amino-terminal and the other carboxy-terminal with respect to the central POU domain. We have studied the structure and function of both activation domains of Oct-2 in more detail.

By truncation analysis, we have shown that the amino-terminal activation domain is a 66-amino-acid (66aa) glutamine-rich domain and the carboxy-terminal domain is a 43-amino-acid (43aa) proline-rich domain. These two mini-domains, by cooperating with one another, are sufficient to activate transcription as well as the parental Oct-2. The positions or combinations of mini-domains, however, are not critical for activity because it was possible to generate a functional activator that contains multiple copies of only one or the other mini-domain as well as one that contains a transposition of the two mini-domains relative to the central POU domain.

To characterize further the structure and function of an activation domain, one of the crucial surfaces in protein-protein interactions involved in transcriptional activation, we have dissected the 66aa glutamine-rich mini-domain by use of a novel "multimerization approach." This multimerization approach involves tandem reiteration of a small portion of a polypeptide to amplify its otherwise weak activity. Our rationale for such an approach was as follows: In general, transcription factors appear to have redundant and modular structures. Specifically, we found that a fusion of the Oct-1 glutamine-rich amino terminus to the Oct-2 glutamine-rich domain increased transcriptional activity of the protein tenfold, and reiteration of such multiple glutamine-rich domains further increased transcriptional activity of the protein by three orders of magnitude compared to the parental Oct-2 protein. This multimerization approach has led to identification of an 18-amino-acid (18aa) segment within the 66aa glutamine-rich mini-domain, which is sufficient for activation upon multimerization.

The 18aa segment was chosen because it is one of several repeated sequence motifs found within the 66aa glutamine-rich mini-domain. When replacing the amino terminus of Oct-2, a single copy of the 18aa segment shows a weak but significant activity, and the activity increases with increasing copy number of the 18aa segment. Multiple copies of the 18aa segment fused to either the Oct-2 POU domain alone or to the heterologous GAL4 DNA binding domain generate functional activators. These reiterated segments thus contain an intrinsic transcriptional activation function. Taken together, these results suggest that the general architecture of transcriptional activators can be defined by multiple and modular small functional units (activation domains) cooperating with each other independently of their precise position or combination.

The future focus of our interest is to understand the mechanism of transcriptional activation by well-defined activation domains such as the 18aa glutamine-rich segment and the carboxy-terminal 43aa proline-rich mini-domain. We have initiated in vitro transcription studies that will allow us to dissect the

protein-protein interactions involved in the activation process. In a reconstituted system composed of multiple fractions derived from a HeLa cell nuclear extract, *E. coli*-produced Oct-2 protein stimulates transcription severalfold better than the Oct-2 POU domain, which itself only slightly stimulates transcription. This result indicates that transcriptional stimulation by Oct-2 in this system relies mostly on the presence of the activation domains that lie outside of the DNA binding domain. In the same reconstituted system, the Oct-2 derivative that contains multiple copies of the 18aa glutamine-rich activation domain efficiently activates transcription. Thus, protein-protein interactions involving the 18aa activation domain are reproduced in vitro. Therefore, we will further refine and characterize our reconstituted system to investigate such interactions.

Ethidium Bromide Provides a Simple Tool for Establishing Genuine DNA-independent Protein Associations

J.-S. Lai

DNA-dependent and -independent associations of DNA binding proteins are important in transcriptional regulation and are the focus of intensive investigation. During the course of characterizing a series of monoclonal antibodies raised against Oct-1 and Oct-2, we noticed four cellular proteins of 68 kD, 70 kD, 85 kD, and 110 kD that coimmunoprecipitate with the Oct-2 POU domain from labeled cell extracts in the absence of any added Oct-2 DNA binding sites, suggesting a DNA-independent association. We discovered, however, that the three larger Oct-2-associated proteins can bind DNA on their own and, subsequently, that the 70-kD and 85-kD proteins are the two heterologous subunits of the human autoantigen Ku, which possesses strong nonspecific DNA binding properties. These observations led us to test whether the association of these three proteins with Oct-2 might indeed be DNA-dependent and result from association with contaminating DNA in the extracts, which could serve to tether the different proteins to one another during the immunoprecipitation.

For the test, we sought a reagent that could easily disrupt protein-DNA association without generally disrupting protein-protein associations. As illustrated by the experiment shown in Figure 2, the DNA intercalator ethidium bromide serves this purpose and shows that the association between Oct-2 and the 70-kD, 85-kD, and 110-kD proteins is indeed DNA-dependent. Figure 2 shows three series of precipitation experiments with an extract from human 293 cells containing ectopically expressed Oct-2. Ethidium bromide does not generally affect protein-protein associations, because increasing ethidium bromide concentration does not affect recovery of several adenovirus E1A-associated proteins (arrowheads) in an E1A immunoprecipitation (compare lanes 2 and 3 with lane 1). In contrast, ethidium bromide does affect protein-DNA association, because it impedes recovery of either Oct-2 or the three nonspecific DNA binding proteins (indicated by dots in the figure) in a DNA affinity precipitation assay (lanes 7–9). In an Oct-2 immunoprecipitation, however, ethidium bromide only affects recovery of the three Oct-2-associated proteins (lanes 4–6), indicating that the association of Oct-2 with the three larger

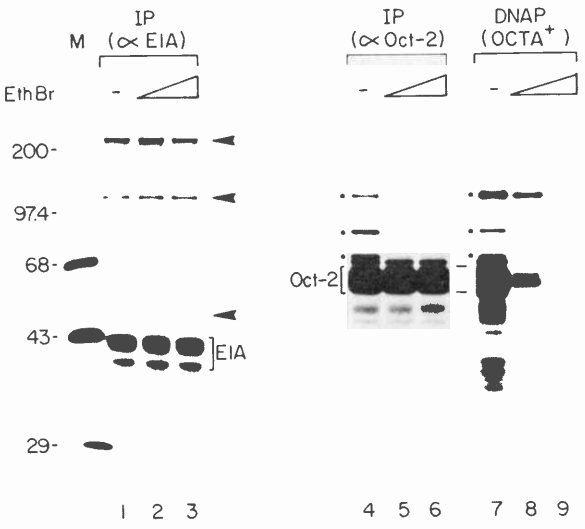

FIGURE 2 Association of 70-kD, 85-kD, and 110-kD proteins with Oct-2 is sensitive to the presence of ethidium bromide. A 293 cell extract containing Oct-2 was immunoprecipitated with the E1A-specific monoclonal antibody M73 (lanes *1–3*) or the Oct-2-specific monoclonal antibody PT1 (lanes *4–6*) or used for DNA-affinity precipitation with multimerized octamer and heptamer motifs (lanes *7–9*). The reactions were done in the absence (lanes *1*, *4*, and *7*) or presence of either 12.5 μg/ml (lanes *2*, *5*, and *8*) or 50 μg/ml (lanes *3*, *6*, and *9*) ethidium bromide. The precipitates were separated by 10% SDS-PAGE and visualized by fluorography. Arrowheads indicate the positions of the E1A-associated proteins, p300, p107/p105^{Rb}, and p60$^{Cyclin\ A}$, respectively. Dots indicate the positions of the three Oct-2-associated proteins.

proteins is DNA-dependent and presumably due to contaminating DNA in the extracts. Consistent with this result, treatment of the immunoprecipitates with DNase also disrupts these associations.

We extended these studies to show that unsuspected DNA contamination can also stabilize association between DNA binding proteins in protein affinity assays when using a glutathione *S*-transferase fusion protein as a probe to assay association with in vitro translated proteins, a commonly used strategy to study protein-protein associations. Thus, these studies suggest that ethidium bromide is an excellent reagent to discriminate between protein associations that are entirely independent or remain dependent on DNA.

VP16 Complex Formation with Oct-1 and HCF: Oct-1 Acquires New *cis*-regulatory Activation Properties through Association with VP16

M. Cleary, S. Stern, M. Tanaka, A. Wilson

Differential transcriptional regulation by homeodomain proteins elicits complex patterns of cell differentiation during development, and yet, when analyzed biochemically, these proteins frequently display very similar, if not identical, DNA binding properties. Therefore, a central question in regulation of development by these proteins is how they can control transcription from different promoters while possessing the same DNA binding specificity. The differential association of VP16 with the octamer motif-binding proteins Oct-1 and Oct-2 serves as a model to elucidate such mechanisms. We, as well as T. Kristie and P. Sharp (Massachusetts Institute of Technology), have shown previously that VP16 engineers formation of the VP16-induced complex by interacting with each of the three other components of the complex: HCF, DNA, and Oct-1. To dissect the complex in greater detail, we are purifying the least well characterized component HCF. This component forms a heteromeric complex with VP16, independently of Oct-1 or the TAATGARAT motif, and we are using this property as one of the strategies to purify the factor.

Association of VP16 with Oct-1 has two important effects on the transcriptional activation properties of Oct-1. The first of these effects results in imparting Oct-1 with activation properties more normally associated with Oct-2. Oct-1 does not readily activate mRNA promoters, but VP16 contains a potent acidic activation domain and the VP16-induced complex can readily activate mRNA-type transcription. Thus, this first effect makes the two normally dissimilar activators Oct-1 and Oct-2 more similar. The second effect, however, displays another mechanism by which two proteins that possess the same DNA binding properties can activate different promoters. This difference is achieved by selective recruitment of Oct-1 to a new regulatory site, the TAATGARAT motif. In the absence of VP16, TAATGARAT motifs that lack any overlapping octamer motif are poor binding sites for Oct-1 and Oct-2. But in the presence of VP16, the affinity of Oct-1 for these sites increases, whereas the affinity of Oct-2 for these sites is unaffected. This differential influence on affinity is reflected in the activity of such a TAATGARAT site in vivo, because it fails to activate either the U2 snRNA promoter in the presence of Oct-1 or an mRNA promoter in the presence of Oct-2, but it can activate mRNA transcription in the presence of both Oct-1 and VP16. Thus, as a result of recruitment to a new site, Oct-1 is able to participate in activation of a new promoter that is resistant to Oct-2 activation because Oct-2 does not associate with either this site or VP16 effectively.

HIV-1 Promoter Sequences Isolated Directly from Infected Individuals Contain a Hot Spot for Tandem Sequence Duplication

W. Phares, D. Taylor

To study the influence of variation in promoter structure on HIV-1 pathogenesis, we have been analyzing the sequence of the HIV-1 promoter isolated directly from individuals infected with HIV-1. Retroviruses like HIV-1 display very high rates of mutation, and in other retroviruses, viral pathogenicity can be influenced by the potency or cell-specific activity of the viral promoter. One type of mutation that can arise in promoter sequences are tandem duplications. Our earlier studies on the SV40 enhancer revealed that selection for improved activity resulted in tandem sequence duplications that duplicate individual subelements called proto-enhancers. Different SV40 proto-enhancer duplications resulted in enhancers with new cell-specific activities. We hypothesize that similar changes in HIV promoter sequences could also influence virus growth and hence the

course of acquired immunodeficiency syndrome or AIDS.

To analyze HIV-1 promoter structure in infected individuals, P. Baron and J. Gold (Memorial Sloan-Kettering Cancer Center and Bronx-Lebanon Hospital Center) provide us with DNA from infected blood samples, and we amplify the HIV-1 promoter sequences directly by polymerase chain reaction (PCR). Characterization of the amplified promoter sequences has revealed a hot spot for tandem sequence duplications (TSDs) immediately upstream of the HIV core enhancer elements. Although numerous different TSDs varying in sequence and size (15–29 bp) have been isolated, they all duplicate a common 12-bp "core" sequence. This pattern of sequence duplication has not been observed in HIV isolates that have been passaged in cell culture and is therefore apparently unique to in vivo viral isolates. The 12-bp core region does not encompass any previously recognized HIV promoter or enhancer element. This type of overlapping duplication pattern is reminiscent, however, of the duplication patterns we observed in the SV40 enhancer, suggesting that an HIV-1 regulatory element (either positive or negative) is being amplified by the TSDs. By examining the patterns of nuclear factor binding to the duplicated and nonduplicated forms of the HIV promoter, we have identified a factor enriched in T-cell lines, a natural host for HIV infection, that binds to the duplicated sequences but binds poorly, if at all, to the nonduplicated form. This result was unexpected and suggests that rather than duplicate a regulatory element the TSDs may create a new element that is recognized by the T-cell factor.

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PROTEIN CHEMISTRY

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The Protein Chemistry laboratory conducts research on signal transduction and cell growth using advanced methods of analytical and preparative biochemistry. These methods include automated protein sequence analysis, high-performance liquid chromatography (HPLC), amino acid analysis, preparative electrophoresis, mass spectrometry, and peptide synthesis. Such procedures allow us to deduce chemical structures of proteins and to synthesize structural and functional domains. In addition, physical analysis of proteins by mass spectrometry, in conjunction with chemical studies, permits the determi-

nation of posttranslational modifications of proteins, such as phosphorylation and acylation. Our goal is to attack biological problems of the control of cell growth by using an arsenal of state-of-the-art biochemical methods. Often, this requires the development of new methods when existing procedures are not adequate to solve the cell biological question. This chemical approach to cell biology is highly complementary to genetic approaches in many of the other laboratories at Cold Spring Harbor, and analytical biochemistry is an essential part of a multidisciplinary approach to problems of normal and abnormal cell growth, as in cancer.

Synthetic Peptide Substrates for Protein Kinases

D.R. Marshak, M. Vandenberg, I.J. Yu, F. Sun, M. Meneilly, G. Binns

Protein kinases are enzymes that catalyze the transfer of phosphates from the γ-phosphate position of ATP to a hydroxyl moiety (serine, threonine, or tyrosine) on proteins. The recognition sequences for protein kinases are surprisingly short; usually only four to six amino acid residues in linear sequence are required for specifying the substrate. Therefore, synthetic peptide substrates have been quite useful as model substrates for protein kinases. Such peptides, usually 10–20 residues in length, can be synthesized by solid-phase methods using automated instrumentation. They are constructed as protected molecules on polystyrene supports and then deprotected and cleaved from the support by treatment with acid. In our laboratory, rigorous purification and characterization are carried out to assure purity and identity of the material. We have documented evidence that products of the side reactions of peptide synthesis can alter the measured kinetic properties of the substrate (Marshak and Carroll 1991). Therefore, we use mass spectrometric measurements to assess the molecular weight of the product to eliminate unwanted, modified side products that arise from incomplete removal of protecting groups or other modifications. Combined with HPLC and amino acid analysis, mass spectrometry has allowed us to prepare peptides with excellent characteristics as kinase substrates, including low K_M values.

We have focused on two particular protein kinases, casein kinase II (CKII) and $p34^{cdc2}$, in our approach to synthetic peptide substrates. Figure 1 shows a comparison of the amino acid sequences of several peptide substrates for CKII and $p34^{cdc2}$. CKII substrates have been prepared based on novel sites of action of the enzymes on nuclear oncoproteins, such as c-*myc*, SV40 large T antigen, and adenovirus E1A (Marshak and Carroll 1991). These peptides have K_M values below 0.1 mM, which suggests that they are high-affinity substrates for the enzyme. Peptide substrates of $p34^{cdc2}$ were developed based on sites of phosphorylation that we determined on SV40 T antigen and the recessive oncogenes, *p53* and retinoblastoma (*RB*). The T-antigen peptide, CSH103, was shown to be specific for $p34^{cdc2}$ among eight classes of protein kinases (Marshak et al. 1991). This peptide can be used as a specific substrate of $p34^{cdc2}$ in whole-cell extracts and gives results similar to those of very involved procedures that require specific precipitation of the enzyme from the extracts: histone H1 phosphorylation, electrophoresis, and autoradi-

Liver Casein Kinase II

His-Glu-Ala-Gly-Phe-Pro-Pro-Ser-Asp-Asp-Glu-Asp-Glu-Glu-Gly

Ser-Glu-Glu-Met-Pro-Ser-Ser-Asp-Asp-Glu-Ala-Thr-Ala-Asp

Glu-Glu-Glu-Thr-Pro-Pro-Thr-Thr-Ser-Ser-Asp-Ser-Glu-Glu-Glu-Gln-Glu-Asp-Glu-Glu-Glu

Mitotic $p34^{cdc2}$ Kinase

Ala-Asp-Ala-Gln-His-Ala-Thr-Pro-Pro-Lys-Lys-Lys-Arg-Lys-Val-Glu-Asp-Pro-Lys-Asp-Phe

Lys-Arg-Ala-Leu-Pro-Asn-Asn-Thr-Ser-Ser-Ser-Pro-Gln-Pro-Lys-Lys-Lys-Pro-Leu-Asp-Gly-Glu-Tyr

FIGURE 1 Synthetic peptide substrates for CKII and for mitotic $p34^{cdc2}$ kinase.

ography. Because CSH103 binds well to phosphocellulose paper, the peptide assay is rapid: 30 minutes compared to 2 days. Recent work has centered on analyzing the substrate specificity of $p34^{cdc2}$ and related protein kinases. By using CSH103 as a starting point, we have altered each amino acid individually to evaluate the details of substrate requirements for the enzyme. In addition, these studies will allow us to identify new forms of the enzyme with alternate substrate specificities.

Molecular Cloning and Expression of Casein Kinase II Subunits

N. Chester, I.J. Yu, D.R. Marshak

CKII is a protein serine/threonine kinase found in all eukaryotic cells. Its ubiquitous distribution among species and tissues implies a function central to all nucleated cells. The enzyme consists of two subunits, α and β, with molecular mass ranges of 37–44 kD and 24–28 kD, respectively, and an apparent subunit composition of $\alpha_2\beta_2$. The α subunit is found in two different forms, known as α and α′, that arise from separate genes. To answer questions about the mechanism of enzyme regulation, we have cloned and expressed DNA molecules coding for the full-length forms of the α and β subunits, using a novel method called the polymerase chain reaction (PCR). Typically, the PCR for a particular DNA is optimized by varying the temperatures used in denaturing and renaturing the DNA templates. This is time-consuming because most automated temperature cyclers are limited to one or two temperatures at one time, although many samples can be run simultaneously. Nick Chester utilized a solvent modifier, dimethylsulfoxide, to alter the apparent melting temperature of the DNA template and related the modifier concentration directly to the apparent change in melting temperature. Therefore, one can set up PCR in samples with a wide variety of concentrations of the modifier, perform one overnight reaction, and identify the optimal conditions for product formation. The predicted, optimal melting temperature in the absence of modifier can be calculated directly from the modifier concentration determined empirically. This greatly reduces the time required for generation of specific DNAs by PCR. The new method permitted the generation of PCR products for the CKII α and β subunits using either one-step or two-step PCR cycles. These DNA products were cloned into both bacterial and mammalian expression vectors for studies of cell physiology.

Cell Cycle Regulation of Casein Kinase II

I.J. Yu, M. Vandenberg, D.R. Marshak

We studied the relationship between the changes of protein kinase activity during the cell cycle. The cellular activities in HeLa extracts of three endogenous kinases, CKII, cAMP-dependent protein kinase (CAK), and histone H1 kinase ($p34^{cdc2}$ kinase), were analyzed. CKII activity was determined by measuring the total [^{32}P]phosphate incorporation into the synthetic peptide RRREEETEEE, which is a specific substrate for CKII (Marshak and Carroll 1991). CAK activity was measured by [^{32}P]phosphate incorporation into Kemptide, a synthetic substrate for CAK. Histone H1 kinase activity was determined by measuring the incorporation of [^{32}P]phosphate into histone H1 or peptide CSH103, which is a specific substrate for $p34^{cdc2}$ kinase (Marshak et al. 1991). As shown in Figure 2, CKII activity is high in G_1 cells and gradually decreases as cells are in S to G_2/M phase. In addition, the CKII activity can be inhibited by addition of heparin, which is a known inhibitor of CKII. The CKII peptide kinase activity is found at high levels in G_1 cellular extracts and lower in S and G_2/M-phase extracts. CAK activity does not change significantly during the cell cycle. In contrast, histone H1 kinase activity is high in G_2/M cells and low in S and G_1 cells. In addition, $p34^{cdc2}$ kinase activity measured by CSH103 also indicated that the kinase activity is high in G_2/M cells and low in G_1 cells.

To develop further our studies of cell-cycle-associated changes in the phosphorylation of c-*myc* and the role of cellular kinase(s), we tested extracts of HeLa cells specifically in G_1 phase (more than 85% by cell sorter analysis), S phase (arrested by hydroxyurea), and M phase (arrested in the presence of nocodazole) for differential levels of c-*myc* phosphorylation and kinase activity. As seen in the case of the elutriated HeLa cells, CKII activity is maximal in G_1, lower during S phase, and slightly elevated in M phase. Histone H1 kinase activity, on the other hand, is maximal during M phase and lower during S phase and G_1. CAK, in contrast with both CKII and histone H1 kinase or $p34^{cdc2}$, shows no sig-

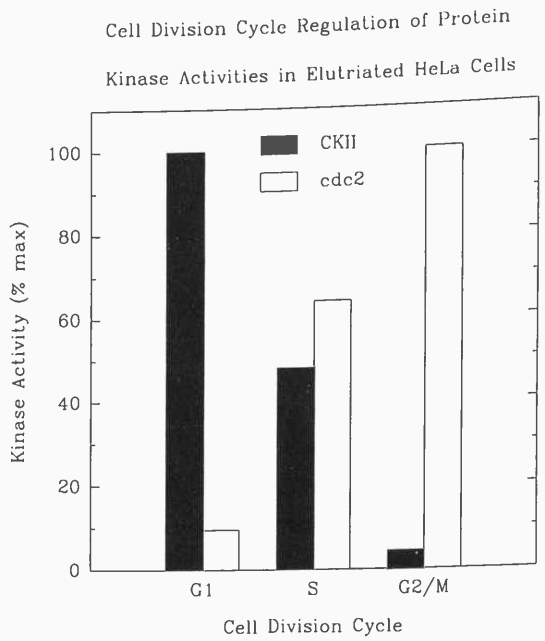

FIGURE 2 Cell division cycle control of CKII and $p34^{cdc2}$ kinase in human cells. HeLa cells, grown in suspension, were subjected to centrifugal elutriation. Peak fractions corresponding to G_1 (2N DNA content), G_2/M (4N DNA content), and S (intermediate DNA content) phases were collected, and extracts were tested for kinase activities with the appropriate synthetic peptide substrate. Results are expressed as a percentage of the maximal activity for comparison.

nificant changes in its cellular activity during G_1, S, or M phase. CKII activity associated with each of the extracts can be competitively inhibited when a synthetic peptide whose primary sequence is derived from a CKII phosphorylation region in c-*myc* is added to the reaction mixture. When the c-*myc* synthetic peptide is added to the reaction mixture, phosphorylation of histone H1 is slightly depressed in M-phase cells. These experiments establish that the phosphorylation of a known substrate of CKII is regulated during the cell division cycle.

Interactions between Casein Kinase II and cdc2

G.L. Russo, I.J. Yu, M. Vandenberg, G. Binns, D.R. Marshak

The biological importance of CKII stems from studies of hormonal and growth regulation for the enzymatic activity. In recent years, CKII has been found to be stimulated by serum, growth factors, and cell differentiation. The molecular basis for this regulation is unknown. In all of these cases, there appears to be no increase in the amount of CKII protein, and inhibitors of protein synthesis do not block the rapid increase (30 min) in CKII activity. CKII is not phosphorylated on tyrosine, but the serine/threonine phosphorylation of the β subunit increases on growth factor stimulation of the enzyme. The bulk of the phosphorylation of CKII appears to be at the amino terminus of the β subunit, which is catalyzed by autophosphorylation. The remaining phosphorylation of the β subunit may be accounted for by $p34^{cdc2}$ kinase. Il Je Yu has conducted a series of studies of the β subunit phosphorylation during the cell division cycle in HeLa cells. This project involves immunoprecipitating β from cell extracts with a specific antibody, followed by electrophoresis and transfer of the protein to a solid supporting membrane. The protein can then be digested with cyanogen bromide, and the peptides are then separated by HPLC. The amino-terminal peptide containing the autophosphorylation site and the carboxy-terminal peptide containing the $p34^{cdc2}$ site are thus separated and can be quantitated by solid-phase, radioactively labeled, sequence analysis. These ongoing studies will establish the regulation of these two sites during the cell division cycle, and we will extend these experiments to other models of cell growth in which CKII is regulated.

A parallel set of studies has been conducted by Gian Luigi Russo to investigate the cell cycle regulation of the phosphorylation of $p34^{cdc2}$. This enzyme has major phosphorylation sites at threonine and tyrosine residues that regulated its activity positively and negatively, respectively. Previous reports of serine phosphorylation have led us to attempt to identify these minor sites of phosphate attachment. We have used a similar strategy of synthetic peptide substrates as models of various domains of the proteins and then examined in vivo and in vitro phosphorylation of $p34^{cdc2}$ by purified kinases. One candidate enzyme for this is CKII. It would be of interest if reciprocal phosphorylations by $p34^{cdc2}$ and CKII were important to cell cycle control. Our next approach will be to determine the functional significance of serine phosphorylations of $p34^{cdc2}$ and whether these are involved in modifying protein-protein interactions among associated subunits of the enzyme.

Subcellular Localization of Casein Kinase II

I.J. Yu, D.R. Marshak [in collaboration with D. Spector, Cold Spring Harbor Laboratory]

The CKII enzyme activity is found both in cytosol and in nuclei, and there are substrates identified in both locations. Cytosolic substrates include proteins involved in translational control (eIF-2, -3, -4B, -5), metabolic regulation (glycogen synthase), and the cytoskeleton (nonmuscle myosin heavy chain, β-tubulin). Substrates found in the nucleus include DNA topoisomerase II, RNA polymerases I and II, oncoproteins such as Myc, Myb, and SV40 large T antigen, and transcription factors such as serum response factor. The extraordinary range of substrates for this enzyme supports the contention that CKII plays a significant role in cell physiology. We developed specific antibodies to synthetic peptide antigens that react with the individual subunits of CKII. These antibodies allowed us to study the subcellular localization of the subunits of the enzyme during the cell division cycle of HeLa cells (Yu et al. 1991). Using immunofluorescence microscopy, we examined asynchronous populations of cells, as well as cells arrested and released from the G_1/S transition by the chemical hydroxyurea. The results indicated that the α and β subunits are localized to the cytoplasm during interphase and throughout the cell during mitosis. Electron micrographs indicate an abundance of α subunit immunoreactivity associated with spindle fibers of the mitotic apparatus. During the G_1 phase of the cell cycle, the α′ subunit is found predominantly in the nucleus. Upon transit across the G_1/S boundary, the α′ subunit appears to disappear from the nucleus and become cytoplasmic. This is concomitant with the down-regulation of the activity in S phase. These dynamics suggest that CKII may affect cell division cycle control by altering its localization to different subcellular compartments.

Structural Analysis and Phosphorylation of Nuclear Proteins

D.R. Marshak, M. Vandenberg, G. Binns, M. Meneilly [in collaboration with T. Curran, Roche Institute, J. Kadonaga, University of California at San Diego, and E. Harlow, Massachusetts General Hospital]

For several years, we have been characterizing the phosphorylation of nuclear oncoproteins by a variety of protein kinases. During the past year, we have focused on three areas of such characterization: (1) the transcriptional control proteins and proto-oncogenes, c-*fos* and c-*jun*; (2) the recessive oncogene product, retinoblastoma; and (3) a transcriptional repressor, histone H1.

The phosphorylation of c-Fos and c-Jun protein was done in collaboration with Tom Curran's group at the Roche Institute. Sites on c-Fos and c-Jun proteins were analyzed by phosphorylating in vitro the proteins produced in bacteria as recombinants. Purified proteins as well as truncated versions with various domains were deleted. Phosphorylation sites on c-Fos occur in regions that have been linked genetically to the regulation of transcriptional control. Notably, sites for the cell-cycle-regulated protein kinase, $p34^{cdc2}$, were identified, suggesting that there might be significant cell cycle regulation of the transcriptional control by c-Fos. Interestingly, the CKII site that was predicted from synthetic peptide studies was not phosphorylated in the intact protein. This is consistent with very low rates of phosphorylation of the peptide in solution.

The recessive oncogene, retinoblastoma (*RB*), was analyzed in collaboration with Ed Harlow's group, now at Massachusetts General Hospital (Lees et al. 1991). RB protein is quite large (105 kD), but it is found in only minute quantities in cells. It is therefore very difficult to identify phosphorylation sites directly from in vivo, metabolically labeled proteins. We took a novel approach of using synthetic peptide model substrates to map phosphorylation sites in vitro and then compared these to maps of immunoprecipitated, in-vivo-labeled RB protein. This approach required that we survey the RB protein sequence, inferred from the cDNA structure, for potential sites of $p34^{cdc2}$ phosphorylation. We identified clusters of potential sites and synthesized moderately long peptides (30–40 residues) covering these sites. Determination of the stoichiometry and positions of the phosphates was accomplished by a combination of mass spectrometry and protein sequencing. The final compilation of data allowed the assignment of five sites of phosphorylation of RB by $p34^{cdc2}$ and excluded several sites from phosphorylation in vivo.

The protein histone H1 is a nuclear protein associated with chromatin but not with the core histones or the nucleosomes. Jim Kadonaga at the University of California, San Diego, had isolated a basic protein from *Drosophila* embryos that acted as a transcriptional repressor for RNA polymerase II transcription. We used a combination of peptide mapping by

HPLC, protein sequencing, and mass spectrometry to establish that the purified transcriptional repressor was histone H1 (Croston et al. 1991). This observation has fueled the theory that part of the activation of gene transcription arises from the derepression of histone H1.

Glycogen Synthase Kinase 3 and Casein Kinase II during Differentiation

W. Benjamin, I.J. Yu, D.R. Marshak

During his sabbatical year, Bill Benjamin has been studying the coordinate regulation of CKII and another protein kinase, glycogen synthase kinase 3 (GSK3), in 3T3-L1 fibroblasts. These cells can be induced to differentiate into adipocytes that synthesize and store lipids and are therefore useful in understanding the metabolic regulation and development of fat cells. Preliminary data available during the past few months indicated that both enzyme activities fluctuate during this differentiation. These two protein kinases may interact at the level of target substrates, as has been established for glycogen metabolism, in which CKII phosphorylation precedes and is required for GSK3 phosphorylation to proceed. Enzymes of fatty acid synthesis may well be coordinately regulated in much the same way. In addition, recent reports that GSK3 may phosphorylate transcription factors suggest that GSK3, like CKII and $p34^{cdc2}$, may be important in the control of gene expression.

New Methods for Phosphoprotein Sequence Analysis

D.R. Marshak, G. Binns

Two methods have been developed to determine complicated phosphorylated protein patterns. These employ two different technologies: the chemistry of automated Edman degradations and the physical analysis of mass spectrometry. Using commercial membranes made of arylamine-modified polyvinylidene difluoride, it is possible to covalently attach phosphopeptides via the carboxylate moiety. This coupling procedure allows the removal of contaminating salts, buffers, and inorganic phosphate, as well as excess coupling reagent, a water-soluble carbodiimide. The innovation comes when these immobilized peptides are loaded into existing protein sequencing instruments. By altering the cycles, we efficiently remove the phosphorylated residues at each cycle using liquid trifluoroacetic acid and a modified solvent consisting of *n*-butyl chloride, ethyl acetate, and neat phosphoric acid. Bypassing the conversion flask permits direct collection of anilinothiazolinone derivatives of each amino acid cycle. Upon scintillation counting, the cycle containing [^{32}P]phosphate is determined. Efficiency of this sequencing method allows the analysis of a few hundred cpm of radioactivity with approximately 20:1 signal:noise ratios. Figure 3 shows an example of this type of sequence analysis. In real terms, it means that one can recover peptides from metabolically labeled, immunoprecipitated, and electrophoretically purified samples. Previous methods were more than 100-fold less sensitive.

The second new method arises from our previous studies of mass spectrometry which showed that manipulation of the peptide matrix could promote fragmentation of the molecule, primarily at the pep-

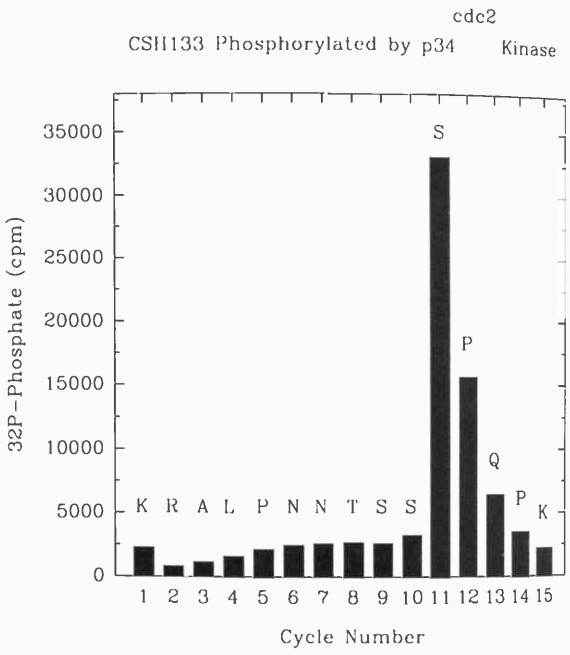

FIGURE 3 Sequence analysis of a phosphorylated peptide using solid-phase methods. Recovery of radioactivity is shown for each sequencer cycle using [^{32}P]phosphate labeled peptide CSH133, based on the sequence of the *p53* recessive oncogene product.

tide bonds. Extending this method to phosphorylated peptides, particularly with the RB peptides described above, has allowed us to determine the sites of phosphorylation directly from the fragmentation pattern in the spectrometer. The predominant ions that occur are the A series, arising from the acylium ion at each peptide bond with loss of carbon monoxide. Other ions, including the Y′′ series, arising from the ammonium ions at each peptide bond, are also seen at lower frequency. The combination of mass spectral methods and chemical sequencing methods in conjunction with synthetic peptide preparation provides a complete set of techniques for analysis of phosphorylated proteins in cells.

Protein Chemistry Core Facility

G. Binns, M. Meneilly, R. Kobayashi, D.R. Marshak

The Protein Chemistry Core facility provides state-of-the-art methodology to staff members at Cold Spring Harbor for the chemical analysis of proteins and the preparation of synthetic peptides. The facility moved to new quarters in the W.M. Keck Structural Biology Laboratory in the Beckman Neuroscience Center in May, 1991. The move involved dismantling and reassembling the intricate analytical instruments, including the mass spectrometer, protein sequencers, peptide synthesizer, and chromatographs. Enormous credit goes to Georgia Binns and Maria Meneilly who principally shouldered these burdens. Downtime was minimized during the move to the new laboratory, and the instruments were recalibrated, tested, and fully operational by the end of July. The new facilities provide space specifically designed for the instruments, as well as support equipment such as specialty gases, conditioning power supplies, air compressors, and chilled water recirculators.

The services provided by the Core facility increased in all areas, on an annual basis, accounting for the downtime of the move. The progress is shown graphically in Figure 4. Protein sequencer runs totaled 172, representing an annualized rate of 11% above that for 1990. The sequences ranged from 4 to 50 residues, averaging 19.5 residues. Many of these runs were at very high sensitivity, from 0.5 to 2.0 picomoles. In one project, Georgia Binns and Adrian Krainer collaborated to obtain structural information on two pre-mRNA splicing factors (Krainer et al. 1991). The repetitive yield curve is shown in Figure 5. Using the reagents and methodologies available, these results are at the physical limit of detection. Further innovations in high-sensitivity sequencing are being pursued by Ryuji Kobayashi (see below).

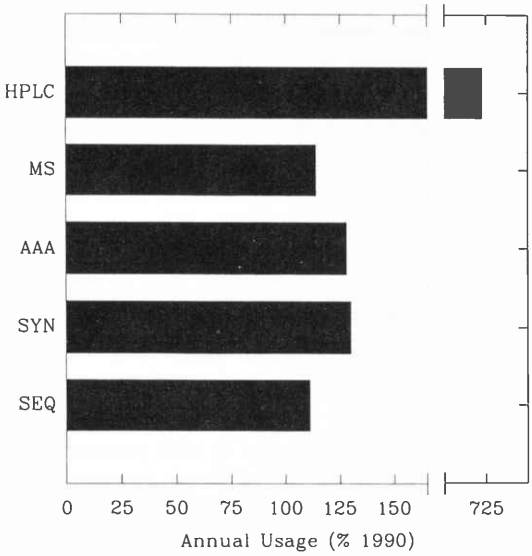

FIGURE 4 Summary of protein chemical services for 1991. The annualized rate for each service is compared with that for 1990. (SEQ) protein sequencing; (SYN) peptide synthesis; (AAA) amino acid analysis; (HPLC) chromatography; (MS) mass spectrometry. The data are expressed as percentages for normalization.

Peptide synthesis, led by Maria Meneilly, accounted for 77 purified and characterized synthetic products, representing a total of 1877 synthesis cycles. This is an annualized rate 30% above that for 1990 and corresponds to two peptides completed per week, the physical limit of the personnel and automation resources now available. The length of these peptides ranged from 3 to 47 residues and averaged 24.4 residues. We look forward to expanding the peptide synthesis functions during 1992. Other areas showed similar increases: amino acid analysis, 28%; mass spectrometry, 14%; and chromatography, 723%. The extraordinary increase in chromatography is due to the introduction of new peptide mapping and sequencing protocols that place a heavier burden on chromatographic separation. In addition, Ryuji Kobayashi has accelerated the rate of protein digestions and chromatographic separations.

The Protein Chemistry Core facility is a member of the Association of Biomolecular Resource Facilities (ABRF), an organization of similar laboratories around the world. As a member of the peptide

synthesis/mass spectrometry subcommittee, Dan Marshak was involved in the production of a worldwide survey of facilities on their abilities to synthesize peptides. Maria Meneilly, Georgia Binns, and Dan Marshak invented a 16-residue, test peptide (VKKRCSMWIIPTDDEA) that was synthesized by 42 facilities. The peptide products, both crude and supposedly purified, were analyzed by many different analytical techniques, including mass spectrometry, and compared with standard pure peptide produced at Cold Spring Harbor Laboratory and Yale independently. All of the products tested had the correct amino acids in the proper order, but the majority of products were either chemically modified, not fully deprotected, or contaminated by side products. These results (Smith et al. 1992) indicate that mass spectrometry is an essential tool in screening synthetic peptides and that the Protein Chemistry facility at Cold Spring Harbor Laboratory is among a very few Core laboratories that provide highly purified and verified peptides. This is essential to successful studies using peptides as substrates for enzymes and in structure-function studies.

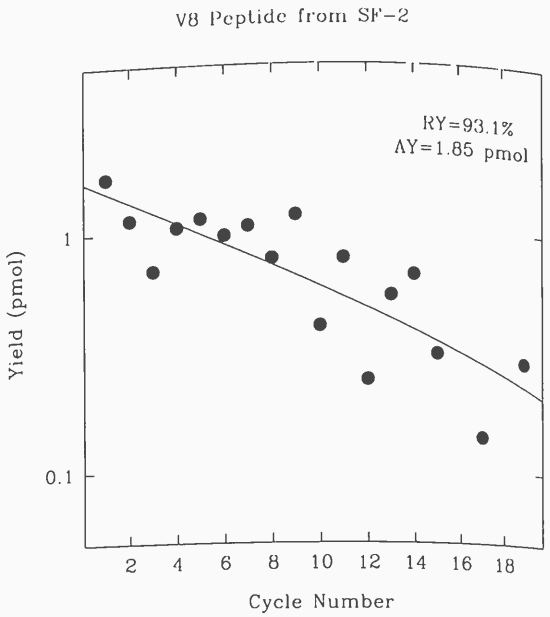

FIGURE 5 Amino acid yields for a protein sequencer analysis of a peptide derived from a pre-mRNA splicing factor purified by A. Krainer.

Methodological Study of Protein Sequence Analysis

R. Kobayashi

I joined the Keck Structural Biology Center at Cold Spring Harbor Laboratory in April, 1991. One goal is to develop a new, highly sensitive method for protein sequence analysis, and a second goal is to collaborate with other Cold Spring Harbor Laboratory scientists involved in cancer research to study protein structure and function.

Since the early days of molecular biology, protein sequence analysis has played a major role in studies on the relationship of protein structure and function. As the technology for identifying and purifying proteins continues to develop, more sensitive techniques will be required for protein structural analysis. Often, proteins of significant biological importance are not easily obtained in sufficient amounts, and this has caused delays in biological discoveries. Furthermore, attempts to overcome the problem have required considerable expense; e.g., a tremendous number of animals may be required to produce sufficient amounts of biologically active substances for study. Sampling of blood and tissues from human sources has severe limitations as well. For most unknown proteins, it is necessary to purify the protein to absolute homogeneity prior to cleavage with a specific protease and isolation of peptides for protein sequence analysis. Often, this can only be achieved by purification using electrophoresis in polyacrylamide gels containing SDS, followed by elution or blotting the sample from the gel. As a consequence, there is often a need to isolate large amounts of protein (50–200 pmoles) that are detectable by methods such as Coomassie blue staining in order to finally obtain peptides at the 10–20 pmole level. With this amount of peptide, the protein sequence can be usually determined with high accuracy. It is often difficult, however, to obtain proteins in these amounts. To improve these matters, further development of protein sequencing methods is necessary, particularly to improve the sensitivity of the protein sequencing chemistry so that smaller amounts of protein can be studied, e.g., the amount detectable only by silver staining.

The method is based on the manual Edman degradation established in early 1980s, previously described by Kobayashi and Tarr, and has been used for protein sequencing at the picomole level of detection for the last 10 years. The major limitation of the current technology is the total dependence on ultraviolet absorption of the sequencing products, although a

fancy computer can help in interpreting the sequencing data. The new method will introduce a fluorescence tag into the Edman chemistry, followed by analyzing the sequencing product by high-performance liquid chromatography with a chemiluminescence detection (HPLC-CL) system. These two innovations will allow two to three orders of magnitude in increased sensitivity. Several reports have demonstrated that amino acid analysis can be achieved at the low- or subfemtomole level with chemiluminescence detection or by improved fluorogenic derivatization with laser-induced fluorescence detection. Therefore, it is feasible that the sensitivity of protein sequencing can be increased to the subfemtomole level using this technology. No one, however, has yet succeeded in protein sequence analysis with chemiluminescence detection. Accomplishing this project will allow protein sequencing using subpicomole amounts of protein. Specifically, it is proposed that an isothiocyanate, 4-(*N*-*t*-butoxycarbonyl) phenylisothiocyanate (Boc-amino-PITC, BAPITC) will be used for Edman degradation instead of the conventional phenylisothiocyanate. The 4-aminophenylthiocarbamyl amino acids, intermediate products of the modified Edman degradation, will then be labeled by a fluorescent probe. The final product, 4-(*N*-1-dimethylaminonaphthalene-5-sulfonyl-amino) phenylthiohydantoin-amino acid derivatives, will be analyzed by the HPLC-CL system.

New developments in chemiluminescence chemistry (see Fig. 6) and the new Edman reagent can both be combined to enhance protein sequencing methodology. I have been developing the techniques and chemistry employed here in related research at the University of Michigan and at Nagasaki University, Japan, prior to arriving at Cold Spring Harbor Laboratory. This research will now be carried out in a biological research environment where this new protein sequencing technology, once developed, will be immediately applied to biologically important peptides and proteins isolated in small amounts as part of the Cancer Research Project at Cold Spring Harbor Laboratory.

Protein Structure of Replication Factors

R. Kobayashi [in collaboration with B. Stillman, Cold Spring Harbor Laboratory]

Since I became a part of the Protein Chemistry Core section in the tumor virus project at Cold Spring Harbor Laboratory, 70% of my effort has been to collaborate closely with other Cold Spring Harbor Laboratory scientists to study protein structure and function. This project will contribute to our understanding of the biochemical mechanism of transformation. I am and will be mainly participating in the study of proteins that have been already purified; methods include fragmentation of proteins to relatively smaller peptides to determine structures, peptide mapping, and protein sequence analysis. HPLC is usually employed to separate and purify peptides fragmented by protease digestion or chemical cleavage. This year, in collaboration with Bruce Stillman's laboratory, I have obtained some protein sequence information on replication factors in human and yeast using currently available methods and instruments in the Protein Chemistry Core facility in the Keck Center. Collaboration with other scientists at Cold Spring Harbor Laboratory will be carried out as well.

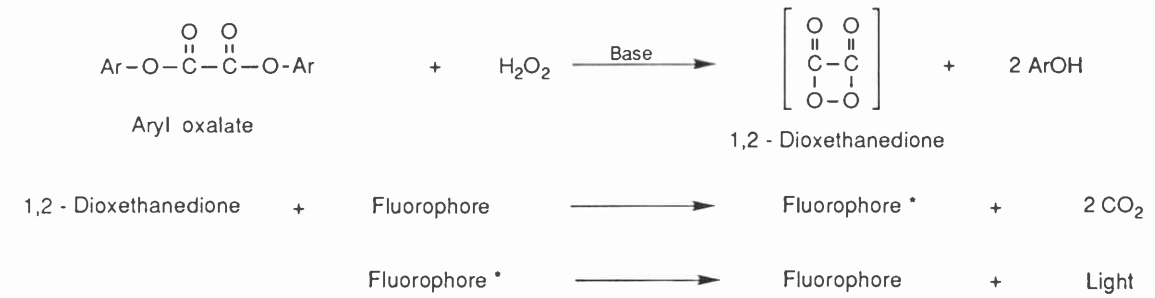

FIGURE 6 Chemiluminescence reaction.

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MOLECULAR GENETICS OF EUKARYOTIC CELLS

Members of the Molecular Genetics of Eukaryotic Cells Section study a variety of topics in cellular and organismal biology, including oncogenes, signal transduction, protein processing, the cytoskeletion, nuclear processing, and immunological responsiveness.

- Jacek Skowronski's laboratory (Transgenic Mice) utilizes gene transfer into the mouse germ line to model aspects of the acquired immunodeficiency syndrome (AIDS) and to study brain function. They have observed that the expression of the HIV-1 *ref* gene leads to the loss of $CD4^+$ T cells, a loss that occurs also during the progression of AIDS in humans.
- Michael Wigler's group (Genetics of Cell Proliferation) studies signal transduction pathways related to the *RAS* oncogenes. New elements of these pathways have been defined both in yeasts and in mammals. The characterization of these new components indicate previously undiscovered homologies between the signal transduction pathways of yeasts and mammals involving *RAS* and links to cytoskeletal organization.
- The laboratory of Dafna Bar-Sagi (Transmembrane Signaling) focuses on the role of RAS and phospholipase A (PLA_2) in signal transduction. She has demonstrated that cross-linking of the immunoglobulin receptors causes translocation of RAS to the cytoskeleton and that PLA_2 is involved in biochemical activation of RAS.
- Mike Gilman's laboratory (Nuclear Signal Transduction) studies the mechanisms by which signal transduction pathways lead to activation of gene expression. Recent experiments suggest a novel role for homeodomain proteins in these pathways.
- Nick Tonk's group, Structure, Function, and Regulation of Protein Tyrosine Phosphatases, studies those important components of signal transduction pathways. Novel forms of phosphotyrosine phosphatases have been identified. Their characterizations suggest their role in the control of cytoskeleton and cell adhesion.
- David Spector's section (Cell Biology of the Nucleus) develops and applies methods for the visualization of nuclear events at the ultra-microscopic level. They have visualized the transport of specific RNA products from the nucleus and have visualized the distribution and time of replication of satellite DNA within the nucleus.
- The group of Jim Garrels (Quest Protein Database Center) develops and applies programs for the analysis of two-dimensional gel electrophoresis separation of cellular proteins. These methods are being used to create a mouse embryo database, in collaboration with Davor Solter's group.
- Scott Patterson's group (Posttranslational Modifications: Regulatory and Targeting) works in close collaboration with the QUEST 2D gel group, utilizing those methods to analyze the posttranslational processing of proteins involved in signal transduction.
- The laboratory of Robert Franza (Quantitative Regulatory Biology) studies cellular proteins involved in the regulation of growth and differentiation of mammalian cells, particularly cells of lymphoid and hematopoietic lineages. Current studies focus on the components of the E1A complex, the c-rel protein, and proteins that regulate immediate early gene expression.
- David Helfman's laboratory (Molecular Cell Biology) focuses on two fundamental problems: the mechanisms of regulated RNA splicing and the function of isoforms of cytoskeletal components. They have identified an RNA-binding protein involved in the differential splicing of tropomyosins and have identified a new family of actin-like molecules.

TRANSGENIC MICE

J. Skowronski R. Mariani N. Peunova L. Usher G. Enikolopov

Gene transfer into the mouse germ line provides a model system in which to study complex biological problems at the organismal level. In particular, transgenic mice provide a powerful tool to assess the disease-inducing potential of viral genes and to generate animal models where complex processes involved in disease induction are reproduced and accessible to study. In the last 3 years, our main focus has been to use this transgenic approach to address mechanisms involved in induction of acquired immunodeficiency syndrome (AIDS) by the human immunodeficiency virus type 1 (HIV-1) and to identify HIV-1 genes involved. Recently, our work has demonstrated that the HIV-1 *nef* gene effects depletion of the $CD4^+$ T cells when expressed in transgenic animals. This is reminiscent of $CD4^+$ T-cell depletion invariably associated with AIDS immunodeficiency in HIV-1-infected individuals. Our current experiments address the mechanism of $CD4^+$ T-cell loss in this transgenic mouse model and the biochemical mechanisms of Nef action. In addition, Grigori Enikolopov and Natalya Peunova are interested in studying signaling pathways in the brain. They have constructed recombinant inhibitors of protein kinases and are applying them to investigate depolarization-induced gene activation in the neuronal cells.

Transgenic Mouse Models for AIDS Research

R. Mariani, L. Usher, J. Skowronski

HIV infection of $CD4^+$ T cells and antigen-presenting cells (APCs) is associated with deregulation of the immune system and the depletion of $CD4^+$ T cells that is invariably associated with the immunodeficient state in AIDS. However, the mechanisms by which immunodeficiency viruses subvert the normal function of infected cells or even the identity of viral genes that mediate these effects in infected $CD4^+$ T cells are not well understood.

In search of HIV-1 genes that may play a direct role in disease induction in HIV-1-infected individuals, we became interested in one of the less-characterized HIV-1 genes, the *nef* gene. The *nef* gene is found in both human (HIV-1 and HIV-2) and simian immunodeficiency viruses, and it encodes related small cytoplasmic proteins of 25–30 kD. Nef polypeptides are well conserved among independent HIV-1 isolates; however, *nef* is not required for virus replication in established cell lines, suggesting that *nef* may have a role in the in vivo infection and perhaps can perturb the normal function of infected cells, thus playing a direct role in disease induction. To test this latter hypothesis, a series of experiments, in which expression of two independent isolates of the HIV-1 *nef* genes was directed to T cells and APCs in transgenic animals, was initiated approximately 2 years ago.

CD3 NEF1 TRANSGENIC MICE

Recently, we have constructed strains of transgenic mice that express the *nef* gene of the HIV-1 NL43 isolate in T cells. This was accomplished by placing the HIV-1 *nef* gene under the control of transcription regulatory elements derived from the murine gene encoding the δ subunit of the CD3/T-cell receptor complex. Six independent lineages of CD3 Nef1 transgenic animals have been established.

Nef protein expression in CD3 Nef1 animals was assessed by immunoblot analysis as shown in Figure 1 (top). Rabbit anti-Nef serum detected two proteins in extracts prepared from the thymus of all six lines of transgenic animals examined, but not in those prepared from the control C3H mice. The major species migrating slightly slower than the recombinant Nef standard has a predicted molecular mass of 25–27 kD and therefore corresponds to the Nef polypeptide. The nature of a smaller and less abundant protein migrating at 20–22 kD and faster than the Nef standard is not known, although it may represent the amino-terminally truncated form of Nef or a degradation product. Quantitation of Nef protein expression, performed by comparison with known amounts of the recombinant Nef, revealed that Nef constituted approximately 0.01–0.03% of total thymic protein in CD3 Nef1 1, 2.2, 3.2, and 5.2 mice and therefore was expressed at a moderate level. In CD3 Nef1 3.1 and 4 animals, Nef was expressed at

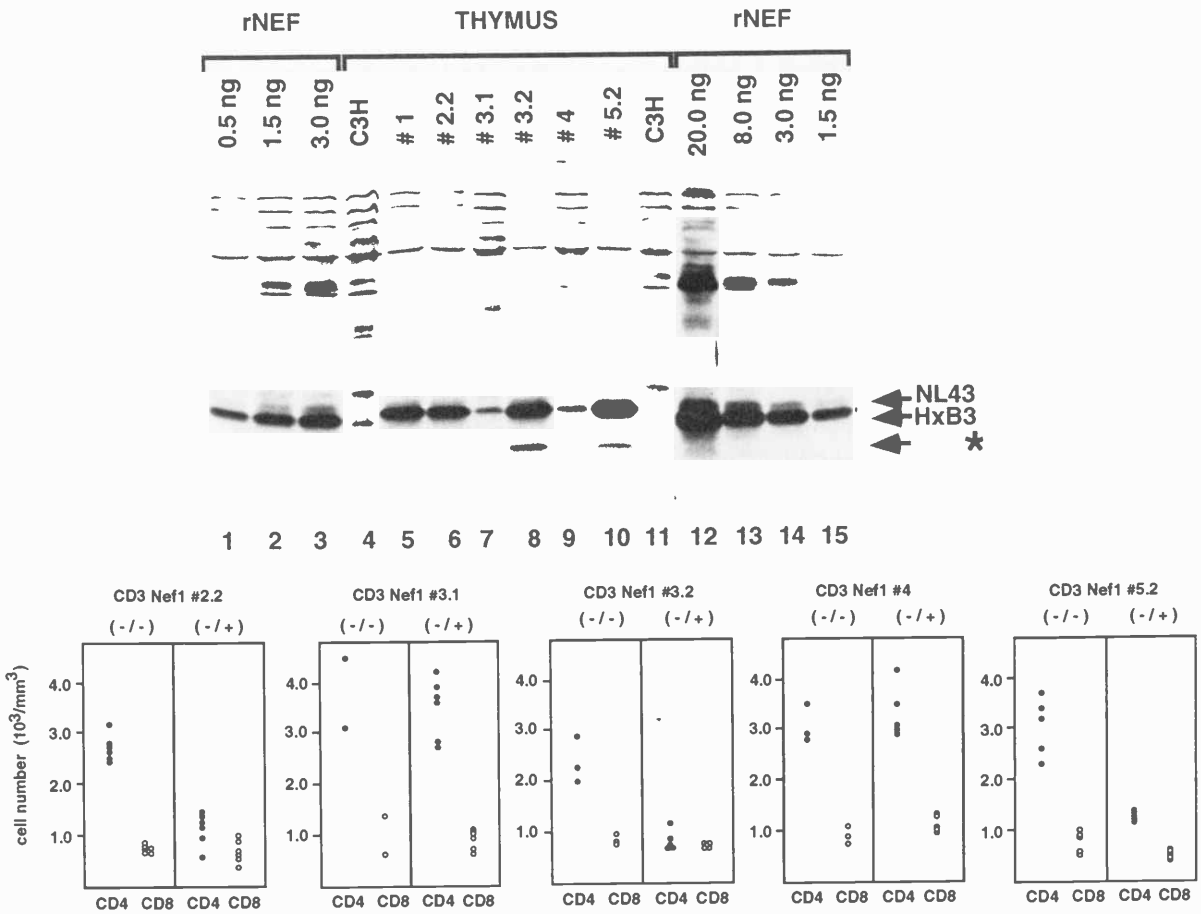

FIGURE 1 CD4$^+$ T cell depletion in CD3 Nef1 transgenic mice. (*Top*) Western blot analysis of Nef protein expression in CD3 Nef1 transgenic mice. Protein extracts prepared from thymus collected from transgenic animals of CD3 Nef1 lines (THYMUS: #1, #2.2, #3.1, #3.2, #4, #5.2), and control C3H animal (C3H) were analyzed by Western blotting. Aliquots of C3H thymic extracts supplemented with recombinant Nef protein (rNEF) were used as positive control and standards for quantitation (rNEF). Blots were probed with affinity-purified rabbit α-*nef* antibodies and antigen-antibody complexes detected with goat α-rabbit IgG/alkaline phosphatase conjugates. Arrows indicate the bands corresponding to the rNef (HxB3) and NL43 Nef (NL43) proteins. Asterisk indicates the 20–22 kD Nef-related protein detected in thymic extracts from transgenic animals. (*Bottom*) T-cell subsets in PBLs of CD3 Nef1 transgenic mice. CD4$^+$ and CD8$^+$ T-cell counts in PBLs of transgenic (–/+) and control (–/–) littermates are shown. The fraction of CD4$^+$ and CD8$^+$ T cells was determined by flow cytometry analysis of leukocytes isolated from peripheral blood and stained with anti-CD4 and anti-CD8 monoclonal antibodies (GK-1.5 PE and 53-6.7 biotin, respectively, followed by streptavidin-FITC). The PBL counts were averages of six to eight hemocytometer counts of blood samples stained with crystal violet. Each mouse analyzed is represented by a pair of circles (*closed circles* for CD4 and *open circles* for CD8, respectively). For each mouse, 30,000 PBLs were analyzed on an EPICS C flow cytometer equipped with an argon laser, and each circle represents a result of a single determination. Residual dead cells and debris was excluded from analysis by electronic gating on forward and 90^0 light scatter.

5–15-fold lower levels (compare lanes 7 and 9 with 6, 8, and 10). Surprisingly, Nef protein was not detected by Western blotting in lymph node extracts despite reproducible transgene expression in the thymus and the demonstrated ability of CD3 transcription regulatory elements to direct expression of other hybrid genes to both thymic and peripheral T cells in transgenic mice at comparable levels.

CD4$^+$ T CELLS ARE PREFERENTIALLY DEPLETED IN CD3 NEF1 ANIMALS

To assess the status of mature T cells, leukocytes isolated from peripheral blood lymphocytes (PBLs) of transgenic and control littermate animals were stained with the anti-T-cell receptor antibody and analyzed by flow cytometry. The total T-cell count in PBLs of the transgenic CD3 Nef1 2.2 and 3.2 animals

was approximately twofold lower than that in control littermates. To confirm and extend these observations, analysis of the $CD4^+$ and $CD8^+$ T-cell subsets was performed in animals from five transgenic lines. As seen in Figure 1 (bottom), significant and highly preferential decrease of the $CD4^+$ T cells was detected in PBLs of Nef1 2.2, 3.2, and 5.2 animals. In these mice, the $CD4^+$ T-cell count is two to three times lower than that in control animals, whereas the number of $CD8^+$ T cells is almost normal. Significantly, this preferential depletion of $CD4^+$ T cells was not detected in the CD3 Nef1 3.1 and 4 mice, which display a considerably lower level of transgene expression, suggesting that a certain threshold of *nef* gene expression is required to elicit the perturbation or that transgenes may be expressed only in a small population of T cells in these latter lineages, thus precluding detection of the perturbation.

NEF PERTURBS THYMIC DEVELOPMENT OF $CD4^+$ T CELLS

To explore the nature of the defect elicited by Nef in transgenic animals, lymph node and thymic T cells from 3–5-week-old high-expressor CD3 Nef1 2.2 and 3.2 mice were studied. Lymph nodes of transgenic animals were smaller and yielded roughly half as many cells as those of control animals. Flow cytometry analysis with antibodies reacting with the CD3, CD4, and CD8 antigens revealed that the $CD4^+$ T cells were preferentially depleted in transgenic lymph nodes.

Transgenic thymuses had a normal appearance and contained normal or a slightly higher number of cells than those of control littermates (1.25- and 1.5-fold more in Nef1 2.2 and 3.2 lines, respectively, than in the controls). Flow cytometry analysis of thymocytes stained for the CD4 and CD8 surface antigens was used to define the four major developmental thymocyte populations, including the immature $CD4^-CD8^-$ double-negative and the $CD4^+CD8^+$ double-positive thymocytes and the mature $CD4^+$ or $CD8^+$ single-positive T cells. Cells were also analyzed for the CD3 antigen, which is expressed at a low level on a subpopulation of the double-positive T cells ($CD3^{low}$) and at a high level on the mature single-positive thymic T cells ($CD3^{high}$). This analysis detected several alterations of both the immature and mature thymocyte populations in transgenic animals, including a drastic reduction in the level of CD4 antigen surface expression and a less pronounced reduction of CD8 antigen surface expression on double-positive immature T cells. Despite altered surface phenotypes, the overall numbers of the $CD4^+CD8^+$ double-positive thymocytes were not decreased, indicating that Nef does not have a direct toxic effect on these cells. In contrast to the apparently normal frequency of double-positive $CD4^+CD8^+$ thymocytes, the $CD4^+$ single-positive T cells were two- to threefold underrepresented in CD3 Nef1 animals. This depletion of the mature T cells was also reflected by a twofold lower content of cells that express high levels of CD3 antigen. These results indicated that *nef* perturbs development of the $CD4^+$ T cell subset preferentially.

The late stages of thymic T-cell development, which are disrupted in CD3 Nef1 transgenic mice, are dependent on interactions of the T-cell receptor (TCR) with its ligands. Surface CD4 antigen interacts with the major histocompatibility complex class II antigens on APCs, and this is required for normal TCR signaling. Thus, it is possible that a *nef*-elicited defect in CD4 antigen function and/or T-cell receptor function is responsible for compromised development of $CD4^+$ T cells in CD3 Nef1 transgenic mice, and an analogous mechanism may be used by HIV-1 to perturb function of infected $CD4^+$ T cells in humans. We are currently defining alterations in normal function of CD3 Nef1 T cells and the mechanism of $CD4^+$ T-cell depletion in these transgenic animals.

Downregulation of Surface CD4 Antigen Is a Frequent Property of HIV-1 *nef* Genes Isolated from HIV-1-infected Individuals

R. Mariani, J. Skowronski

Our work has shown that the HIV-1 NL43 *nef* expression in transgenic mice is associated with low levels of CD4 antigen expression on the T-cell surface and depletion of a significant fraction of their $CD4^+$ T cells. Modulation of the CD4 antigen surface expression, observed also by other investigators in experiments in established T cell lines (Garcia et al., *Nature 350:* 508 [1991]), may be critical to the effects of *nef* gene expression. It was therefore important to determine whether modulation of surface CD4 antigen expression is a common property of different HIV-1 *nef* genes isolated directly from infected individuals. To address these questions, we have cloned

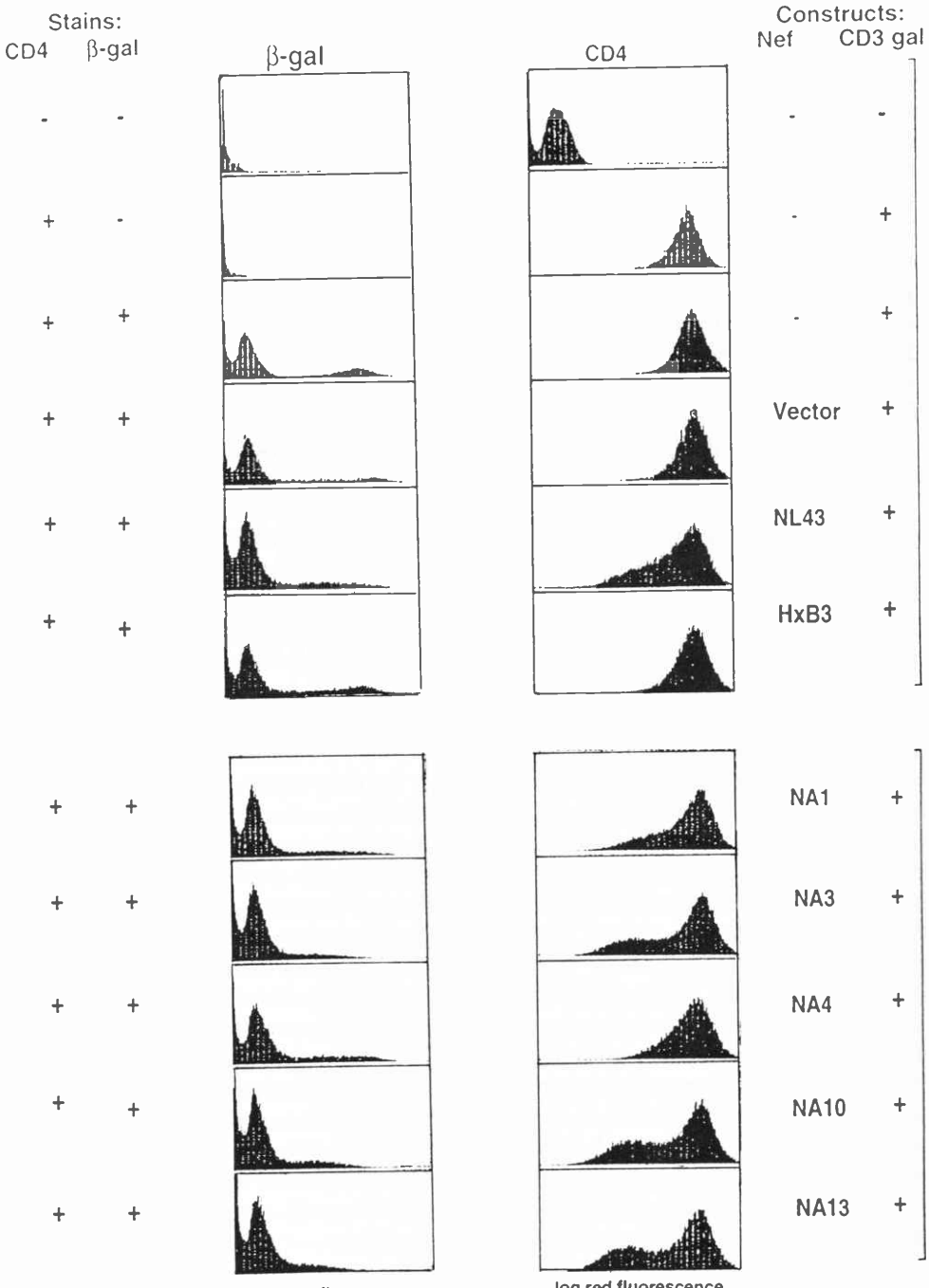

FIGURE 2 Transient assay for downregulation of CD4 antigen on the surface of human CEM T cells. CEM cells electroporated with various CD3 Nef expression constructs (CD3 NA) and/or the CD3 *gal* reporter gene (right side of the panel) are shown. Cells were stained for the surface CD4 antigen and/or *E. coli* β-galactosidase (*left*). Histograms of the CD4 stain and of the β-galactosidase stain are shown on the right and left panels, respectively. Vector, NL43, and HxB3 represent the CD3 expression vector per se, and CD3 vectors that direct expression of the HIV-1 NL43 and HxB3 *nef* genes, respectively. NA1 and NA13 are selected CD3 *nef* constructs that direct expression of new isolates of the HIV-1 *nef* gene cloned from the peripheral blood of an HIV-1-infected individual.

several new isolates of the HIV-1 *nef* directly from infected individuals.

We have developed a novel transient assay that permits us to quantitate effect of the *nef* on $CD4^+$ antigen expression on the surface of human CD4 CEM T cells. Nef expression vectors (CD3 NA vectors) were introduced into CEM T cells by electroporation. To provide an internal control for electroporation efficiency, the CD3 *gal* reporter gene that directs expression of the *Escherichia coli* β-galactosidase gene was coelectroporated into CEM cells; 40–48 hours after electroporation, surface CD4 antigen was detected with a monoclonal antibody labeled with phycoerythrin (red fluorescence). The β-galactosidase activity was detected in live cells with the fluorescein-labeled substrate (green fluorescence). As shown in Figure 2, both the cell surface expression of the CD4 antigen and β-galactosidase activity were detected and quantitated simultaneously on a cell-by-cell basis by FACS. Several new HIV-1 *nef* genes isolated from HIV-1-infected individuals were tested in this assay. It appeared that expression of most of these new clones (11 of 15 clones that were tested so far) resulted in downregulation of CD4 on a subpopulation of electroporated cells. Two-color analysis of electroporated cells demonstrated that those cells that show decreased expression of CD4 also show high levels β-galactosidase expression. Therefore, these cells represent a fraction of CEM cells that was successfully coelectroporated with both constructs. Different *nef* gene isolates had different abilities to reduce the level of surface CD4 in CEM cells, and three general classes of HIV-1 *nef* genes were defined in these experiments. First, some of the new isolates (see, e.g., NA10 and NA13 in Fig. 2) appeared to be much more potent in modulating surface CD4 antigen than the NL43 *nef* gene, which is associated with a low frequency of $CD4^+$ T cells in CD3 Nef1 transgenic mice. Genes of the second class had an effect similar to that of the NL43 *nef*. Finally, yet another group of isolates had no measurable effect of the surface expression of the CD4 antigen. Results of these studies indicated that the effect of Nef on surface CD4 antigen expression is a frequent property of HIV-1 *nef*. The observed modulation of surface CD4 antigen by Nef is, most likely, relevant to AIDS, because it has been observed with *nef* genes cloned directly from HIV-1-infected individuals, rather than from HIV-1 propagated (and perhaps selected) in vitro. Results of our studies in transgenic mice suggest that modulation of surface CD4 antigen may be critical to $CD4^+$ T-cell depletion in AIDS. We therefore will test whether *nef* genes in AIDS patients showing fast or slow rate of disease progression differ in their ability to modulate expression of the CD4 antigen. In the next year, we will also initiate experiments to address the biochemical mechanism(s) that mediates effects of *nef* gene expression in T cells.

Dissection of Signaling Pathways in Neuronal Cells with Recombinant Inhibitors of Protein Kinases

N. Peunova, G. Enikolopov

We are interested in how gene activity in the nucleus is changed when the membrane of the neuronal cell is excited by an incoming stimulus, such as depolarization, a neurotransmitter, or a combination of both.

We have developed a new approach to investigate the components of the signaling machinery in neuronal cells based on the use of the recombinant pseudosubstrate inhibitors of protein kinases. Protein kinases are involved in the modulation of synaptic plasticity in the brain and are directly implicated in the transduction of the synaptic signal from the membrane to the nucleus. Several protein kinases are regulated by an autoinhibitory mechanism involving a pseudosubstrate domain. We have prepared recombinant constructs encoding polypeptides that act as highly specific pseudosubstrate inhibitors of the corresponding protein kinases. We are using them as tools to dissect signaling events in neuronal cells and to investigate the points of overlap between different signal transduction pathways.

This year, our efforts were concentrated on increasing the potency of recombinant inhibitors and on applying them to study gene activation by membrane depolarization. We have prepared recombinant expression constructs encoding pseudosubstrate inhibitors of cAMP-dependent protein kinase (PKA), protein kinase C (PKC), and Ca^{++}/calmodulin-dependent protein kinase II (CaMK). We were able to augment the activity of the recombinant inhibitors (50–100-fold in some cases) while retaining their specificity toward the cognate kinases by changing particular residues in the inhibitory regions and by fusing them to other protein domains. This increase probably reflects both increased K_i and stability of the polypeptides in the cell. This approach worked particularly well for inhibitors of PKA, where, by swapping the autoinhibitory domain of the regulatory

subunit of PKA with a fragment of heat-stable PKA inhibitor (PKI), we increased the inhibitory activity of the constructs 300–600-fold. Tagging the inhibitory polypeptides with an epitope derived from the influenza virus hemagglutinin (HA) gene allowed us to visualize the chimeric products, in addition to increasing strongly their inhibitory abilities.

We made an additional modification of our expression vectors with the goal of uncoupling the cytoplasmic and nuclear effects of protein kinase inhibitors and making their action regulatable. We fused the inhibitory domains to the steroid hormone-binding domains of the estrogen receptor and glucocorticoid receptor, thus making the action of the inhibitors hormone-dependent.

We used the chimeric inhibitors of protein kinases to investigate the signal transduction pathways activated by depolarization in the neuronal cell line PC12. Depolarization of the excitable membrane of a neuronal cell leads to the activation of voltage-gated ion channels, an influx of calcium ions into the cell, and rapid induction of gene transcription. We wanted to know which protein kinases, transcription factors, and regulatory DNA sequences mediate depolarization-induced gene activation. We have addressed these questions by cotransfecting a series of derivatives of the c-*fos* promoter linked to the CAT reporter gene (from M. Gilman and L. Berkowitz) along with a set of recombinant inhibitors, depolarizing the membrane of the cells, and monitoring the changes in transcription of the c-*fos* plasmids. These experiments confirmed the observation that cAMP-response element (CRE) can act as a Ca^{++}-responsive element but showed that other elements contribute to the Ca^{++} response as well. Next, we cotransfected truncated variants of c-*fos* promoter plasmids with the recombinant protein kinase inhibitors and monitored whether inhibition of a particular kinase can block *fos* induction going through a particular DNA element. Our results demonstrate that CaMK, acting through the CRE element of the promoter, and PKC, acting through the serum response element (SRE), mediate the Ca^{++}-dependent induction of *fos* transcription. Thus, our data directly implicate CaMK and PKC in the depolarization-induced gene activation. To understand which transcription factors are involved, we have tested whether the chimeric proteins containing the DNA-binding domain of the yeast transcriptional activator GAL4 fused to the amino terminus of the CREB protein (constructed by L. Berkowitz and M. Gilman) can confer Ca^{++}-dependent inducibility on a reporter gene containing GAL4-binding sites in the promoter region. Depolarization of the cell membrane strongly activated the reporter gene, suggesting that the CREB is a target of the CaMK-mediated branch of the pathway. We are currently exploiting this approach to investigate other Ca^{++}-mediated events in neuronal cells, including the intracellular Ca^{++} release and synergistic interactions of the Ca^{++}- and cAMP-dependent signaling pathways.

Mobilization of Intracellular Calcium Potentiates cAMP-dependent Gene Activation

G. Enikolopov, N. Peunova

The increase of intracellular Ca^{++} concentration in neurons either by influx through the voltage-gated ion channels or by release from intracellular stores is involved in the regulation of many aspects of neuronal function. We are interested in how the Ca^{++}-dependent signaling pathways cross-talk and overlap with other pathways when a neuronal cell receives two different stimuli.

We used a c-*fos*-CAT reporter construct transfected into a neuronal cell line (PC12) to monitor the rapid induction of transcription by agents that elevate the calcium levels, in combination with other stimuli. We have used various procedures to promote the Ca^{++} concentration increase—membrane depolarization, agonist-dependent activation of calcium channels, neurotransmitter-mediated mobilization of intracellular Ca^{++}, direct release from the intracellular stores. Different ways of delivering Ca^{++} ions into the cells activate c-*fos* transcription to different extents. Activation of extracellular Ca^{++} influx by membrane depolarization and by mobilization of Ca^{++} after acetylcholine receptor stimulation can directly activate the c-*fos* promoter. When Ca^{++} ions are discharged from different intracellular stores by thapsigargin or ionomycin, direct induction of c-*fos* was not observed, but these treatments greatly potentiated the response of the *fos* gene to cAMP. When accompanied by the mobilization of intracellular calcium, cAMP-dependent induction of the *fos* gene is further enhanced up to tenfold.

Using a series of the c-*fos*-promoter deletion mutants, we have mapped the CRE site (located at −56 to −71) of the promoter as the site that mediates the potentiation effect, although other regions of the gene, located both upstream and downstream, con-

tribute to the enhanced response. Experiments with GAL4-CREB chimeric proteins and reporter constructs containing GAL4-binding sites have demonstrated that this effect can be conferred by CREB transcription factors. We are using combinations of recombinant inhibitors of protein kinases and of CREB mutants to investigate how the phosphorylation of CREB and the possible recruitment of other transcription factors are involved in the calcium-mediated enhancement of the cAMP response. Convergence of two different signaling pathways in the neuronal cells, seen as potentiation of cAMP-induced gene induction by the elevated Ca^{++} level may explain the mechanisms of integrating the impulse activity and synaptic activity in the nucleus of the neuron.

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GENETICS OF CELL PROLIFERATION

M. Wigler R. Ballester, G. Bolger, J. Field, T. Michaeli, Y. Wang, D. Young, J. Camonis, M. Kawamukai, N. Lisitsyn, K. Sen, E. Chang, J. Gerst, G. Heisermann, V. Jung, S. Marcus, A. Polverino, R. Swanson, L. Van Aelst, A. Vojtek, H.-P. Xu, M. Barr, C. Nicolette, K. O'Neill, G. Asouline, J. Brodsky, J. Douglas, M. Riggs, L. Rodgers

Our laboratory continues to study signal transduction, with the main focus on RAS-dependent signal transduction. In the yeast *Saccharomyces cerevisiae*, RAS proteins regulate adenylyl cyclase. The mechanism by which they do this is unknown, and this is not their only function. Since activation of adenylyl cyclase is not the function of mammalian RAS, the role of RAS in *Schizosaccharomyces pombe* has become a main effort within the group. The command of both *S. pombe* and *S. cerevisiae* systems gives us some clearer perspectives on the evolution of signal transduction pathways and provides us with very powerful tools for the identification and examination of mammalian genes encoding components of signaling pathways.

Signal Transduction in *S. cerevisiae*

R. Ballester, J. Fleld, T. Michaeli, J. Camonis, J. Gerst, G. Heisermann, V. Jung, A. Polverino, R. Swanson, L. Van Aelst, A. Vojtek, H.-P. Xu, M. Barr, M. Riggs, L. Rodgers

We have been studying the cellular components of *S. cerevisiae* that regulate RAS. Among these are two closely related proteins, IRA1 and IRA2 (Tanaka et al., *Mol. Cell. Biol. 9:* 757 [1989]; Tanaka et al., *Mol. Cell. Biol. 10:* 4303 [1990]). Individually, each has been shown to down-regulate RAS function. These proteins are structurally and functionally related to mammalian GAP, the RAS GTPase-stimulating protein (Tanaka et al., *Mol. Cell. Biol. 9:* 757 [1989]; Tanaka et al., *Mol. Cell. Biol. 10:* 4303 [1990]; Bal-

lester et al., *Cell 59:* 681 [1989]). Although GAP has also been shown to be capable of down-regulating RAS in both mammalian and yeast systems (Ballester et al., *Cell 59:* 681 [1989]; Zhang et al., *Nature 346:* 754 (1990]), there is some experimental evidence supporting the idea that GAP may also be an effector of RAS (Yatani et al., *Cell 61:* 769 [1990]). To test this hypothesis for IRA, both *IRA* genes were disrupted in yeast. The resulting phenotype was that expected for cells with an activated *RAS* allele: heat shock and starvation sensitivity. This phenotype was suppressible by overexpression of the genes encoding cAMP phosphodiesterases. These results suggest that the *IRA* genes do not encode effectors that link RAS to adenylyl cyclase. We cannot eliminate this hypothesis completely, however, since a third homolog of these GAP-like genes may yet be discovered in the genome of yeast, and one can imagine that each of the three might encode redundant RAS effectors. Speculations such as this, which invoke the unseen and undiscovered, will be more readily examined in the future when the complete genomic sequences of organisms favored for genetics will be known. Moreover, we have not eliminated the possibility that the *IRA* genes are required for the alternate function of RAS.

Strains lacking *IRA2* have the typical phenotype of cells with activated RAS, and we have isolated genes that when present on multicopy vectors can suppress this phenotype. Along with the expected genes, including *IRA2* itself, the *PDE* genes, encoding the cAMP phosphodiesterases, and *JUN1*, we found five other genes. These five fail to suppress the mutationally activated $RAS2^{val19}$ allele and hence probably act through the regulation of wild-type *RAS*. One of these genes encodes a carboxy-terminal truncated form of the CDC25 protein that is a RAS-nucleotide exchange catalyst (Broek et al., *Cell 48:* 789 [1987]; Powers et al., *Mol. Cell. Biol. 9:* 390 [1989]). This last result is consistent with the idea that the truncated form of CDC25 interferes with proper CDC25 function, since disruption of the genomic *CDC25* locus is known to suppress the phenotype of an *IRA* disruption. Thus, either the truncated form of CDC25 titrates a factor required for activation of wild-type CDC25 or it forms an ineffective complex with the wild-type CDC25 protein. The other four genes fall into two groups. The two genes of group I also suppress the heat-shock sensitivity of $ira1^{-}$ $ira2^{-}$ $cdc25^{-}$ strains. This result points to the existence of another mode for the regulation of RAS that is not dependent on the *IRA* genes or *CDC25*.

It is not known how RAS proteins stimulate adenylyl cyclase. Last year, we described CAP, an adenylyl-cyclase-associated protein that appears to be required for RAS to be fully capable of activating adenylyl cyclase (Field et al., *Cell 61:* 319 [1990]). Much of our effort to understand RAS/adenylyl cyclase interactions has focused on CAP, and this work is described in a separate section below.

Some time ago, Drs. Kataoka and Powers isolated a number of suppressor mutations of the $RAS2^{val19}$ phenotype (heat shock and starvation sensitivity). Among these were *supH*, or *RAM1*, required for the processing of RAS protein (Powers et al., *Cell 47:* 413 [1986]), and *supC*, encoding CAP, as discussed below (Field et al., *Cell 61:* 319 [1990]). We have recently isolated the gene corresponding to *supF*. It shows no clear homology with proteins in the databases. Complete disruption of *supF* does not completely block the phenotype of $RAS2^{val19}$ and has no effect on the phenotype of cells overexpressing the *TPK* genes, which encode the cAMP-dependent protein kinases (Toda et al., *Cell 50:* 277 [1987]). We hypothesize that *supF* encodes either a protein that facilitates RAS function (e.g., by affecting processing or localization) or a component of an alternate pathway for RAS action. These hypotheses are under study.

Signal Transduction in *S. pombe*

Y. Wang, D. Young, M. Kawamukai, K. Sen, E. Chang, V. Jung, S. Marcus, R. Swanson, H.-P. Xu, G. Asouline, M. Riggs, L. Rodgers

We have continued and expanded an intensive study of the RAS and adenylyl cyclase signaling pathways in *S. pombe* with the hope that the *S. pombe* systems may bear greater resemblance to mammalian cells and with the expectation that knowledge of the two highly diverged yeasts will lead to a greater understanding of each. Figure 1 diagrams some of the hypothetical wiring of the *S. pombe* systems inferred from our work and the work of other investigators. The figure also embodies some speculative ideas about homologies in these systems and those of *S. cerevisiae* and mammals.

S. pombe contains an adenylyl cyclase, encoded by the *cyr1* gene, that has considerable homology with the *S. cerevisiae* enzyme (Young et al., *Proc. Natl. Acad. Sci. 86:* 7989 [1989]). In *S. pombe*, cAMP levels modulate sexual responses (Beach et

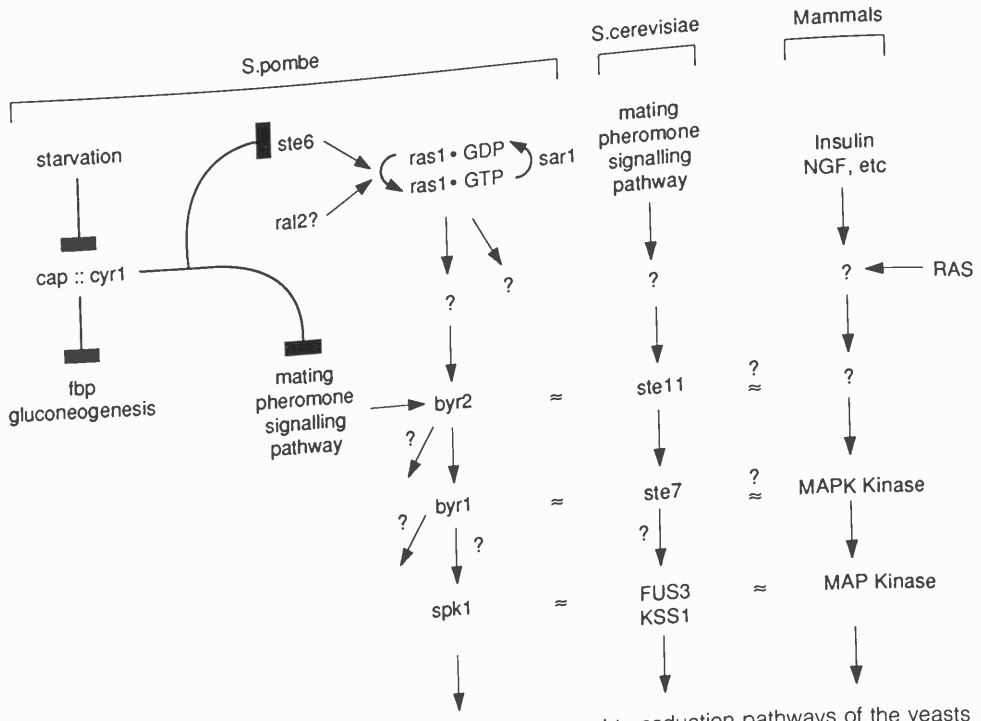

FIGURE 1 Diagram summarizing relationships among signal transduction pathways of the yeasts *S. pombe* and *S. cerevisiae* and mammalian cells. (≈) Homologies between proteins; (→) positive (activating) interactions; (*closed rectangles*) negative (inactivating) interactions; (?) proposed interactions or homologies.

al., *Curr. Genet. 10:* 297 [1985]; Kawamukai et al., *Cell Regul. 2:* 155 [1991]). Cells that overexpress cyr1 are relatively sterile, and cells that lack cyr1 conjugate prematurely. The *S. pombe* cyclase is not regulated by ras1 (Fukui et al., *Cell 44:* 329 [1986]; Nadin-Davis et al., *EMBO J. 5:* 2963 [1986]), the *S. pombe* RAS homolog, but we have inferred that cyr1 is subject to regulatory constraints similar to those of *S. cerevisiae* CYR1 (Kawamukai et al., *Cell Regul. 2:* 155 [1991]). First of all, the *S. pombe* cyr1 contains a leucine-rich repeat, a region homologous to the leucine-rich repeat of the *S. cerevisiae* CYR1 (Young et al., *Proc. Natl. Acad. Sci. 86:* 7989 [1989]). Overexpression of this region in *S. pombe* interferes with cyr1, as does overexpression of the comparable region in *S. cerevisiae* (Field et al., *Science 247:* 464 [1990]). Moreover, the *S. pombe* cyclase binds a protein, cap1, that is homologous to *S. cerevisiae* CAP. Disruption of *S. pombe cap1* causes an apparent failure of *S. pombe* cyr1 to be regulated properly. We do not know what factors activate *S. pombe* cyr1, but it is a question of some importance, since data presented in a subsequent section lead us to speculate that mammalian cells have a cyr1 homolog.

S. pombe contains a gene, *sar1*, that encodes a protein that is homologous to the yeast IRA proteins and the mammalian *GAP* and *NF1* gene products (Wang et al., *Cell Regul. 2:* 453 [1991]). *sar1* was selected as a multicopy suppressor of the activated $ras1^{val17}$ allele. Like its counterparts in *S. cerevisiae*, sar1 appears to down-regulate ras1, and there is no evidence whatsoever that it functions as a ras1 effector. Again, we cannot exclude the possibility that *S. pombe* contains a functional homolog of sar1, but cells with disruptions of sar1 are still ras1-sensitive. sar1 function differs from that of IRA1, IRA2, and GAP in that it is capable of inhibiting mutationally activated ras1. In this respect, sar1 resembles NF1, the product of the von Recklinghausen's neurofibromatosis locus, which can inhibit mutationally activated Ha-ras (Ballester et al., *Cell 63:* 851 [1990]).

S. pombe cells that lack *ras1* are sterile: Haploid cells fail to conjugate and diploid cells cannot undergo sporulation (Fukui et al., *Cell 44:* 329 [1986]; Nadin-Davis et al., *EMBO J. 5:* 2963 [1986]). We have screened for genes that on high copy are capable

of suppressing these defects. One such gene, previously identified by other investigators, is *byr1* (Nadin-Davis and Nasim, *EMBO J. 7:* 985 [1988]). *byr1* encodes a protein kinase and is capable of inducing sporulation in *ras1*$^-$/*ras1*$^-$ diploid. We identified a second gene, *byr2*, that also encodes a protein kinase and also can induce sporulation in *ras1*$^-$/*ras1*$^-$ diploids (Wang et al., *Mol. Cell. Biol. 11:* 3554 [1991]). Genetic interactions suggest that the byr2 product lies upstream of the byr1 product. We have found that both *byr1* and *byr2* can weakly suppress the sterility of *ras1*$^-$ haploids.

byr1 is most homologous to the *S. cerevisiae* gene *STE7*, and *byr2* is most homologous to the *S. cerevisiae* gene *STE11*. Both STE7 and STE11 are kinases that function on the mating pheromone pathway in *S. cerevisiae* (Teague et al., *Proc. Natl. Acad. Sci. 83:* 7371 [1986]; Rhodes et al., *Genes Dev. 4:* 1862 [1990]). Hence, the mating pathways of these two diverged yeasts are at least partially conserved. We can speculate further on these relationships. In both *S. pombe* and *S. cerevisiae*, there are other kinases required for sexual function: in *S. pombe*, spk1 (Toda et al., *Genes Dev. 5:* 60 [1991]), and in *S. cerevisiae*, FUS3 and KSS1 (Elion et al., *Cell 60:* 649 [1990]; Courchesne et al., *Cell 58:* 1107 [1989]). These kinases are related to the MAP/ERK protein kinases that have been identified in mammalian cells as response elements in extracellular signaling mediated through receptor protein tyrosine kinases (Cantley et al., *Cell 64:* 281 [1991]; Cobb et al., *Curr. Opin. Cell Biol. 3:* 1025 [1991]). In mammalian cells, the phosphorylation and activation of the MAP/ERK kinases is RAS-dependent (Thomas et al., *Cell 68:* 1031 [1992]). If we postulate that the KSS1, and FUS3, and the spk1 kinases are regulated by the STE7 and byr1 kinases, respectively, we can imagine drawing a set of homologies between the yeast and mammalian signal transduction pathways as shown in Figure 1. The implication of this speculation, if correct, is that the RAS effector pathways in mammals and *S. pombe* may be homologous.

We have isolated another gene, *byr3*, that can induce sporulation in *ras1*$^-$/*ras1*$^-$ diploid yeast. This gene encodes a protein with seven zinc-finger-binding domains and is homologous to a mammalian protein that was isolated based on its DNA-binding activity (Rajavashisth et al., *Science 245:* 630 [1989]). In collaboration with Dr. Rajavashisth (University of California, Los Angeles), we are examining the conservation of function between these two proteins.

Mammalian Genes That Interact with Yeast Signaling Pathways

R. Ballester, J. Camonis, E. Chang, J. Gerst, C. Nicolette, L. Van Aelst, A. Vojtek, G. Asouline, M. Riggs, L. Rodgers

Yeast may be used to select mammalian cDNAs encoding products that interact with yeast signaling pathways (Lee and Nurse, *Nature 327:* 31 [1987]; Colicelli et al., *Proc. Natl. Acad. Sci. 86:* 3599 [1989]; Colicelli et al., *Proc. Natl. Acad. Sci. 88:* 2913 [1991]). We have employed *S. cerevisiae* strains containing RAS2^{val19} to select mammalian cDNAs that suppress the RAS2^{val19} phenotype. Three cDNA genes were so isolated: *JC99*, *JC265*, and *JC310* (Colicelli et al., *Proc. Natl. Acad. Sci. 88:* 2913 [1991]). All three also suppress the phenotype of *ira*$^-$ strains but not the phenotype of cells that overexpress either adenylyl cyclase or the cAMP-dependent protein kinase catalytic subunits. Sequence determinations indicate that *JC99* and *JC265* are in the same gene family. Otherwise, the three genes show no significant relationship to other genes in the databases.

In an attempt to understand how these mammalian genes function, we have expressed them in *S. pombe* and in mammalian cells. Preliminary studies indicate that expression of JC310 in NIH-3T3 cells blocks the mitogenic and morphological responses of these cells to phorbol esters, but not the activation of protein kinase C itself. These results are compatible with the hypothesis that JC310 blocks aspects of RAS function in mammalian cells, for it is known that RAS function is required for the morphologic and mitogenic effects of protein kinase C. This points to a possible evolutionary conservation in the function of JC310. However, expression of JC310 appears to have no evident phenotypic effects in either wild-type or mutant strains of *S. pombe.*

Expression of JC99, on the other hand, has profound effects in *S. pombe.* Expression has minimal effects on wild-type and *ras1*$^-$ cells, slightly promoting sporulation in *ras1*$^-$/*ras1*$^-$ diploids and haploid sporulation in *ras1*$^-$ or wild-type cells; but expression strongly suppresses the sexual dysfunction in cells that have activated the cAMP-signaling pathways. Cells that overexpress adenylyl cyclase or that lack the regulatory subunit of the cAMP-dependent protein kinase are virtually sterile. Moreover, they are elongated and heat-shock-sensitive. Expression of JC99 reverses these phenotypes, yet does not suppress the phenotypes of altered glucose metabolism

associated with activation of the cAMP pathway. Hence, in *S. pombe*, JC99 acts downstream from the cAMP pathway, where it interacts with gene products involved in sexual function. We do not yet understand how to reconcile these results with the effects of JC99 in *S. cerevisiae*, where it does alter phenotypes due to activation of the cAMP pathway but is unable to effect strains with activating mutations in adenylyl cyclase or the cAMP-dependent protein kinases. If expression of JC99 has effects in mammalian cells, these are not yet clear to us.

We have also selected several mammalian cDNAs on the basis of their ability to complement loss of ras1 function in *S. pombe*. These genes are still under study.

CAP, and Adenylyl-cyclase-associated Protein

J. Field, D. Young, M. Kawamukai, J. Gerst,
A. Vojtek, M. Riggs, L. Rodgers

The year before last we reported the cloning of *S. cerevisiae CAP* (Field et al., *Cell 61:* 319 [1990]). This gene encodes a product required for full responsiveness to activated RAS. CAP is bifunctional (Gerst et al., *Mol. Cell Biol. 11:* 1248 [1991]). The amino-terminal domain binds adenylyl cyclase and is required for cellular RAS responsiveness. The carboxy-terminal domain is required for a variety of other cellular functions. Cells that lack the carboxyl terminus of CAP are temperature-sensitive, have disrupted actin cables, are enlarged and round, bud randomly from the cell surface, and show a variety of nutritional abnormalities, including valine toxicity, failure to grow in rich medium, and exquisite sensitivity to starvation (Gerst et al., *Mol. Cell. Biol. 11:* 1248 [1991]; Vojtek et al., *Cell 66:* 497 [1991]). The amino and carboxyl termini of CAP appear to act independently and are connected by a proline-rich middle portion that to date appears to be phenotypically silent.

To understand the carboxy-terminal function of CAP, we have sought genes that when overexpressed could suppress the phenotypes that result from its loss. One such gene is profilin, a low-molecular-weight protein that binds actin monomers and also binds phosphoinositides (Vojtek et al., *Cell 66:* 497 [1991]). Overexpression of profilin corrects the temperature-sensitive growth defects and the cytoskeletal, morphological, and nutritional abnormalities of cells lacking the carboxyl terminus of CAP, but not the RAS responsive abnormalities of cells lacking the amino terminus of CAP. Moreover, cells lacking profilin display growth and morphological abnormalities that closely resemble the phenotype of cells lacking the carboxyl terminus of CAP. These results point to an intimate relationship between the function of CAP and the function of profilin, a relationship that is strengthened by the discovery of a mammalian CAP (see below).

Another multicopy suppressor of the loss of the carboxy-terminal function of CAP is a gene we have called *SNC1*. *SNC1* encodes a small protein that has striking homology with the VAMP/synaptobrevin family of vertebrate synaptic-vesicle-associated proteins. These are low-molecular-weight proteins with very hydrophobic carboxy-terminal domains that are thought to function in neurotransmitter release. The presence of VAMP homologs in yeast suggests a more general function, perhaps in membrane vesicle fusion. We do not understand how the function of SNC1 relates to the function of CAP.

Curiously, SNC1 expression suppresses the loss of the carboxyl terminus of CAP only in cells with an activated RAS pathway; activation of the cAMP pathway is not sufficient. A similar result was obtained in the suppression of loss of carboxy-terminal CAP by human profilin: Effective suppression was observed only in RAS-activated strains. These results suggest that RAS, acting through its alternate pathway, in some way effects the functions of CAP, profilin, and SNC1.

The *S. cerevisiae CAP* gene has an *S. pombe* homolog (Kawamukai et al., *Mol. Biol. Cell 3:* 167 [1992]). The existence of this protein was first inferred from the presence of a cross-reacting antigen in preparations of adenylyl cyclase purified from *S. pombe*. The gene was independently cloned in two ways: by immunological screening of *S. pombe* cDNA expression libraries in *Escherichia coli* and by complementation screening of a *cap*$^-$ *S. cerevisiae* strain with cDNAs from *S. pombe*. *S. pombe* and *S. cerevisiae* have homologous carboxy-terminal function. Disruption of the carboxy-terminal domain of *S. pombe* cap1 causes temperature sensitivity and morphological abnormalities. Disruption of the amino-terminal domain causes loss of proper regulation of adenylyl cyclase. Thus, it is likely that in both yeasts, amino-terminal CAP function is required for the

proper regulation of adenylyl cyclase, whereas carboxy-terminal function is somehow related to cell morphology and growth.

Recently, Makoto Kawamukai, upon his return to Japan, cloned a human homolog of CAP. Even more recently, the isolation and purification of an actin-binding protein from pig platelets and the sequence of tryptic peptides were published (Gieselmann and Mann, *FEBS Lett. 298:* 149 [1992]). This sequence showed near identity to the human CAP. Since the amino-terminal domain of the mammalian CAP has homology with those of yeast, we infer that we may find a form of mammalian adenylyl cyclase that is homologous to the yeast adenylyl cyclases. Since the mammalian CAP is associated with actin, and can function in yeast, we infer that the mammalian CAP also regulates the cellular cytoskeleton and that yeast CAP, like yeast profilin, may regulate actin function.

Mammalian cAMP-specific Phosphodiesterases

T. Michaeli, G. Bolger, K. O'Neill, M. Riggs, L. Rodgers

Selection for mammalian cDNAs that could suppress the phenotypes of *S. cerevisiae* strains with the $RAS2^{val19}$ mutation led to the cloning of mammalian cDNAs encoding phosphodiesterases. Several of these showed homology with the *Drosophila melanogaster dunce* gene. We have now explored the structure of this gene family in humans and rats. By utilizing polymerase chain reaction (PCR) primers and genomic DNA as a template, we have identified a total of four genes in this family, both from rats and humans. Functional cDNA sequences of three of these four genes have been cloned from humans, although we do not know whether the 5′ extremities of the coding regions have been reached. These genes encode high-affinity cAMP-specific phosphodiesterases which are inhibitable by rolipram. Using yeast lacking endogenous cAMP phosphodiesterases, which also have a heat-shock-sensitive phenotype, we have cloned a human cDNA encoding a novel form of cAMP phosphodiesterase. This gene, given the provisional name TM22, encodes a high-affinity cAMP-specific phosphodiesterase that is not inhibitable by either rolipram or milrinone. It therefore encodes a previously undetected form of cAMP phosphodiesterase.

Genomic Difference Cloning

N. Lisitsyn

Sensitive methods for the detection of sequence differences between like genomes are potentially of great utility in understanding the genetic basis of disease. Such methods have potential application to the discovery of novel pathogens and the definition of genetic lesions in tumor cells. We previously published a method that allows the enrichment of difference sequences between two genomes by factors of several hundred (Wieland et al., *Proc. Natl. Acad. 87:* 2720 [1990]). We have modified this approach in two fundamental ways. First, we simplify the genomes to be compared by taking an "isomorphic representation" of each. This entails making an equal sampling of both genomes and examining sequence differences between these simplified DNA populations. Second, after rounds of subtractive hybridization, we further enrich difference sequences by melting, annealing, and selecting for double-stranded DNA. For sequences enriched by a factor of N, this step can, theoretically, give another N-fold enrichment. Using this approach, and model systems in which we have added λ DNA or *E. coli* DNA at single-copy levels to human DNA, subtracted against the same DNA without the added sequences, we have enriched the difference sequences by as much as 100,000-fold.

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TRANSMEMBRANE SIGNALING

D. Bar-Sagi M. Boyer, N. Gale, P. Gesteland, L. Graziadei, K. Degenhardt, S. Kaplan, Y. Yeh

We are interested in signal transduction pathways involved in growth control. Our research focuses on the role of Ras proteins in signal transduction. Since their discovery in 1982, cellular *ras* genes have been the focus of intense research because their mutated alleles are prevalent in various carcinogen-induced animal tumors as well as in a significant proportion of human tumors. In addition, *ras* genes have attracted much interest because they have been highly conserved during evolution and are therefore presumed to be involved in a basic cellular function. The identity of this function is unknown. The proteins encoded by *ras* genes bind guanine nucleotide, possess an intrinsic GTPase activity, and are localized to the inner face of the plasma membrane. On the basis of these properties, it has been postulated that Ras proteins are intermediates in a transmembrane signaling pathway, acquiring a signal from an upstream element and passing it on to a downstream target. During the past year, we have continued to study the mechanism of signal transduction by Ras. Through the use of signaling events in the immune system, we have obtained some insights into the possible identity of the Ras signaling pathway.

Interaction of Ras Proteins with the Cytoskeleton

M.J. Boyer, D. Bar-Sagi

Our laboratory has recently demonstrated that Ras cocaps with surface immunoglobulin receptors (sIg) when these receptors are cross-linked by multivalent ligands. The capping of sIg is an energy-dependent process that is mediated by the contractile activity of the peripheral cytoskeleton. Furthermore, it has been shown that in the course of their ligand-induced redistribution, sIg molecules form a physical association with the underlying cytoskeleton. Since the redistribution of Ras exactly mimicked the redistribution of sIg, and since both redistributions were inhibited by cytoskeletal disrupting agents, we hypothesized that Ras proteins may become associated with the cytoskeleton following ligand-receptor interaction. The strategy we used to examine the possible association of Ras with the cytoskeleton is based on the use of differential solubilization by nonionic detergents of cellular proteins. This technique enables the fractionation of cytoskeletal attached components (detergent insoluble) from noncytoskeletal attached components (detergent soluble). Thus, we compared

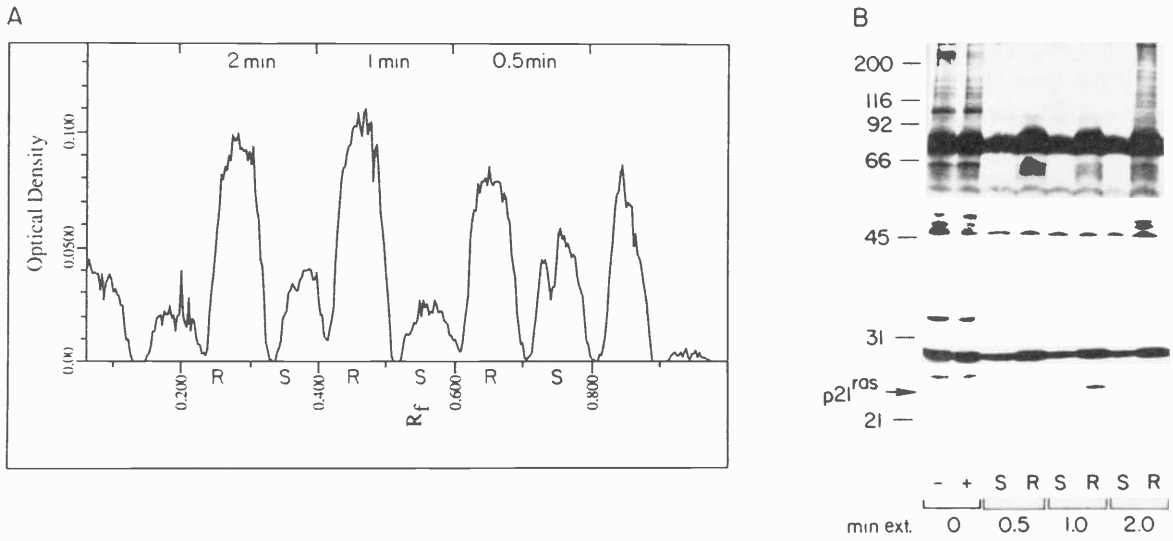

FIGURE 1 Detergent extraction of Ras from resting and stimulated B cells. [^{35}S]Methionine-labeled cells were stimulated with anti-immunoglobulin antibodies and detergent-extracted for various intervals. The amount of Ras polypeptides in the detergent lysate was determined by immunoprecipitation. (*A*) Densitometric scan of the Ras band from respective samples in the gel shown on panel *B*. (*B*) Autoradiogram of immunoprecipitates carried out in detergent extracts from resting (R) and stimulated (S) cells which were detergent-extracted for the indicated intervals (min ext.). Control immunoprecipitations (0 min ext.) to establish the specificity of the reaction were carried out on resting cells using anti-Ras antibodies that were either unblocked (+) or blocked (–) by preincubation with purified Ras protein. Note that the amount of Ras in the detergent-soluble extract is significantly reduced in stimulated cells, indicating that a portion of Ras molecules became associated with the detergent-insoluble fraction.

the detergent solubility of Ras in resting and stimulated B cells. The detergent extraction protocol that we employed involved the use of nonionic detergent in high-sucrose buffer. The amount of detergent-extracted Ras was determined by immunoprecipitation. Our results to date indicate that the amount of Ras in the detergent soluble extract is significantly reduced in stimulated cells, indicating that a portion of Ras molecules became associated with the detergent insoluble fraction (Fig. 1). This is to our knowledge the first demonstration of an interaction between Ras and the cytoskeletal framework. Ongoing efforts in the laboratory are directed at identifying the cytoskeletal components with which Ras interacts and the possible functional significance of this interaction.

Participation of Ras Proteins in T-cell Activation

N. Gale, P. Gesteland, D. Bar-Sagi

T lymphocytes can be activated to produce lymphokines, to express new cell surface molecules, and, eventually, to proliferate. This process is triggered by ligand receptor interactions occurring at the plasma membrane. As a consequence of these cell-surface interactions, signals are transmitted across the plasma membrane to the cell interior. The T-cell antigen receptor (TCR) plays a central role in these transmembrane signaling events. Several lines of evidence from our laboratory and others suggest a role for Ras in transduction of signals that lead to T-cell activation. These include: (1) Ras proteins undergo a coincident redistribution with cell-surface receptors in Jurkat T cells upon mitogenic stimulation with phytohemagglutinin (PHA), a plant lectin that achieves its mitogenic potential by cross-linking surface receptors, including the T-cell receptor. (2) Overexpression of oncogenically activated alleles of Ras in Jurkat cells results in an enhanced ability of these cells to produce interleukin-2, a specific functional marker of the activation program. This effect is observed only when the cells are stimulated with a combination of calcium ionophore and phorbol myristate acetate (PMA), a potent activator of protein kinase C. (3) Activation of Jurkat cells with a number of agents, including antibodies to specific components

of the T-cell receptor complex, PHA, or PMA, results in a rapid increase in the amount of GTP bound to Ras. It has been shown that the formation of the active Ras-GTP complex is regulated by the GTPase-activating protein (GAP). GAP stimulates the otherwise weak intrinsic GTPase activity of Ras proteins and therefore promotes the return of Ras to an inactive GDP-bound form. Upon activation of T cells, however, GAP is inhibited, thus leading to the accumulation of active GTP-bound Ras. To understand how GAP may function as a linker between Ras and extracellular signals, we have been investigating the mechanisms of inactivation of GAP. Two types of signals for the inactivation of GAP have been postulated: (1) indirect inhibition mediated by protein kinase C activation and (2) direct inhibition by biologically active lipids, specifically, arachidonic acid and some of its metabolites. Arachidonic acid is produced in cells by the activity of phospholipase A_2 (PLA_2) and is the rate-limiting precursor in the synthesis of the eicosanoids, including the prostaglandins. Therefore, it has been our aim to test whether PLA_2 activity plays a role in the modulation of GAP and Ras activities. To test this hypothesis, we have employed an inhibitor of PLA_2, para-bromophenacyl bromide (P-BPB). P-BPB is a histidine-modifying reagent that alters a critical histidine within the catalytic pocket of PLA_2. Our results to date show that inhibition of PLA_2 activity by P-BPB blocks the biochemical activation of Ras proteins. This effect is dose-dependent and is believed to be due to the inhibition of arachidonic acid production and not its metabolites, because inhibition of the major pathways of arachidonic acid metabolism has no effect on the ability to activate Ras. These results suggest that PLA_2 participates in the cascade of signals leading to the biochemical activation of Ras proteins. Future experiments in our laboratory will be aimed at further characterizing the biochemical steps involved in this process.

Role of Ras Proteins in Signaling via the Immunoglobulin Receptor

L. Graziadei, D. Bar-Sagi

Immunofluorescence studies performed previously in the laboratory have demonstrated a spatial and temporal coincidence between the ligand-induced migration of surface antigen receptors (sIg) and Ras in mitogenically stimulated B lymphocytes. We wanted to investigate the functional significance of this phenomenon. An increase in the proportion of Ras molecules in the GTP bound or "active" conformation has been used by other investigators as an indicator of functional activation of Ras. We have used this approach to determine whether Ras proteins are involved in signaling through sIg. We utilized an immunoprecipitation protocol in which cellular guanine nucleotide pools were metabolically labeled with ^{32}P. Cells were then stimulated through their sIg using the appropriate species-specific anti-immunoglobulin antibody. Cellular Ras proteins were immunoprecipitated, and bound nucleotides were eluted. Thin-layer chromatography was used to distinguish GDP from GTP.

Initial experiments were performed on the lymphoma B-cell line, WEHI-231. The immunoglobulin receptors expressed on the surface of WEHI-231 cells are of the IgM isotype (sIgM). Antibody stimulation of these cells through their sIgM led to a significant increase in the proportion of GTP-bound Ras molecules (Fig. 2). In a typical experiment, resting cells showed 21% of Ras molecules complexed to GTP, whereas after a 30-minute stimulation on ice, this value increased to 56%. These results indicate that sIg receptors are capable of signaling to cellular

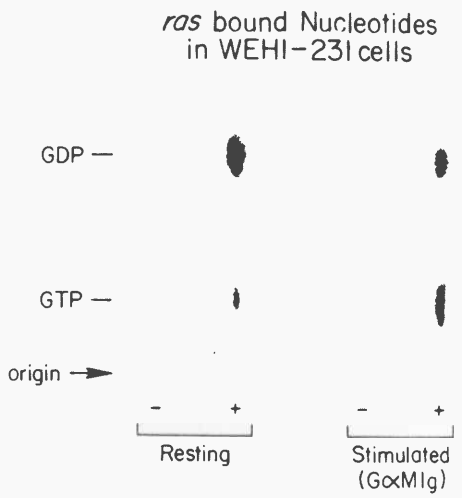

FIGURE 2 Effect of B-cell activation on guanine nucleotides bound to Ras. TLC of the guanine nucleotides eluted from immunoprecipitates of Ras from ^{32}P-orthophosphate-labeled WEHI-231 cells. Immunoprecipitation was carried out with a monoclonal anti-Ras antibody (+) or without the addition of specific antibody (-). Cells were stimulated for 10 min with $F(ab')_2$ derivatives of anti-IgM antibodies. The positions at which GTP and GDP standards ran are indicated.

Ras molecules. Since B cells undergo a distinct and progressive rearrangement of sIg molecules upon stimulation, we wanted to see if Ras activation correlated with a particular morphological stage of receptor rearrangement. Immunoglobulin receptors on cells that are stimulated on ice are capable of undergoing only patching (the earliest, passive clustering of cross-linked sIg). Under these conditions, maximal stimulation of Ras molecules was achieved after 10 minutes, with significant increases in the portion of GTP-bound Ras detected as early as 2 minutes after stimulation. This indicates that sIg-induced activation of Ras precedes sIg redistribution. To assess whether the activation of Ras is particular to one class of immunoglobulin receptor, we have employed the A20 B-cell line that expresses sIg of the IgG isotype. Experiments such as those described above have revealed that in these cells, the proportion of GTP-bound Ras molecules also increased in response to stimulation through their sIg. This indicates that Ras proteins are involved in signaling from both IgM and IgG isotypes. We are currently investigating the biochemical mechanisms linking stimulation of immunoglobulin receptors and Ras activation.

Analysis of Tyrosine Phosphorylation Events Associated with B-cell Activation

L. Graziadei, Y. Yeh, D. Bar-Sagi

As described above, studies carried out in our laboratory have indicated a possible link between the ligand-induced redistribution of the B-cell antigen receptor (sIg) and the activation of Ras proteins. Although sIg has no intrinsic tyrosine kinase activity, receptor activation leads to the inducible phosphorylation of a number of cellular proteins on tyrosine residues. Candidate kinases have been identified and include the *lck*-encoded protein, which is a member of the *src* family of tyrosine kinases. Since members of this tyrosine kinase family have been postulated to act upstream of Ras, we wanted to investigate the relationship between ligand-induced protein tyrosine kinase (PTK) activity and Ras activation. To this end, we have employed the tyrosine kinase inhibitor, genistein. We have found that treatment of small, dense resting murine primary B lymphocytes with genistein partially inhibited the activation of *Ras* proteins induced by cross-linking of sIg. The mechanisms underlying this inhibitory effect are being characterized further. In addition, we have found that inhibition of tyrosine phosphorylation by genistein affects ligand-induced sIg redistribution. Under normal conditions, cross-linking of sIg is the inductive event for three distinct, sequential stages of receptor relocalization, which include patching, capping, and receptor internalization. Stimulation of cells in the presence of genistein had no effect on the redistribution patterns of sIg at the patching and capping stages. However, at the stage in which most control cells had undergone receptor internalization, genistein-treated cells still displayed prominent caps of sIg on the cell surface (Fig. 3). A comparison between the kinetics of sIg redistribution in control and genistein-treated cells revealed that genistein does not effect the rate at which cells enter or complete patching and capping. This indicates that whereas inhibition of receptor-induced tyrosine phosphorylation has no effect on the kinetics of B-cell sIg patching and capping, PTK activity is required for proper internalization of capped surface receptors.

B-cell antigen-receptor-induced tyrosine phosphorylations have been studied in some detail and can be divided into early and late events. Western blotting demonstrated that inducible phosphorylation is visible as early as 30 seconds after stimulation and persists for up to 90 minutes after receptor/ligand interaction on ice. As an initial step toward identifying tyrosine phosphorylation events relevant to sIg internalization, we wanted to investigate the time dependence of the genistein effect. Toward this end, cells were pretreated for 30 minutes with stimulatory anti-Ig antibody to allow initial PTK activation and then treated with genistein. Under these conditions, genistein was equally effective in inhibiting sIg internalization. Therefore, it appears that persistent tyrosine phosphorylation is crucial for internalization of capped sIg receptors. Since ligand-induced internalization of sIg is essential for B cells to function as antigen-presenting cells, we are interested in further defining the relationships between tyrosine phosphorylation and sIg internalization.

Posttranslational Modification of Ras Proteins

K. Degenhardt, D. Bar-Sagi

We have recently shown that the carboxyl methyl group on the carboxy-terminal cysteine residue of Ras proteins turns over with a half-life of approxi-

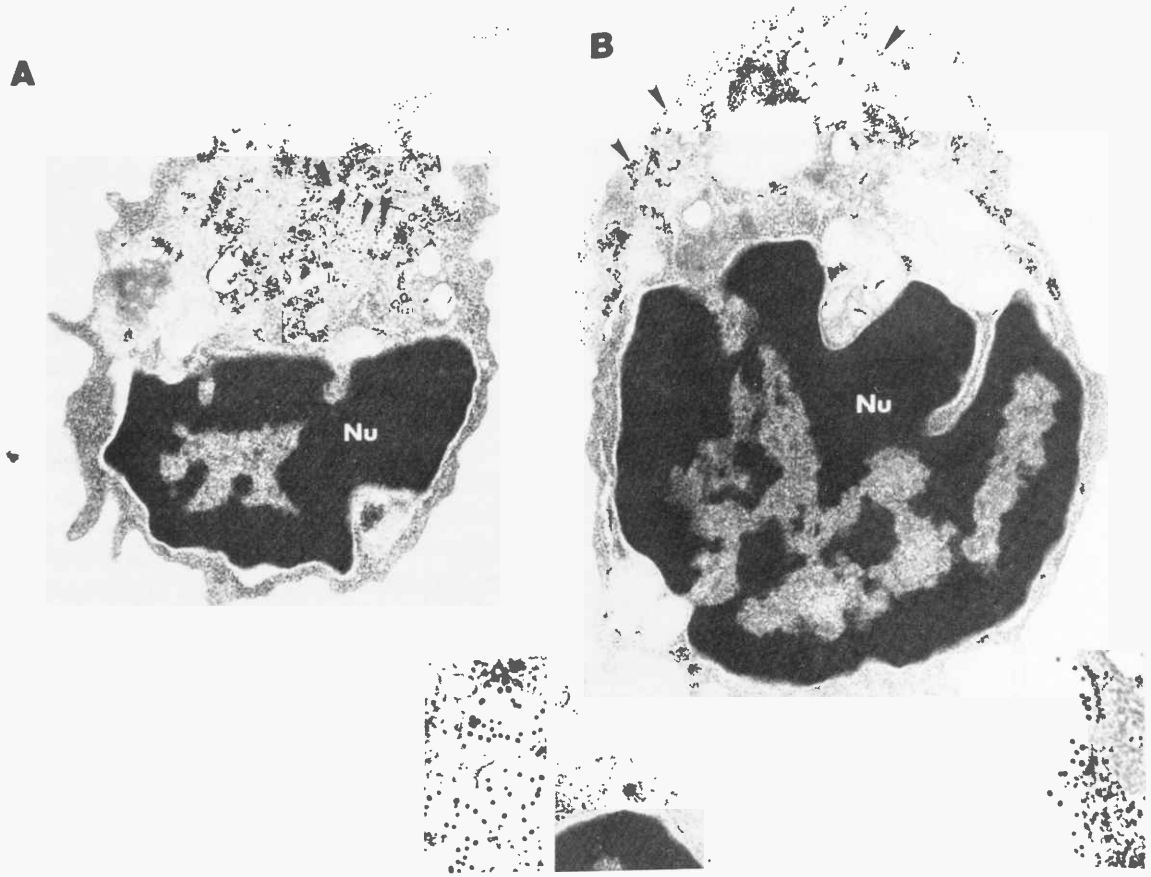

FIGURE 3 Distribution of immunoglobulin receptors in primary murine B lymphocytes following ligand-induced activation. Control cells (*A*) or cells pretreated with the tyrosine kinase inhibitor genistein (*B*) were stimulated for 45 min with 15 nm of colloidal-gold-conjugated goat anti-mouse IgG. Localization of sIg was determined by electron microscopy. In control cells, essentially all sIg molecules (visualized as gold particles) are internalized and present in endocytic vesicles (panel *A*, arrowheads and inset). In cells treated with genistein, receptor internalization is completely blocked, and all sIg molecules remain capped at the cell surface (panel *B*, arrowhead and inset). (Nu) Nucleus. Magnifications: (*A,B*) 26,000x; (*insets*) 64,000x.

mately 60 minutes (Fig. 4). The biological function of this dynamic methylation event is currently under investigation in our laboratory. To study the carboxyl methylation of Ras, we have established an in vitro methylation system that specifically assays for methyl esterification occurring on the α carboxyl group of the carboxy-terminal cysteine residue of Ras proteins. This assay utilizes a crude membrane fraction supplying both Ras and the methyl transferase and an exogenous methyl donor [methyl-^{3}H]*S*-adenosyl-L-methionine ([^{3}H]SAM). Although many membrane proteins are carboxyl methylated in this system, the methylation of Ras can be specifically assayed by the immunoprecipitation of Ras polypeptides using an anti-Ras monoclonal antibody (Y13-259). To study the functional significance of the carboxyl methylation of Ras proteins, we will need to identify the carboxyl methyl transferase that catalyzes this reaction. To this end, we have initiated subcellular fractionation experiments. Through fractionation of total cellular membranes over a sucrose gradient, we have found that the majority (78%) of protein carboxyl methylation activity is associated with the plasma membrane fraction. Furthermore, we have found that the in vitro carboxyl methylation of Ras is stimulated by guanine nucleotides. This observation suggests the involvement of a guanine-nucleotide-binding protein in the regulation of Ras carboxyl methylation. Along similar lines, initial experiments have been targeted to investigate receptor-

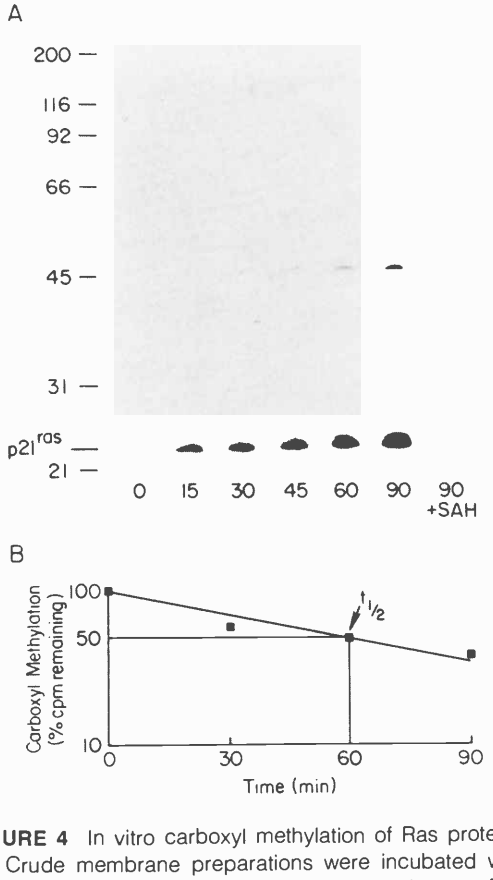

FIGURE 4 In vitro carboxyl methylation of Ras proteins. (*A*) Crude membrane preparations were incubated with [^{3}H]SAM for the indicated intervals in minutes. Ras polypeptides were isolated by immunoprecipitation and fractionated on a SDS-polyacrylamide gel. As can be seen, carboxyl methylation of Ras is time-dependent and is inhibited by the addition of *S*-adenosyl homocysteine, a specific inhibitor of methyltransferases. (*B*) Turnover of carboxyl methyl group on Ras. Membranes were methylated for 30 min and chased for the indicated intervals in buffer containing an excess of unlabeled SAM. Ras polypeptides were immunoprecipitated and analyzed by SDS-PAGE. Quantitation of ^{3}H-labeled methyl ester on Ras versus time of chase indicates a $t_{1/2}$ of 60 min for the carboxyl methyl turnover.

mediated alterations in Ras carboxyl methylation. To this end, we have studied the effects of serum stimulation on Ras carboxyl methylation in quiescent NIH-3T3 cells. Serum stimulation is a receptor-mediated process in which Ras has been implicated. Results from these experiments show that under the conditions examined thus far, the carboxyl methylation of Ras is independent of serum stimulation. We plan to continue our efforts in investigating the biological significance of carboxyl methylation of Ras using this in vitro system.

Role of Ras Proteins in Exocytotic Secretion

L. Graziadei, S. Kaplan, D. Bar-Sagi

For a number of years, we have been studying the role of Ras proteins in the regulation of exocytotic secretion in mast cells. In earlier studies, we used the microinjection approach to examine the effects of Ras proteins on exocytosis in rat peritoneal mast cells. The results of these studies can be summarized as follows: Microinjection of the *ras* oncogene protein into mast cells induces exocytotic degranulation. In contrast, microinjection of similar amounts of the proto-oncogenic protein had little apparent effect on mast cells. Degranulation induced by the injection of the *ras* oncogene protein occurred in the absence of an external stimulus and required the presence of external calcium. The ultrastructural features of the exocytotic degranulation in mast cells injected with the *ras* oncogene protein were similar to those seen when mast cells are activated by soluble ligand.

To investigate further the mechanisms underlying the stimulatory effect of Ras proteins on exocytosis, we have initiated studies on the effects of overexpression of *ras* proto-oncogene and oncogene proteins on the ligand-induced exocytotic secretion in the mast cell line, RBL-2H3. In these cells, exocytotic secretion is induced by the interaction of an appropriate ligand with surface IgE receptors. To obtain stable expression of the normal and activated forms of the *ras* gene, RBL-2H3 cells were transfected using the DEAE-dextran procedure. The plasmids used for these transfection experiments contain the coding sequences of the oncogenic and proto-oncogenic forms of the human Ha-*ras* gene. In these plasmids, the expression of the Ha-*ras* gene as well as the *neo*^{R} gene is under the transcriptional control of murine leukemia virus long terminal repeat (MLV LTR). Transfected clones were isolated on the basis of G418 resistance. RBL-2H3 cells expressing the normal Ha-*ras* were morphologically indistinguishable from nontransfected cells. In contrast, the morphology of RBL-2H3 cells expressing the Ha-*ras* oncogene was significantly altered; the cells were very flat and displayed extensive ruffling activity (Fig. 5). Measurements of IgE-mediated secretion from the various transfectants revealed a twofold enhancement of the stimulated secretion in cells expressing the Ha-*ras* oncogene. This enhancement did not result from changes in either number or affinity of the IgE receptor. These results suggest that Ras proteins may be

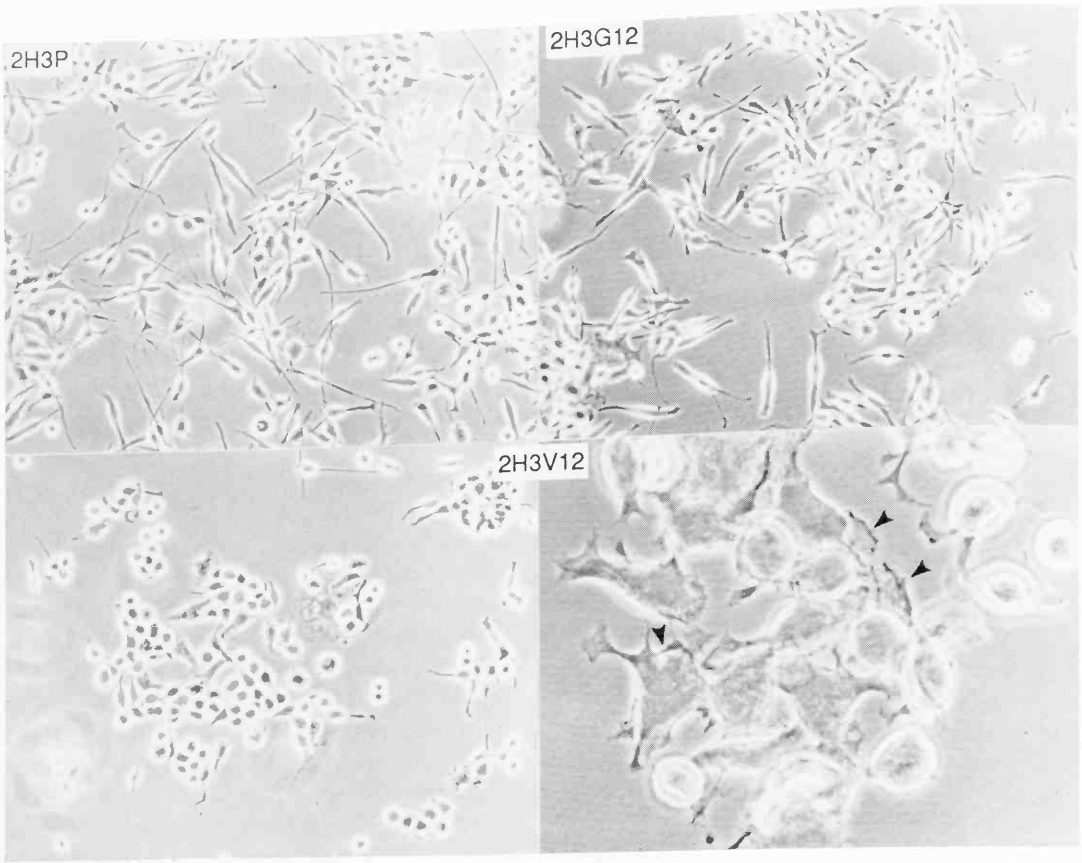

FIGURE 5 Morphologies of RBL-2H3 cells transfected with vector (2H3P), *ras* proto-oncogene (2H3G12) or *ras* oncogene (2H3V12). Cells expressing the *ras* oncogene display altered morphology: They are flat, and, at a higher magnification (right panel), extensive ruffling activity is detected (arrowheads).

involved in the signaling events that control IgE-mediated exocytotic secretion. Consistent with this notion is our recent observation that stimulation of the IgE receptor induces an increase in the GTP-bound form of Ras. Studies are currently under way to characterize the biochemical basis of the effect of Ras proteins on the exocytotic response.

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NUCLEAR SIGNAL TRANSDUCTION

M. Gilman C. Alexandre, R. Attar, L. Berkowitz, R. Graham, D. Grueneberg, G. Lee, A. Majid, S. Natesan, K. Riabowol, A. Ryan, H. Sadowski, K.-A. Won

Our major focus continues to be the mechanisms by which extracellular signals are communicated to the nucleus to control the proliferation and development of eukaryotic cells. We view this as a two-part problem. First, we need to understand the simple mechanics of the process. What are the individual molecular steps by which signals are relayed from the cell surface to nuclear targets? Second, we need to understand where the biological specificity in these signals is encoded. How do cells know what signal they have received and respond appropriately?

Our general approach to these questions has revolved around studies of the c-*fos* proto-oncogene. This gene is rapidly activated at the transcriptional level by a variety of extracellular stimuli, including growth and differentiation factors, hormones, and neurotransmitters. Induction of c-*fos* transcription occurs within seconds or minutes of exposure to these signaling molecules and does not require the cell to synthesize new proteins. Thus, activation of the c-*fos* gene is a primary response to the signal transduction pathways activated by these factors, and our working hypothesis is that the c-*fos* gene itself is a physical target for these signals.

Consequently, our general strategy has been to identify the sequences that mediate the response of the c-*fos* gene to specific signal transduction pathways. Then, using these sequences as reagents, we identify and characterize the cellular proteins that interact with them. Eventually, we use these proteins as reagents to identify the next proteins up the signaling chain. As described below, we have made varying degrees of progress working our way up the different signal transduction pathways that activate c-*fos* transcription. For cAMP, for example, we think we understand at least in a general way the entire route through which cAMP activates c-*fos* transcription. For other pathways, however, we know much less.

These experiments address the first question, the mechanics of signaling, but in addition, our recent results have suggested possible answers to the second question, the specificity of signaling. For example, we have generated c-*fos* promoter derivatives that respond to only subsets of signals. These results show that in some cases, the specificity of signaling is due to the use of completely distinct *cis*-acting regulatory elements. In other cases, different signals target different nuclear proteins that interact with a common sequence element. A second insight into the specificity of signaling comes from our recent discovery that homeodomain proteins may participate in the regulation of c-*fos* and other genes by extracellular signals. Since homeodomain proteins play a role in establishing the specificity of cell identity in embryonic development, we suspect that the interaction of these proteins with the proteins that control c-*fos* expression may be an important aspect of how the specificity of signaling is achieved. Third, we have begun to study a family of genes that may be downstream targets of the c-*fos* gene product, which is itself a transcription factor. These are the G_1 cyclins, which have key regulatory roles in controlling the entry and exit of cells from the proliferative cycle. By understanding how the transcription of these genes is controlled, we hope to get a picture of how cells are able to discriminate mitogenic from nonmitogenic signals.

Most of the work in the laboratory is focused on the serum response element (SRE), a relatively simple sequence that nevertheless integrates a great deal of signaling information. We describe below several of our approaches to understanding the function of this element and the proteins that interact with it. We begin, however, by describing our work with two signal transduction pathways that activate c-*fos* transcription independently of the SRE.

Regulation of c-*fos* Transcription by cAMP

L. Berkowitz

Transcription of the c-*fos* proto-oncogene is rapidly induced as a primary response to signals that increase the intracellular concentration of the second messenger cAMP. We learned early on that induction by cAMP was not prevented by mutations that destroyed the SRE. Using deletions and point mutations, we

identified three sequence elements in the c-*fos* promoter that mediate induction by cAMP. All of these elements contain the consensus sequence identified in the cAMP response elements (CREs) of other genes, although their transcriptional strengths and affinities for cellular CRE-binding proteins vary. We found that microinjection of double-stranded oligonucleotides corresponding to the major c-*fos* CRE prevented the induction of endogenous c-*fos* expression by cAMP in fibroblasts, showing that these elements are indeed required in vivo for this induction.

Our search for intracellular mediators of c-*fos* induction focused first on the cAMP-dependent protein kinase A (PKA). We found that microinjection of the purified catalytic subunit of PKA was sufficient to induce c-*fos* transcription, indicating that protein phosphorylation was involved in this induction. To identify the putative target for PKA, we focused on the previously characterized CRE-binding protein, CREB. CREB is phosphorylated in vitro by PKA and can activate transcription of a CRE-containing gene. We isolated CREB cDNAs by the polymerase chain reaction (PCR) and discovered that two different CREB mRNAs are produced in cells, leading to the synthesis of proteins that differ by the insertion of 14 amino acids. To study the activity of CREB in the presence of cAMP, we reprogrammed its DNA-binding specificity by attaching the DNA-binding domain of the yeast transcriptional activator GAL4. This allowed us to measure CREB transcriptional activity without interference from endogenous CREB or related proteins. These experiments showed that the transcriptional activity of CREB is directly modulated in response to cAMP and that some but not all of this effect requires the characterized PKA phosphorylation site. That residual cAMP-inducible activity remains in the absence of this site suggests that CREB contains other sites for PKA or that other regulatory mechanisms are able to influence the transcriptional activity of the protein.

We have raised both polyclonal and monoclonal antibodies to CREB and have made a series of mutants to identify important regulatory sites in the protein. In collaboration with G. Enikolopov (see this Section), we have also examined the role of CREB in the regulation of gene expression by calcium in PC12 cells. We find that CREB is indeed able to mediate calcium-responsive transcription and that this activity is only partially abolished by mutation of the PKA phosphorylation site, again suggesting that alternative mechanisms exist for activating CREB.

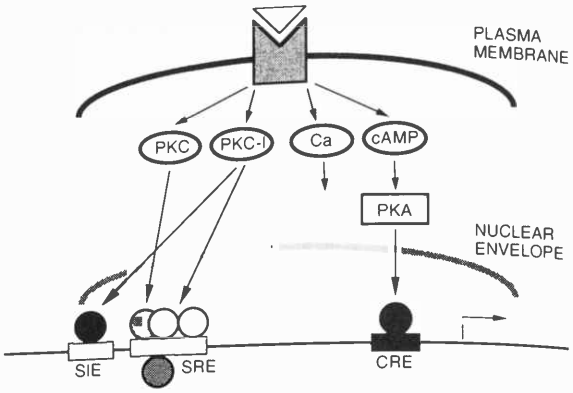

FIGURE 1 Our current view of the intracellular signaling pathways that control c-*fos* transcription. At least four distinct signal transduction pathways can activate c-*fos* transcription. cAMP acts by releasing the catalytic subunit of cAMP-dependent protein kinase (PKA), which migrates to the nucleus, where one of its actions is to phosphorylate the transcription factor CREB. Phosphorylation of CREB activates its transcriptional stimulatory function. Intracellular calcium acts on the c-*fos* gene through at least two or three discrete targets. In PC12 cells, calcium appears to activate CREB via a pathway similar to that used by cAMP. In T cells, in contrast, calcium acts to enhance initiation of c-*fos* transcription through as yet uncharacterized *cis*- and *trans*-acting elements and to promote the efficient elongation of c-*fos* transcripts, perhaps through a regulatory element in the body of the c-*fos* gene. Polypeptide growth factors that act through receptors with intrinsic or tightly linked protein–tyrosine kinase activity transmit at least two distinct signals to the c-*fos* gene. One signal, via protein kinase C (PKC), acts through a protein complex assembled at the c-*fos* serum response element (SRE) that consists of SRF and TCF. Other signals, independent of PKC (PKC-I), act through distinct SRF-containing complexes. At least one of these activates a latent DNA-binding protein, termed SIF, that binds just 5′ to the SRE. Several other proteins interact at the SRE; some, such as $p62^{DBF}$ and ZBP, bind directly to the SRE, whereas we believe that others, such as homeodomain proteins and the p65 subunit of NFκB, interact with SRF. For details, see the individual sections.

Regulation of c-*fos* Transcription by Intracellular Calcium

G. Lee

Intracellular calcium is an important second messenger in many cells, particularly in neuronal cells where it is a second messenger for neurotransmitters and in T cells where it is important in the activation of T cells by antigen. One of the cardinal properties

of calcium in T cells is its ability to act synergistically with activators of protein kinase C. This synergy can be seen at the level of the biological response of T cells: Calcium and PKC together but neither alone can fully activate T cells. In addition, it can be seen at the level of c-*fos* induction. PKC activators and calcium ionophores, at concentrations that have negligible effects on c-*fos* transcription singly, elicit large inductions of c-*fos* transcription when used together. We are interested in defining the mechanisms of this synergy and understanding how calcium signals are transmitted to the c-*fos* gene.

To study the regulation of c-*fos* transcription by calcium in T cells, we have used nuclear run-on assays. We find that whereas treatment with the PKC activator PMA induces transcription of sequences at the 5′ end of the c-*fos* transcription unit, transcription does not proceed efficiently into the body of the gene. In the presence of calcium, however, there is a dramatic transient enhancement in the transcription of downstream sequences, with little change in the transcription of the 5′ region of the gene. Thus, part of the synergistic action of calcium is due to the enhanced elongation of transcripts initiated in response to PMA. We are currently considering and testing two different models for this elongation enhancement. One model is that there is a specific block to transcript elongation as seen in the c-*myc* gene. Alternatively, transcription of the c-*fos* gene in the absence of calcium may be poorly processive, and a calcium-induced modification of the transcription complex enhances the general elongation properties of RNA polymerase. To distinguish between these models, we are performing nuclear run-on assays with short probes from different segments of the c-*fos* gene.

At higher concentrations, calcium is capable of inducing c-*fos* transcription alone by a second distinct mechanism. This mechanism has proven difficult to study because, in contrast to the synergistic interaction with PMA, direct induction by calcium is not faithfully recapitulated by transfected c-*fos* genes, despite the presence of long 5′- and 3′-flanking sequences. Thus, direct induction of c-*fos* transcription by calcium must require sequences not present on our clones or it may require integration of the c-*fos* gene into cellular chromatin. One mechanism of c-*fos* transcription we can clearly rule out is that it is mediated by CREB, as seen in PC12 cells. In contrast to PC12 cells, a promoter containing a CRE is not inducible by calcium in T cells, nor is CREB activity changed in response to calcium. This last observation suggests that there are significant cell-type-specific differences in the activity of CREB.

Distinct Protein Complexes at the SRE Mediate Growth Factor Induction of c-*fos* Transcription

R. Graham, M. Gilman

The serum response element (SRE) is required for the response of the c-*fos* gene to at least two distinct signal transduction pathways, one dependent on PKC and one or more independent of PKC. In addition, the SRE is the target for the rapid repression of c-*fos* transcription that follows induction. Given the complexity of SRE function, it is not surprising that the SRE binds several different cellular DNA-binding proteins. One of these proteins, SRF, appears to be required for all activities of the SRE. As described in more detail below, our working hypothesis is that SRF works by recruiting an array of distinct proteins to the SRE and that it is these accessory proteins that carry signaling information to the SRE. One such accessory protein is a 62-kD protein termed ternary complex factor (TCF), recently identified as a member of the ETS family. TCF specifically recognizes the SRF-SRE complex, probably contacting both SRF and the SRE. Indeed, we were able to generate a set of mutant SREs that bound SRF with wild-type affinity but failed to support formation of the SRF-TCF ternary complex. When tested for inducibility in vivo, these mutants lost response to only one of the two signal transduction pathways the target the SRE. They failed to respond to the PKC pathway but responded at wild-type levels to PKC-independent signals. We concluded from these experiments that these two signal transduction pathways act through distinct protein complexes at the SRE: PKC acts through the SRF-TCF ternary complex, whereas PKC-independent signals act through SRF alone or in conjunction with other unidentified accessory factors. We describe in the next section a candidate for such an accessory factor for PKC-independent signals.

Regulation of c-*fos* Transcription by Receptor Tyrosine Kinases

H. Sadowski

Nuclear signaling by polypeptide growth factors or hormones such as platelet-derived growth factor

(PDGF), whose receptor possesses ligand-induced tyrosine kinase activity, is complex. In the case of PDGF, at least three rapid signals can be sent to the nucleus, two as a consequence of phospholipase-C activation (PKC and calcium) and at least one that does not involve PKC. As described above, mutagenesis of the c-*fos* promoter has revealed that at least two of these pathways act through proteins binding to the SRE. PKC-dependent signals act through a ternary complex composed of SRF and TCF, whereas PKC-independent signals act either through SRF alone or in conjunction with other accessory proteins. These accessory proteins may either bind directly to specific sequence elements within the promoter (close to or distant from the SRE) or be recruited to the SRE by regulated protein-protein interactions.

We have been investigating whether the previously described v/c-*sis* (PDGF)-inducible DNA-binding factor (SIF; Hayes et al., *Proc. Natl. Acad. Sci. 84:* 1271 [1987]) is such an accessory protein. The SIF-binding element (SIE) is approximately 20 bp upstream of the SRE and coincides with an epidermal growth factor (EGF)-inducible in vivo footprint of the c-*fos* promoter in A431 cells. We have found that in BALB/c 3T3 cells, mutations in the c-*fos* promoter that selectively disrupt ternary complex formation but still allow SRF binding lose the PKC-dependent component of the PDGF signal, whereas constructs lacking an intact SRF-binding site are essentially unresponsive to PDGF. Our preliminary experiments also suggest that constructs with mutations that abolish SIF binding lose a portion of the PDGF response (presumably, the PKC-independent component), whereas a construct with mutations abolishing both SIF binding and ternary complex formation is largely uninducible by PDGF, despite the presence of an intact binding site for SRF. Surprisingly, its activity is comparable to a construct entirely lacking an SRF-binding site. Therefore, the PKC-independent component of the c-*fos* induction by PDGF appears to involve interactions between the SIE and the SRE, most likely through the proteins that bind to these sites.

We have concentrated on the biochemical characterization of the SIE-binding activity. Of the several growth factors tested, only PDGF (both AA and BB homodimers) causes potent and rapid induction of SIE-binding activity in BALB/c 3T3 cells, as measured by mobility shift assays. However, small increases in SIE-binding activity in extracts from EGF-treated cells are detectable. In addition, SIE-binding activity is rapidly induced by insulin in NIH-3T3 cells expressing the human insulin receptor and maintained at this high level in the continued presence of insulin (at least 60 min). Furthermore, in A431 cells, EGF causes a massive induction of SIE-binding activity that is maximal within 1 minute. Expression of the *neu* oncogene, which encodes a constitutively activated receptor tyrosine kinase, leads to constitutively high levels of SIE-binding activity. As expected, neither PMA, a direct activator of PKC, nor calcium ionophore induces SIE binding in any of the cell lines tested, whereas the phosphotyrosine phosphatase inhibitor sodium orthovanadate enhances both basal and tyrosine kinase activated levels of SIE binding. Further studies are in progress to determine whether other tyrosine kinases (receptor and non-receptor type) induce this activity, but our results suggest that the SIE and SIE-binding activity may be a general pathway through which several receptor tyrosine kinases activate c-*fos* transcription in a PKC-independent manner.

Experiments aimed at addressing the mechanism of activation of the SIE-binding factor have been performed, although no conclusive results have yet emerged. Interestingly, SIE-binding activity is abolished by treatment of extracts with potato acid phosphatase. We are testing the possibility that the SIE-binding activity contains phosphotyrosine, as the rapid kinetics of induction (detectable increases in binding in both cytosol and nuclear extracts within 20 sec of growth factor addition) suggests that this activity is very closely linked to the earliest events in signal transduction. Using UV cross-linking and microscale DNA-affinity precipitation, we have identified the SIE-binding protein(s) as a doublet of 90–94 kD. Whether these represent different posttranslationally modified forms of the same protein or different gene products is not yet clear.

Purification and Characterization of the SRE-binding Protein p62^{DBF}

S. Natesan

Previous work by Bill Ryan in our laboratory identified a novel 62-kD SRE-binding protein that we termed p62^{DBF}. p62^{DBF} binds directly to the 5′ side of the SRE in an asymmetric fashion, in contrast to the symmetrical binding of SRF. We have purified this protein from HeLa cells, using a combination of

conventional and affinity chromatography. Currently, we are scaling up the purification procedure to obtain sufficient amounts of the protein for sequence analysis.

In the course of our studies of $p62^{DBF}$, we have discovered some unexpected properties of the protein. First, despite the highly overlapping binding sites of $p62^{DBF}$ and SRF, we believe that the two proteins can occupy the SRE simultaneously. In fact, in the presence of $p62^{DBF}$, the rate of association of SRF with the SRE is greatly enhanced, a phenomenon we also observe with the homeodomain protein Phox1, as detailed below. Second, we have learned that $p62^{DBF}$ bends the DNA when bound to the SRE. This activity may contribute to its ability to enhance the interaction of SRF with the SRE. Indeed, we suspect that the direction of the bend may be determined by the sequence of the AT-rich core in the center of the SRE. We are testing this hypothesis using circularly permuted probes and phasing analysis, as described by Kerppola and Curran (*Cell 66:* 317 [1991]).

Cloning of SRE-ZBP, a Novel SRE-binding Protein

R. Attar

As described above, the SRE is a target for multiple signals that activate and repress c-*fos* transcription. To understand the complex mechanisms by which the SRE regulates the c-*fos* transcription, we have attempted to identify and clone previously unidentified SRE-binding proteins. We screened a HeLa cell cDNA expression library for phage expressing proteins that specifically bound an SRE oligonucleotide. We isolated a phage that encoded a *lacZ* fusion protein that specifically bound this oligonucleotide but not a mutant site. The fusion protein binds specifically to the SRE in both a Southwestern blot and a mobility-shift assay.

Analysis of the partial DNA sequence of this clone revealed that it was a previously unidentified gene that belongs to the family of zinc-finger-containing proteins related to the *Drosophila Krüppel* gene. The clone contains seven tandem repeats in the carboxyl terminus that match the zinc-finger consensus for this gene family. In the zinc-finger region, the clone shares up to 65% sequence identity with several human and mouse *Krüppel*-related genes. We named that protein SRE-ZBP (*s*erum *r*esponse *e*lement-zinc finger containing *b*inding *p*rotein). This gene is expressed at very low levels in most human cell lines, but at high levels in the hepatoma cell line HepG2. In HeLa cells, expression of SRE-ZBP is induced by serum stimulation with a maximum level at 6 hours of stimulation. Antibodies raised against SRE-ZBP do not recognize any previously characterized SRE-binding proteins. These observations, together with the methylation interference protection pattern obtained with the cloned protein, suggest that this clone identifies a novel protein that interacts with the 3′ side of the SRE. Immunofluorescence assays performed on transfected COS cells show that SRE-ZBP is localized in the nucleus. Preliminary results suggest that the cellular SRE-ZBP protein has an apparent molecular weight of 70,000. The presence of zinc fingers in this clone strongly suggests that it is a DNA-binding protein and probably is a transcription factor. Our current efforts are directed at understanding the role of this protein in the regulation of c-*fos* transcription.

A Human Homeodomain Protein That Interacts with SRF

D. Grueneberg

Our studies of the organization of the SRE and the function of SRF suggest that SRF works primarily by recruitment of accessory proteins with distinct functions. In this regard, SRF resembles the yeast protein MCM1, to which it is closely related. MCM1 also acts in conjunction with accessory proteins, including the DNA-binding proteins MATα1, MATα2, STE12, and SFF. Because SRF and MCM1 are so closely related in structure and function, we have attempted to use a genetic approach in yeast to identify novel accessory proteins for SRF. We have constructed a yeast strain in which the expression of a selectable marker (HIS3) is regulated by the UAS of the *STE3* gene. The element binds the complex of MCM1 and MATα1 and is active in MATα cells. Thus, in wild-type cells, this *STE3::HIS3* reporter gene confers a His^+ phenotype. When the *MATα1* gene is deleted from this strain, expression of the reporter is lost, leading to a His^- phenotype. Using this latter strain as a recipient, we introduced a human cDNA library on a yeast expression plasmid and selected for human cDNAs that restore the His^+ phenotype. Our hope

was that we would identify cDNAs that encode proteins able to cooperate with MCM1 to activate the reporter gene and that such proteins would interact with SRF as well.

In our first screen using this strain, we isolated clones for the same human gene more than 30 times. Sequence analysis revealed that the gene contains a homeodomain that shares 70% homology with the homeodomain of the *Drosophila paired* gene, which is an embryonic development gene of the pair-rule class. Genetic and biochemical studies in yeast (in collaboration with Cyrille Alexandre) indicated that this protein, which we have called Phox1, interacts with MCM1 to activate the *STE3::HIS3* reporter gene. Moreover, studies with purified bacterially expressed proteins show that Phox1 is also capable of interacting with SRF. The consequence of the Phox1-SRF interaction is the enhancement of binding of SRF to the SRE. This is a specific interaction that Phox1 shares with the closely related *Drosophila* homeodomain proteins but not with more distantly related members of the homeodomain family. By examining the kinetics of the SRF-SRE interaction in the presence and absence of Phox1, Sridaran Natesan has shown that Phox1 accelerates the rate of both association and dissociation of the SRF-SRE complex. Our interpretation of these data is that the formation of a stable SRF-SRE complex requires an isomerization from an initial complex into the final complex detected in our DNA-binding assays and that this isomerization is greatly enhanced by Phox1. Mutagenesis of the Phox1 homeodomain suggests that this enhancement does not require sequence-specific DNA-binding activity. Thus, the ability to enhance the binding of SRF appears to be a novel function of the homeodomain that almost certainly involves direct protein-protein interactions between the homeodomain and SRF. We believe that the interaction of homeodomain proteins with members of the SRF/MCM1 family will prove to be important in development.

Interaction of SRF with Members of the κB/*rel* Family

C. Alexandre

In a repeat of the genetic selection in yeast described in the previous section, we have identified additional human cDNAs that activate the MCM1-dependent reporter gene. Six independent isolates encoded the p65 subunit of the heterodimeric transcription factor NFκB. NFκB binds to the consensus sequence GGGRNNYYCC found in the immunoglobulin enhancer, the interleukin-2 (IL-2) receptor gene, the long terminal repeat (LTR) of human immunodeficiency virus type 1 (HIV-1), and elsewhere. Both p65 and the p50 subunit of NFκB belong to the *rel/dorsal* family. These proteins are transcriptional activators that shuttle between the cytoplasm and nucleus. Indeed, activation and nuclear translocation of NFκB occurs in response to many of the same signals that activate c-*fos* transcription. It is the p65 subunit that regulates the translocation of NFκB together with the cytoplasmic inhibitor IκB. Moreover, p65 carries a potent *trans*-activation domain. Thus, it is intriguing to us that p65 may interact with SRF: This offers a strong clue as to how the SRF complex may be activated in response to extracellular signals.

Our current work focuses on a genetic analysis of p65 function in yeast and biochemical studies of the protein in vitro. In yeast, genetic evidence suggests that p65 activates the *STE3::HIS3* reporter via interaction with MCM1. Mutations in the reporter that prevent the binding of MCM1 also blocked activation by p65. In contrast, reporters carrying multiple copies of a consensus NFκB-binding site were not activated by p65. We are currently investigating whether p65 interacts with SRF.

SRF Family Proteins in *Drosophila*

A. Ryan [in collaboration with Martin Klingler and J. Peter Gergen, SUNY Stony Brook]

Our discovery that human SRF interacts with human and *Drosophila* homeodomain proteins suggests that interactions between these proteins may be important in development. We have chosen to test this hypothesis in the *Drosophila* embryo, where the roles of homeodomain proteins have been most extensively studied. A further advantage of studying the *Drosophila* embryo is the availability of both classical and molecular genetic tools to manipulate the embryo. Thus, we have begun to clone *Drosophila* genes related to human SRF. Our goal is to study the genetics, expression pattern, and function of these genes in embryogenesis.

Meanwhile, we are taking a more direct approach to address the role of SRF in the *Drosophila* embryo. Earlier work in our laboratory has shown that it is

possible to inactivate SRF in vivo by microinjection of excess SRE oligonucleotides into cells. These oligonucleotides presumably titrate SRF and prevent it from binding to its natural target sites in the genome. In fibroblasts, microinjection of SRE oligonucleotides blocks the induction of c-*fos* transcription by serum. Since we know that *Drosophila* embryos contain a protein with the same DNA-binding specificity as human SRF, we should be able to neutralize this activity in embryos by injection of SRE oligonucleotides. The effect would be to phenocopy an SRF loss-of-function mutation. Our preliminary data suggest that injection of SRE oligonucleotides results in specific perturbations of embryonic development that we have not yet fully characterized.

Linking Growth Factors to Cell Cycle Control: G_1 Cyclins

K.-A. Won

How the initial intracellular signaling events triggered by growth factors are linked to the regulatory machinery that controls cell cycle progression in mammalian cells is still poorly understood. The transition from the G_0 to the G_1 phase of the cell cycle involves quantitative and qualitative changes in the expression of many genes and their products. Within minutes after growth factor or serum stimulation and independently of protein synthesis, a set of genes, termed the immediate early genes, becomes transcriptionally active. Yet, not all signals that activate the immediate early genes cause cells to enter or leave the cell cycle. To understand how cells are able to discriminate among such signals, we are studying the regulation of a family of genes whose expression may be critical to the control of cell cycle progression—the G_1 cyclins.

Yue Xiong and David Beach here at Cold Spring Harbor Laboratory have cloned a family of human cyclin genes termed D-type cyclins. One of these genes, cyclin D1, appears to correspond to the oncogene *bcl1*, whose chromosomal locus is amplified and translocated in many tumors. We have examined the expression of the cyclin D1 and D3 genes in primary human fibroblasts undergoing the G_0/G_1 transition. We find that transcription of these genes is induced by mitogenic growth factors, with increased mRNA levels appearing 2–4 hours after stimulation and peak levels just prior to the onset of S phase. Cyclin D expression is induced by all signals that are mitogenic for these cultures, consistent with the idea that cyclin D expression is required for the G_1/S transition. However, the genes are also activated by signals such as phorbol esters that are not mitogenic in these cells, suggesting that cyclin D expression is not sufficient for entering S phase.

We are now studying the promoter/regulatory regions of the cyclin D1 and D3 genes with the goal of identifying the *cis*- and *trans*-acting factors that regulate the expression of these genes in response to growth and differentiation factors. Among the *trans*-acting regulators, we expect to find products of the immediate early genes. Our goal is to understand how signaling information is processed to generate the decision to enter or exit the cell cycle.

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STRUCTURE, FUNCTION, AND REGULATION OF PROTEIN TYROSINE PHOSPHATASES

N.K. Tonks S. Brady-Kalnay, R. Del Vecchio, A. Flint, P. Guida, Jr., A. Samatar, H. Sun, Q. Yang

The phosphorylation of tyrosyl residues in proteins is a key component of the control of many fundamental physiological processes. Our laboratory is particularly interested in the role of tyrosine phosphorylation in transducing an extracellular signal into an intracellular response, such as proliferation or differentiation. Most laboratories have focused their studies on the protein tyrosine kinases. However, phosphorylation is a reversible, dynamic process in which the net level of phosphate observed in a target substrate reflects not only the activity of the kinases that phosphorylate it, but also the competing action of protein phosphatases that catalyze the dephosphorylation reaction. We direct our attention to the expanding family of protein tyrosine phosphatases (PTPases) that, like the kinases, comprise both transmembrane, receptor-linked forms and nontransmembrane cytosolic species (Fig. 1). The structures of the PTPases indicate important roles in the control of processes such as cell adhesion, cytoskeletal function, and the cell cycle. Clearly, their characterization will generate a more sophisticated understanding of the precise physiological roles of tyrosine phosphorylation.

IDENTIFICATION OF NOVEL PTPASES

We utilized polymerase chain reaction (PCR) from primers corresponding to conserved segments within the catalytic domains to amplify PTPase-related cDNA from a HeLa cell library. This approach revealed the presence of four known PTPases termed LAR, RPTPα, PTP1B, and TCPTP and three novel isoforms termed PTPH1, H2, and H3. These PCR products are being used to screen libraries for full-length cDNAs.

The sequence of the first of these novel isoforms (PTPH1) was published recently (Yang and Tonks 1991). Its structure can be described in terms of three segments. (1) The amino-terminal segment displays homology with the domains in the cytoskeleton-associated proteins band 4.1, ezrin, and talin that direct their interaction with proteins at the interface between the plasma membrane and the cytoskeleton. (2) The central segment is not homologous to any factor in the database but does bear a number of putative sites of phosphorylation by casein kinase II and $p34^{cdc2}$. (3) The catalytic domain is located at the carboxyl terminus, and we have now demonstrated intrinsic activity. The structure of this PTPase is indicative of a potential involvement in the control of cytoskeletal integrity. Since many of the substrates for the transforming protein tyrosine kinase $pp60^{v\text{-}src}$ are cytoskeleton-associated proteins, we are currently investigating whether expression of PTPH1 reverts the phenotype of a *src*-transformed cell to that of its nontransformed counterpart. Perhaps PTPH1 will be a useful probe with which to determine the precise role of tyrosine phosphorylation in generating the morphology of a transformed cell. By analogy with band 4.1 and talin, which bind to glycophorin and the integrin β subunit, respectively, through their amino-terminal domains, we are also trying to identify the proteins that interact with the homologous amino-terminal domain in PTPH1. Their identification and characterization should provide important information as to the mechanisms of regulation of PTPH1 activity and may provide indicators of possible substrates of physiological importance.

PTPH2, also referred to as RPTPμ (Gebbink et al., *FEBS Lett. 290:* 123 [1991]), is a transmembrane PTPase the extracellular segment of which displays similarity to that of the neural cell adhesion molecules (NCAMs), containing an Ig-like domain and a fibronectin type-III-like domain (Fig. 1). It is the intracellular segment that is of particular interest. The juxtamembrane segment, separating the transmembrane domain from the first of the two catalytic domains is some 70 residues longer than the equivalent segment in other receptor PTPases and displays sequence homology with the intracellular segment of the cadherins (Fig. 2).

Cadherins are adhesion molecules that participate in the mechanisms of coordinated association of cells

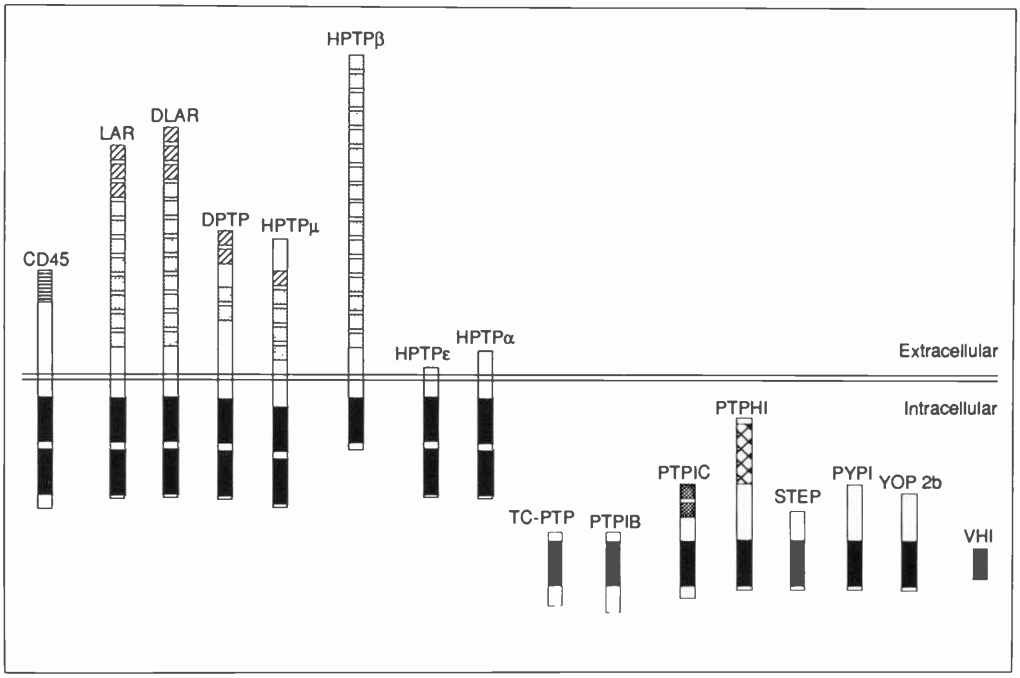

FIGURE 1 Structural organization of protein tyrosine phosphatases (for review, see Fischer et al., *Science 253:* 401 [1991]). Conserved catalytic domains are shown in black. The PTPases can be categorized as transmembrane, receptor-like, or nontransmembrane molecules. The receptor-like species can be subdivided into four types on the basis of the structure of their extracellular segments. Type I represents the CD45 family, multiple isoforms of which arise from differential splicing of a primary mRNA transcript of a single gene; three exons encoding sequences at the extreme amino terminus (horizontal shading) are differentially expressed. Type II, containing immunoglobulin-like (*diagonal lines*) and fibronectin type-III-like (*stippled*) domains, includes LAR, DLAR, DPTP, and HPTPμ. Type III bears multiple fibronectin type III repeats such as in HPTPβ, which is also characterized by the presence of only one PTPase domain in its intracellular segment. Type IV, such as HPTPα and HPTPε, has very small, glycosylated extracellular segments. Multiple nontransmembrane forms have also been identified. In TCPTP and PTP1B, a carboxy-terminal noncatalytic segment appears to play a role in modulating activity and controlling subcellular localization. Several PTPases bearing noncatalytic amino-terminal segments have now been identified. These segments include two SH2 domains in PTP1C, a band-4.1 homology domain in PTPH1, and structures apparently unrelated to sequences in the database in STEP, PYP1, and YOP2b. The vaccinia protein VH1 is smaller than the other PTPase-related segments and presumably encodes only a catalytic domain. This is not an exhaustive list and the family continues to expand. A number of partial cDNAs for additional PTPases have also been identified but are not included.

during tissue development and morphogenesis (for review, see Takeichi, *Science 251:* 1451 [1991]). Essentially all cells that associate into tissues express cadherins, and reduction in the level of expression of E cadherin has been linked with tumor invasiveness and metastasis. Cadherins are concentrated in adherens junctions where they are linked to cortical actin bundles. Four subclasses of cadherin have been well characterized at a structural level: LCAM (liver cell adhesion molecule) in chickens and E(epithelial), N(neural), and P(placental) cadherins in mammals. However, the repertoire of cadherins is also now expanding rapidly. Their structure can be described in terms of three segments. The extracellular segment is glycosylated and comprises at least three repeated structural motifs containing stretches of acidic residues involved in binding Ca^{++}. Cadherin binding is homophilic and Ca^{++}-dependent; binding specificity is governed by the amino-terminal 113 residues of the extracellular segment. There is a single transmembrane domain and an intracellular segment of approximately 150 residues. It is the intracellular seg-

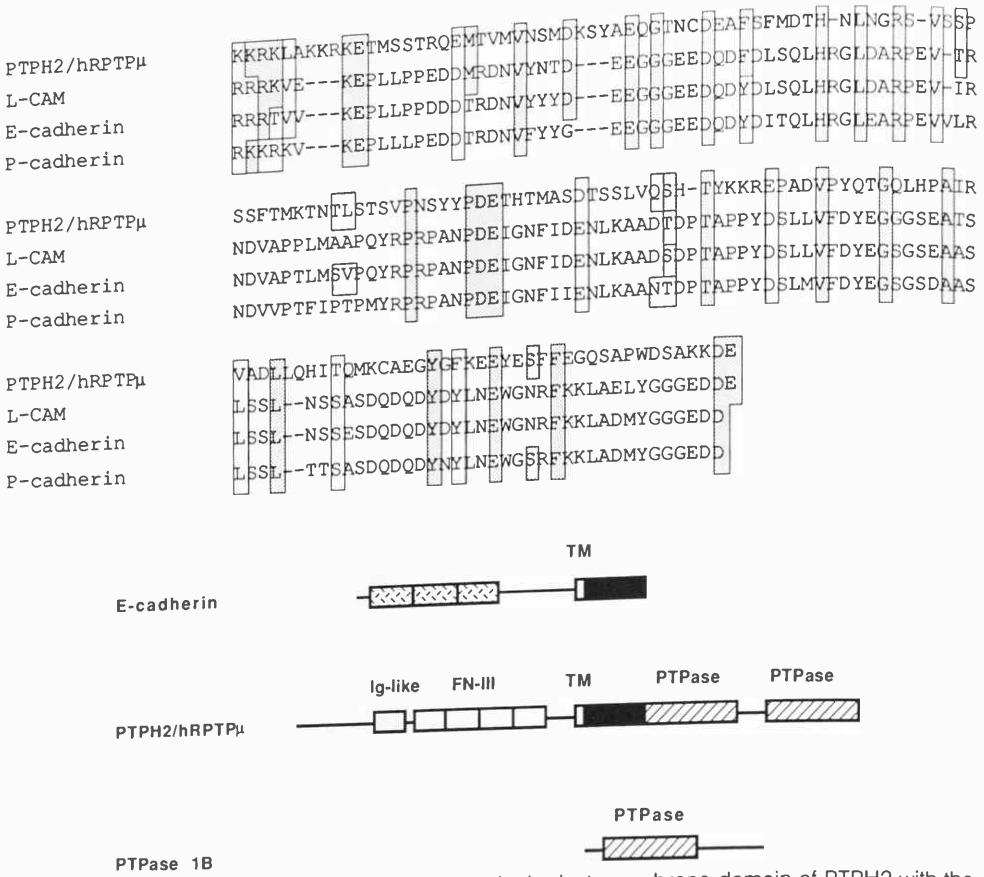

FIGURE 2 (*Top*) Alignment of amino acid residues in the juxtamembrane domain of PTPH2 with the conserved cytoplasmic domains of cadherins. Identities are highlighted in shaded boxes and conservative substitutions in open boxes. (*Bottom*) Schematic diagram illustrating the structure of PTPH2 as well as its relationship to the cadherins and the low-M_r PTPase, PTP1B. Diagonal lines denote PTPase catalytic domains. The segment of homology between PTPH2 and the cadherins is represented in black. The transmembrane segments also share eight identities. Using the ALIGN program, the mutation data matrix for scoring and a gap penalty of 10 to compare residues 742–917 in PTPH2 (Gebbink et al., *FEBS Lett. 290:* 123 [1991]) with 553–722 in LCAM (Gallin et al., *Proc. Natl. Acad. Sci. 84:* 2808 [1987]) and 555–724 in E cadherin (Ringwald et al., *EMBO J. 6:* 3647 [1987]) scores of 7.3 and 7.5, respectively, were obtained. These alignment scores are expressed in units of standard deviation from the average background scores of 100 randomly generated sequences. Scores greater than 5 indicate homology.

ment that is most highly conserved, displaying about 90% identity among the cadherins. Extensive analysis by site-directed mutagenesis has shown that in E and N cadherin, deletions in the intracellular segment, particularly in the carboxy-terminal 72 amino acids, abolish adhesion despite the presence of an intact extracellular segment. The intracellular segment interacts with cortical actin indirectly, a process that is mediated by the binding of cytoplasmic proteins termed catenins. Catenin α (102 kD) displays homology with vinculin, catenin β (92–94 kD) is homologous to plakoglobin, and catenin γ (~80 kD) remains to be characterized. Deletions of the carboxy-terminal sequences of cadherin also abolish the binding to catenins. Thus, the association of the cadherin cytoplasmic domain, the domain that displays homology with PTPH2, with catenins is essential for the interaction of cadherins with the cytoskeleton and their adhesive function.

A second type of intercellular junction, desmosomes, is also characterized by the presence of cadherin-related proteins termed desmogleins and

desmocollins (for review, see Magee and Buxton, *Curr. Opin. Cell. Biol. 3:* 854 [1991]). Like the classical cadherins, these are transmembrane proteins with glycosylated extracellular segments comprising repeated Ca^{++}-binding motifs. Their intracellular domains are distinct from, but nevertheless structurally related to, those of the cadherins displaying approximately 20–30% identity, similar to the degree of identity between the juxtamembrane domain of PTPH2 and the cadherins. Interestingly, these domains in the desmogleins/desmocollins also interact with cytoskeletal elements, in this case, intermediate filaments, a process mediated by catenin-like molecules including plakoglobin. However, the details of these interactions remain to be described in full.

Homophilic interactions between NCAMs are of lower affinity than those involving cadherins. In this case, the role of the cytoplasmic segment is unclear; in fact, this segment is truncated in the small cytoplasmic domain (sd) form of NCAM, whereas the small surface domain (ssd) form is linked to the outer surface of the membrane by a phosphatidyl inositol–glycan anchor and lacks both transmembrane and intracellular segments. No association with the cytoskeleton has been documented, although there are reports of the triggering of signal transduction pathways involving G-protein-mediated changes in the levels of second messengers such as Ca^{++} by NCAM and N cadherin (Doherty et al., *Cell 67:* 21 [1991]). PTPH2 may provide a link between cell adhesion and signal transduction pathways involving reversible tyrosine phosphorylation. Interestingly, the adherens junctions have recently been identified as major sites of tyrosine phosphorylation within the cell and sites at which members of the *src* family of tyrosine kinases are concentrated (Tsukita et al., *J. Cell. Biol. 113:* 867 [1991]). Furthermore, treatment of chick lens cells with inhibitors of PTPase activity leads to a pronounced accumulation of phosphotyrosine at adherens junctions, implying a ready reversibility of the phosphorylation reaction and a rapid turnover of tyrosine phosphate at these sites (Volberg et al., *Cell. Reg. 2:* 105 [1991]). We also suggest that the cadherin-related juxtamembrane domain of PTPH2 may regulate the interaction of this immunoglobulin superfamily molecule with the cytoskeleton as well as controlling its potential adhesive function in a manner analogous to that of the cadherins.

Finally, PTPH3 is similar to, but distinct from, the gene product of the previously described HPTPβ (see Fig. 1). At the moment, we have a cDNA clone encoding the intracellular segment containing a single PTPase domain, a transmembrane segment, and a partial extracellular segment comprising eight repeated fibronectin type-III-like domains (16 such domains exist in HPTPβ). Repeated fibronectin type III domains have been observed in a variety of receptors including those for interleukin-2 (IL-2), IL-4, and IL-6, GM-CSF, prolactin, erythropoietin, and growth hormone (Patthy, *Cell 61:* 13 [1990]). However, the identity of ligands for this, or any other receptor-PTPase, remains an important area of study still to be addressed.

How many PTPases are there? Despite the recent explosion in the number of members of the family to be identified, it seems safe to assume that at present we are only reviewing the tip of the iceberg. We are currently searching for novel PTPases in a number of systems. Of particular note, we have identified by PCR 15 distinct PTPase-related sequences in a *Xenopus* oocyte cDNA library and have confirmed the presence of mRNA for each species. Some represent *Xenopus* homologs of known PTPases, but several appear to be novel. In addition, in collaboration with Bob Horvitz at the Massachusetts Institute of Technology, we have applied the PCR protocols to the identification of PTPases in *Caenorhabditis elegans.* Thus far, ten distinct PCR fragments have been isolated, and chromosomal mapping is in progress. Studies with these systems are currently in their infancy, but we hope that they will yield interesting information in the coming months.

REGULATION OF PTPASE ACTIVITY

A striking characteristic of the members of the PTPase family is their absolute specificity for phosphotyrosyl residues in proteins, coupled with a high affinity for substrate and a specific activity some one to three orders of magnitude in excess of that of the protein tyrosine kinases in vitro (Tonks et al., *J. Biol. Chem. 263:* 6731 [1988]). Consequently, the PTPases have the potential to control effectively the level of phosphotyrosine within the cell and to keep the activity of the kinases in check. One might anticipate that the activity of the PTPases would therefore be tightly controlled so as to permit the normal function of the PTKs. The identification of such control mechanisms represents a major focus of

my laboratory and may illustrate new tiers of regulation of the level of cellular phosphotyrosine.

A general theme among the PTPases is becoming apparent, as typified by the molecules described above; within the protein, distinct functional motifs may control activity by restricting intracellular localization or targeting to interaction with regulatory factors. The structures of PTPH1, H2, and H3 suggest that they may be involved in a link between adhesion phenomena, changes in cytoskeletal integrity, and tyrosine phosphorylation. We are currently trying to clarify potential regulatory proteins with which they may interact in vivo.

The first PTPase isolated in homogeneous form was the cytosolic enzyme termed PTP1B. It was purified from human placenta as a truncated catalytic subunit of 37 kD. Isolation of cDNA for PTP1B, and the closely related TCPTP, has indicated that in vivo, the protein comprises an amino-terminal catalytic domain and a carboxy-terminal segment of 11 kD not present in PTP1B as purified from placenta, which appears to be important for both regulating activity and controlling intracellular localization. We noted that this regulatory segment bears a number of putative sites of phosphorylation for the serine/threonine kinases, $p34^{cdc2}$, and casein kinase II. We have therefore directed our attention toward the possibility that phosphorylation of PTP1B may be of regulatory significance. Indeed, PTP1B is a phosphoprotein in vivo. More interestingly, its phosphorylation state is altered in a cell-cycle-dependent manner (Fig. 3); our data suggest that there is enhanced phosphorylation of PTP1B as cells enter mitosis. Currently, we are examining the effects of phosphorylation on activity and attempting to characterize the kinase involved. Our most recent observations suggest that in immunoprecipitates of PTP1B from HeLa cells, a serine/threonine kinase that recognizes histone H1 as a substrate coprecipitates with the phosphatase. Furthermore, there are up to fivefold higher levels of kinase activity in PTP1B immunoprecipitates from nocodazole-arrested (mitotic) HeLa cells than in such immunoprecipitates from asynchronous populations. Experiments directed toward identifying this kinase are under way.

PTPASES AND INSULIN ACTION

In collaboration with Dr. J. Sommercorn and his colleagues at the National Institutes of Health in Phoenix, Arizona, we have been investigating whether a defect in the level of expression or activity of a PTPase may contribute to the mechanism of noninsulin-dependent diabetes mellitis (NIDDM). This is a life-threatening disease that affects about 10% of the general population over the age of 40 in the United States. Due to its association with aging and the fact that the population as a whole is getting older, the prevalence of NIDDM is likely to increase.

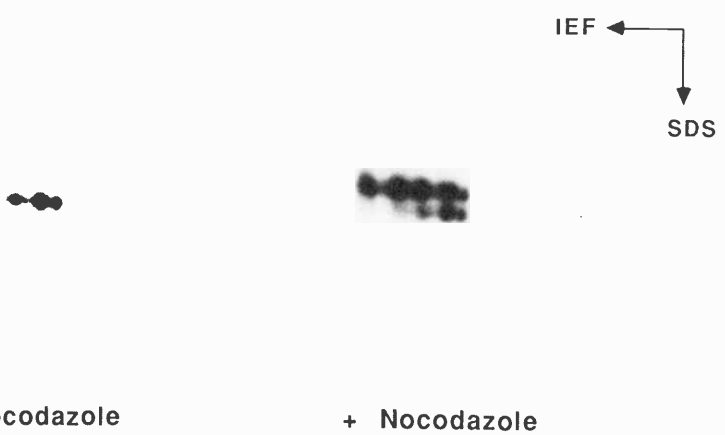

FIGURE 3 Immunoprecipitation of PTP1B from ^{32}P-labeled HeLa cells. PTP1B was immunoprecipitated using a monoclonal antibody, FG6 (generated by Dr. Dave Hill, Applied Biotechnology, Inc., Cambridge, Massachusetts) and subjected to two-dimensional gel analysis. The figure highlights the section of the gel containing PTP1B, identified by autoradiography.

The Phoenix group studies this disease in Pima Indians, of which 50% of adults over 35 years of age are diabetic. Studies of the development of NIDDM indicate that prior to becoming diabetic, subjects first develop impaired glucose tolerance, characterized by a reduced capacity of skeletal muscle to respond to insulin. Insulin resistance limits the ability of the hormone to influence the activities of various muscle enzymes, including S6 kinase, type I protein serine/threonine phosphatase, and glycogen synthase, which are thought to mediate insulin action intracellularly. However, several lines of study suggest that the defect is not in the insulin receptor itself, but rather an early post-receptor step in the pathway of signal transduction stimulated by insulin. Recently, we have shown a higher basal level of particulate PTPase activity in resistant subjects than in insulin-sensitive subjects. In addition, insulin infusion in vivo produced a rapid inhibition of soluble PTPase activity measured in extracts of skeletal muscle from insulin-sensitive subjects that was impaired in subjects who were insulin resistant (McGuire et al., *Diabetes 40:* 939 [1991]). Both of these effects may contribute to an antagonism of signaling through the insulin receptor. PCR-based strategies have identified five distinct PTPases in the skeletal muscle samples, and one of these appears to be novel. We are presently trying to obtain full-length clones for the novel enzyme and are looking for alterations in the level of expression of the PTPases in insulin resistance. In addition, antibodies are being generated to the various PTPases with the aim of looking for changes in activity of a particular isoform.

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CELL BIOLOGY OF THE NUCLEUS

D.L. Spector S. Huang, S. Henderson, R. O'Keefe, L. Jimenez-Garcia, R. Derby, G. Lark, S. Landon

Studies in our laboratory are focused on the structural and functional organization of the mammalian cell nucleus. Our research program evolves around two specific areas of study: (1) understanding the organization of factors associated with pre-mRNA splicing and the RNA substrates with which the factors interact and (2) elucidating the organization of specific DNA sequences (genes and chromosomal regions) in the interphase nucleus. The use of the electron microscopy core facility has continued to expand, and the use of fluorescence and confocal laser scanning microscopy has significantly increased over the past year. With the recent addition of the Zeiss Microscopy Center, our core services have been expanded to include the microinjection and examination of living cells by time-lapse microscopic cinematography. A large number of collaborations are under way with the excellent technical expertise of Robert Derby.

Organization of Chromosome-specific DNA Sequences in the Interphase Nucleus

S.C. Henderson, R. O'Keefe, D.L. Spector

We have previously identified five distinct patterns of DNA replication during S phase in asynchronous and synchronous cultures of mammalian cells by conventional fluorescence microscopy, confocal laser scanning microscopy, and immunoelectron microscopy. During early S phase, replicating DNA (as identified by 5-bromodeoxyuridine incorporation) appears to be distributed at sites throughout the nucleoplasm, excluding the nucleolus. In Chinese hamster ovary (CHO) cells, this pattern of replication peaks at 30 minutes into S phase and is consistent with the localization of euchromatin. As S phase continues, replication of euchromatin decreases, and the peripheral regions of heterochromatin begin to replicate. This pattern of replication peaks at 2 hours into S phase. At 5 hours, perinucleolar chromatin and peripheral areas of heterochromatin peak in replication. Seven hours into S phase, interconnecting patches of electron-dense chromatin replicate. At the end of S phase (9 hr), replication occurs at a few large regions of electron-dense chromatin. Similar or identical patterns have been identified in a variety of mammalian cell types.

To determine the position and timing of chromosome-specific α-satellite DNA sequences, we have developed a method to evaluate simultaneously the position of a specific gene sequence and the pattern of DNA replicating at the time of fixation in S phase. α-satellite DNA, which is associated with the chromosome centromere, consists of chromosome-specific "monomers" of approximately 171 base pairs that are tandemly repeated for several kilobases. Bromodeoxyuridine (BrdU)-labeled HeLa cells were hybridized in situ with a probe to α-satellite DNA from the X chromosome. The localization of the X-chromosome α-satellite DNA was visualized relative to each of the BrdU-labeling patterns in both synchronous and asynchronous populations of cells by superimposition of optical sections (sampled in each channel of fluorescence) generated by confocal laser scanning microscopy. Due to the disposition of centromeres within the three-dimensional space of the nucleus, usually α-satellite DNA from only one centromere was detected in any one optical section. At times of DNA synthesis characterized by the first (Fig. 1a), second (Fig. 1b), fourth (Fig. 1d), and fifth (Fig. 1e) patterns of DNA replication, the X-chromosome α-satellite DNA was visualized as a bright white spot, which did not overlap with the pattern of BrdU labeling (gray regions) in all cells examined. Only at the time characterized by the third pattern (Fig. 1c) of DNA replication, which occurs during mid S phase, did the hybridization to the X-chromosome α-satellite DNA colocalize with the BrdU labeling. Therefore, the replication of the X-chromosome α-satellite DNA appeared to occur during mid S phase, which is characterized by the third pattern of DNA replication. The replication of α-satellite DNA during mid S phase appears to be true for other chromosomes as well. Probes to α-satellite

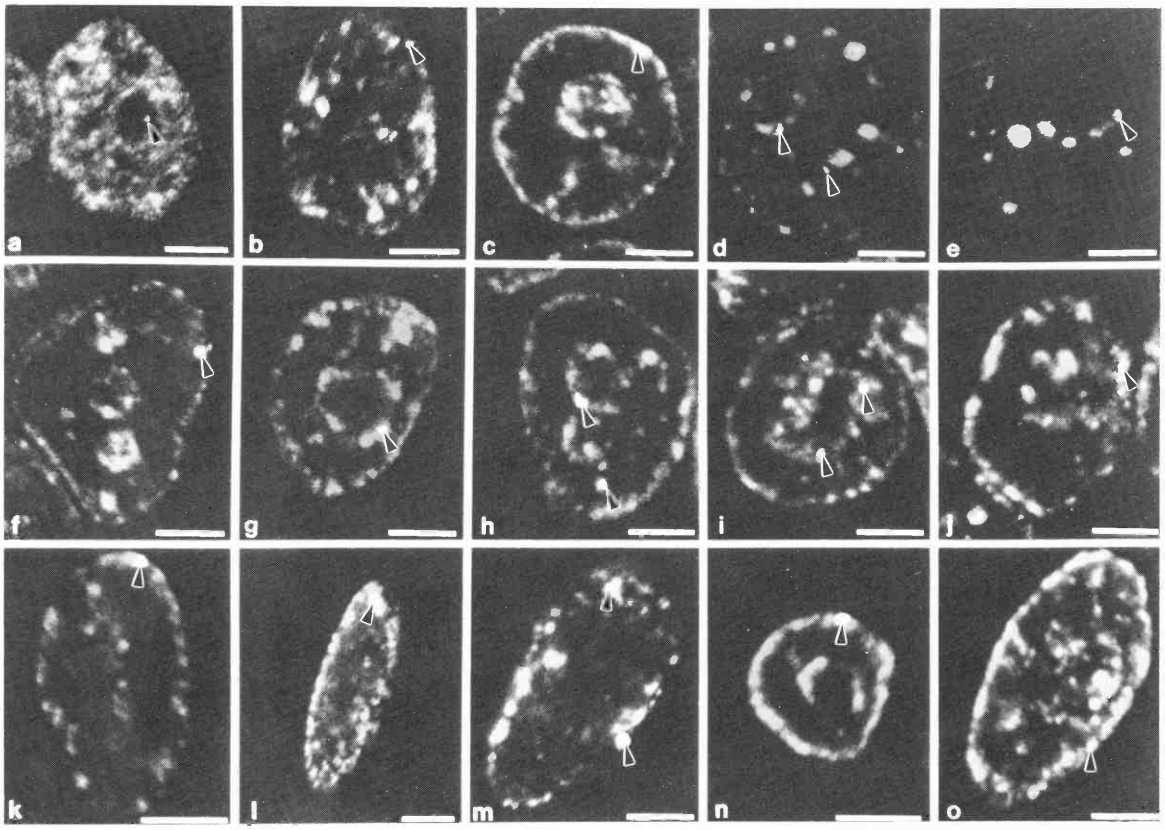

FIGURE 1 Replication of centromeric (α-satellite) DNA (*arrowheads*) occurs during mid S phase of human cells. Human cells were labeled with BrdU and hybridized in situ with biotinylated DNA probes to chromosome-specific α-satellite DNA. BrdU incorporation and hybridization of probes were visualized by labeling with FITC-conjugated anti-BrdU and Texas Red-conjugated avidin, respectively. Regions of DNA replication are shown in gray and localization of α-satellite DNA is shown in white. Optical sections of synchronized HeLa cells, labeled with BrdU and hybridized with a probe to the X-chromosome α-satellite DNA, show that the X-chromosome centromere replicates during mid S phase, which is characterized by the third pattern of BrdU incorporation (*c*). No colocalization of the X centromere and DNA replication occurs in either the first (*a*), second (*b*), fourth (*d*), or fifth (*e*) patterns of BrdU incorporation. The replication of centromeric (α-satellite) DNA in HeLa cells during mid S phase (characterized by the third pattern of BrdU incorporation) also occurs for α-satellite DNA of chromosomes 1 (*f*), 3 (*g*), 10 (*h*), 17 (*i*), and 18 (*j*). Furthermore, the replication of X-chromosome α-satellite DNA occurs during mid S-phase (third pattern of BrdU incorporation) in a variety of human cell types including human umbilical vein endothelial (HUVE) cells (*k*), Detroit 551 (fibroblast) cells (*l*), WI-38 (fibroblast) cells (*m*), SW-620 (adenosarcoma) cells (*n*), and MG-63 (osteosarcoma) cells (*o*). Bars, 5 μm.

DNA from chromosomes 1 (Fig. 1f), 3 (Fig. 1g), 10 (Fig. 1h), 17 (Fig. 1i), and 18 (Fig. 1j) as well as chromosomes 2, 8, and 12 (data not shown) colocalized with the third pattern of DNA replication. α-satellite DNA from homologous chromosomes does not necessarily replicate at precisely the same time, although α-satellite DNA from both centromeres replicates within the same pattern. Often, the replication of α-satellite DNA from one member of a chromosome pair precedes the other, yet both replicate during mid S phase (the third pattern of DNA replication). The replication of α-satellite DNA during mid S phase appears to be independent of cell type, nuclear size or shape, or ploidy. In a variety of human cell types, HUVE (normal human umbilical vein endothelial cells, Fig. 1k), Detroit 551 (normal diploid fibroblasts, Fig. 1l), WI-38 (normal diploid fibroblasts, Fig. 1m), SW-620 (human adenocarcinoma, Fig. 1n), MG-63 (human osteosarcoma, Fig. 1o), X-chromosome α-satellite DNA replicates during the third pattern of BrdU labeling (mid S phase). This pattern differs between cell types in that the ex-

tent of perinucleolar BrdU incorporation is less in some cell types (i.e., fibroblasts vs. epithelial cells). However, in all cases, regardless of chromosomal location or cell type, the α-satellite DNA is found associated with either the surface of the nucleolus or the nuclear periphery. This technique is currently being applied to analyze the timing of replication of single copy genes. In addition, studies are under way to determine if chromosome-specific α-satellite DNA sequences and single-copy genes occupy a similar three-dimensional position in an interphase cell population at different times during the cell cycle.

Transport of Nascent c-*fos* Transcripts and Associations with Nuclear Regions Enriched in Pre-mRNA Splicing Factors

S. Huang, D.L. Spector

We have used in situ hybridization and immunocytochemistry to compare the nuclear localization of a specific nascent pre-mRNA and the essential non-snRNP splicing factor SC-35. We have chosen to evaluate c-*fos* pre-mRNA at the single-cell level because induction of the c-*fos* gene is well characterized at the level of transcription. The c-*fos* gene is highly conserved among vertebrates, and its protein product serves a role in coupling external stimuli such as mitogens to long-term transcriptional responses, leading to cell proliferation. The c-*fos* gene encodes a nuclear protein that forms a part of the AP-1 transcription factor complex and is thought to regulate the transcription of a diverse complement of genes associated with cellular growth and differentiation. Using in situ hybridization combined with confocal laser scanning microscopy, we observed c-*fos* transcripts in the nuclei of cells within 5 minutes of serum stimulation of quiescent NIH-3T3 cells, and by 15 minutes poststimulation, the c-*fos* signal peaked. Our findings, at the single cell level, of the transient expression of c-*fos* transcripts are consistent with previous biochemical studies that evaluated run-off transcripts from nuclei isolated at various time points poststimulation. Prior to serum induction, no c-*fos* RNA is detected. In our experiments, hybridization with a biotinylated genomic c-*fos* probe showed that the c-*fos* transcripts were present as two discrete dots in the nuclei of NIH-3T3 cells. The dots that we observe with the c-*fos* probe appear to represent transcripts from each of the two c-*fos* alleles in this cell line. Using a digoxigenin-labeled intron-specific c-*fos* probe, we have shown that pre-mRNA is a component of this dot localization pattern. RNase A pretreatment of cells results in no hybridization signal. In addition, pretreatment of cells with the RNA polymerase inhibitor actinomycin D results in no hybridization signal. We interpret the intensely stained dots to represent the active sites of transcription and the localization of transcripts that are still attached to the DNA backbone. This possibility is likely in light of our findings that DNase I digestion decreased the size of the dots. We interpret these results to mean that upon removal of the cellular DNA, a subset of the c-*fos* transcripts that were being transcribed and were therefore still attached to the DNA were removed as well. Upon further analysis by confocal laser scanning microscopy, we have determined that the dots extend through the depth of the nucleus, forming "paths." By using high-voltage electron microscopy, we have found that the c-*fos* "path" extends out and comes into direct contact with the nuclear envelope (Fig. 2). This is the first study to demonstrate the pathway taken by nascent transcripts of a mammalian cellular gene and to show a direct association of these nascent RNA transcripts with the nuclear envelope. We propose that the path-like distribution represents the movement of nascent transcripts from the site of transcription to the nuclear envelope. The vectorial transport of c-*fos* RNA transcripts toward the nuclear envelope indicates a highly organized and regulated means of transport of mRNA through the nucleus to the nuclear pores.

When we compared the localization of c-*fos* transcripts with the localization of SC-35, an essential splicing factor, we found the transcripts to be in close proximity to a portion of the speckled nuclear regions enriched in SC-35 and small nuclear ribonucleoproteins (snRNPs) (Fig. 3). In fact, the regions enriched in transcripts and splicing factors were observed to be intermingled and/or surrounding each other. The possibility that this association is a random event is unlikely in light of the fact that the nuclear speckles were determined to occupy approximately 17% of the nuclear volume and that the c-*fos* transcripts occupied less than 1% of the nuclear volume of NIH-3T3 cells. These data are in agreement with our previous study in CHOC400 cells which showed that the speckled pattern occupied approximately 18% of the nuclear volume. This study is the first to localize specific nascent transcripts in a mammalian

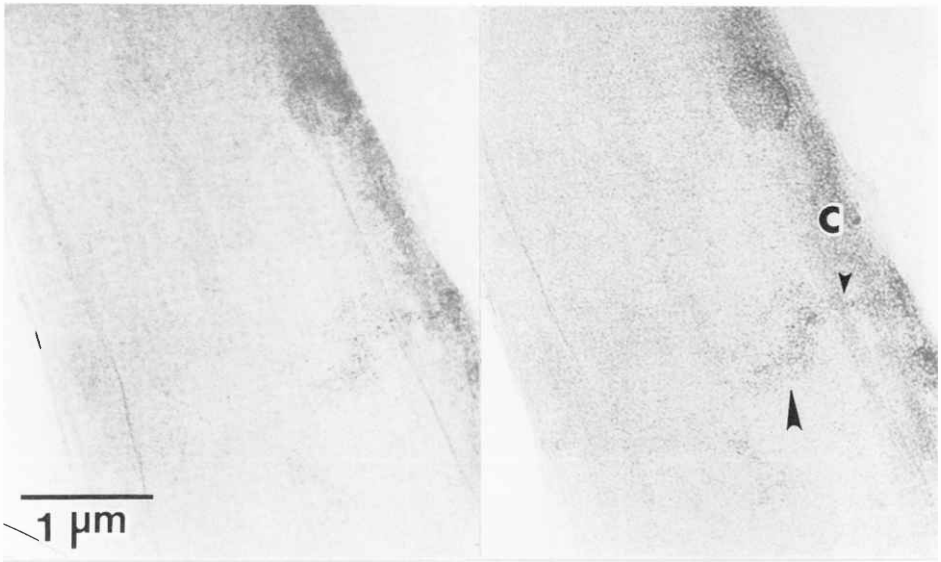

FIGURE 2 c-*fos* transcripts extend in a "path" from their site of transcription to the nuclear envelope. Stereo-pair electron micrograph of a cell cut in cross-section showing the localization of c-*fos* transcripts visualized by peroxidase-conjugated streptavidin. The 1-μm-thick section was examined at 600 kV using a high-voltage electron microscope (HVEM). The transcript path (*large arrowhead*) comes into direct contact with the nuclear envelope. The path extends into the cytoplasm (*small arrowhead*) and transcripts are observed in the cytoplasm (C) on the side of the nucleus where the path of the transcripts exited. Section was not poststained.

cell and to demonstrate a direct link between nascent RNA transcripts and nuclear speckles that are enriched in pre-mRNA splicing factors. These results suggest that the sites of interaction between the c-*fos* RNA transcripts and the speckles are the sites where the c-*fos* pre-mRNA is spliced. We are currently studying the nuclear organization of other cellular transcripts to elucidate general mechanisms of RNA movement through the nucleoplasm.

Differences in snRNP Localization between Transformed and Nontransformed Cells

D.L. Spector, G. Lark, S. Huang

We have examined the localization of snRNPs in a variety of mammalian cells and have observed differences in the organization of these pre-mRNA splicing components in transformed versus immortal or defined passage cells. Cells of defined passage number and immortal cell lines exhibit a speckled staining pattern after immunolabeling with anti-Sm, anti-B′′, or anti-m_3G antibodies (Fig. 4a). In addition, 1–6% of the cells, in a given population, exhibit one to three round "foci" in addition to the speckled labeling pattern. Antibody-labeled transformed cells exhibited one to four large, intensely stained "foci," in 20–98% of the cells, in addition to the speckled labeling pattern (Fig. 4b). In addition to localizing snRNP antigens, we have examined the localization of U1 and U2 snRNAs by in situ hybridization using 2′-*O*-alkyl oligonucleotide probes (provided by A. Lamond, EMBL, Heidelberg). We have found that these snRNAs, which are essential for pre-mRNA splicing, colocalize with the snRNP antigens, supporting the notion that we are localizing intact snRNP particles. When immortal cells (REF-52) that had been transformed by adenovirus (REF-52 Ad5.4) were examined, these cells exhibited an increase in the percentage of cells containing one to two intensely stained "foci," in addition to the speckled labeling, from 6% to 95%. To determine the structural identity of these intensely stained "foci," we examined cell sections by electron microscopy. We have identified

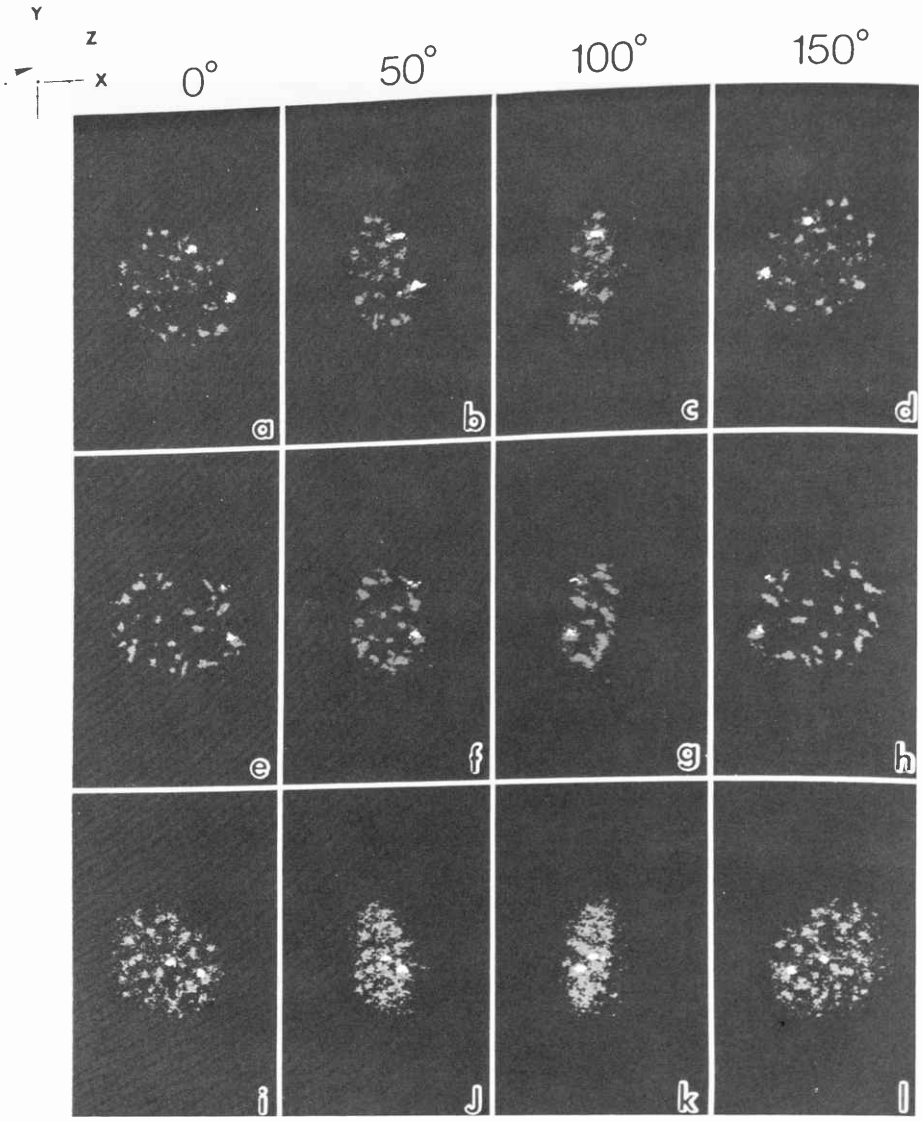

FIGURE 3 c-*fos* transcripts are closely associated with nuclear regions enriched in the essential splicing factor SC-35. Cells were hybridized with a biotinylated c-*fos* genomic probe (white regions) and subsequently immunolabeled with anti-SC-35 antibodies (gray regions). Three-dimensional reconstructions of optical sections of cell nuclei were obtained by confocal laser scanning microscopy. Three cells are shown at 0°, 50°, 100°, and 150° of rotation. *a–d*, *e–h*, and *i–l* represent three different cells.

these structures as coiled bodies that can be visualized in the nucleoplasm of cells with or without antibody labeling (Fig. 5). Coiled bodies are nuclear inclusions first identified at the light microscopic level in 1903 as "accessory bodies" in neuronal cells (Cajal, *S.R. Trab. Lab. Invest. Biol. 2:* 129–221). However, a functional role for this nuclear inclusion is presently unknown. This study is the first to correlate directly an increase in the number of cells containing coiled bodies in a given cell population with the transformed phenotype. However, the fact that snRNP-enriched coiled bodies are not present in all cells of a given population, which still must process pre-mRNA, suggests that this nuclear inclusion prob-

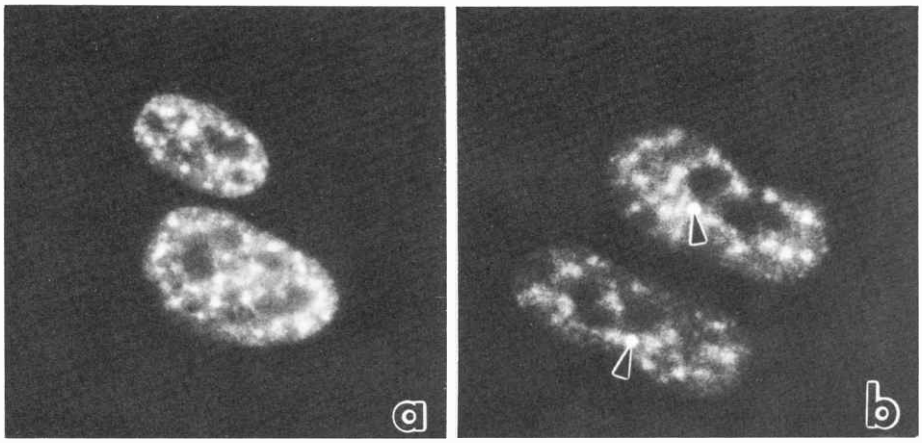

FIGURE 4 In cells of defined passage number, such as Detroit 551 (*a*), snRNPs are localized in a speckled pattern which form interconnections and extend from the surface of the nucleolus to the nuclear envelope-lamina. In 293 cells (*b*), snRNPs are concentrated in a speckled pattern, in addition to being present in foci (*arrowhead*).

ably does not play a central role in pre-mRNA splicing. On the basis of this study, we conclude that the organization of snRNPs within the mammalian cell nucleus is a reflection of the physiology of the cell and that this organization can change upon transformation. The localization of these key components of the pre-mRNA splicing machinery (snRNPs) to speckled nuclear regions in all cell types examined suggests that these regions may be involved in pre-mRNA splicing.

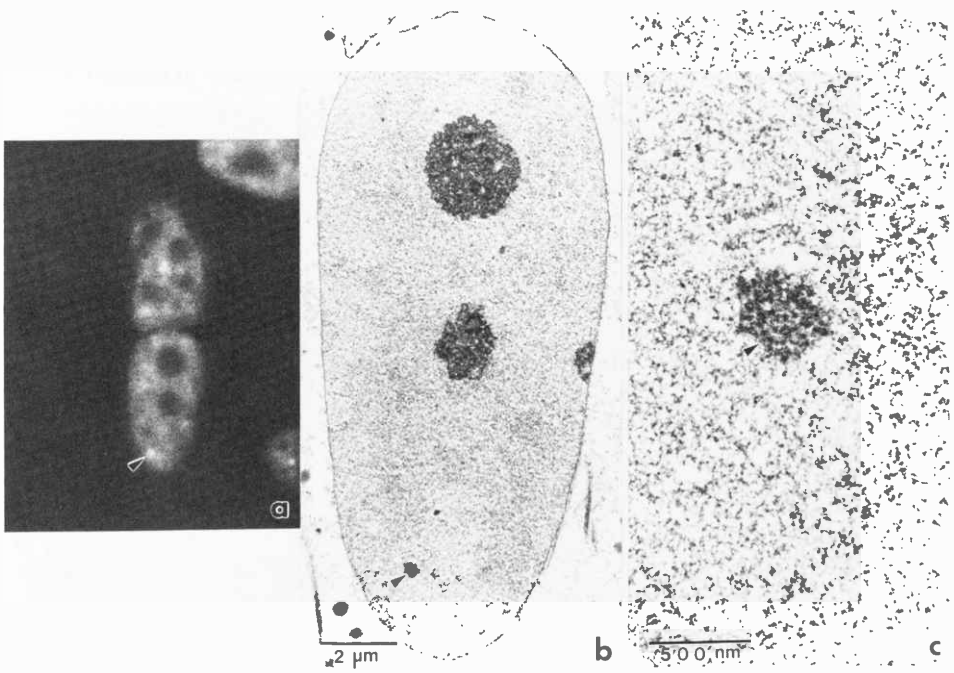

FIGURE 5 "Foci" are coiled bodies. HeLa cells were immunolabeled with anti-Sm monoclonal antibody, photographed, and then prepared for electron microscopy. The "focus" shown in panel *a* is identified as a coiled body in the same nucleus (panels *b,c* arrowheads).

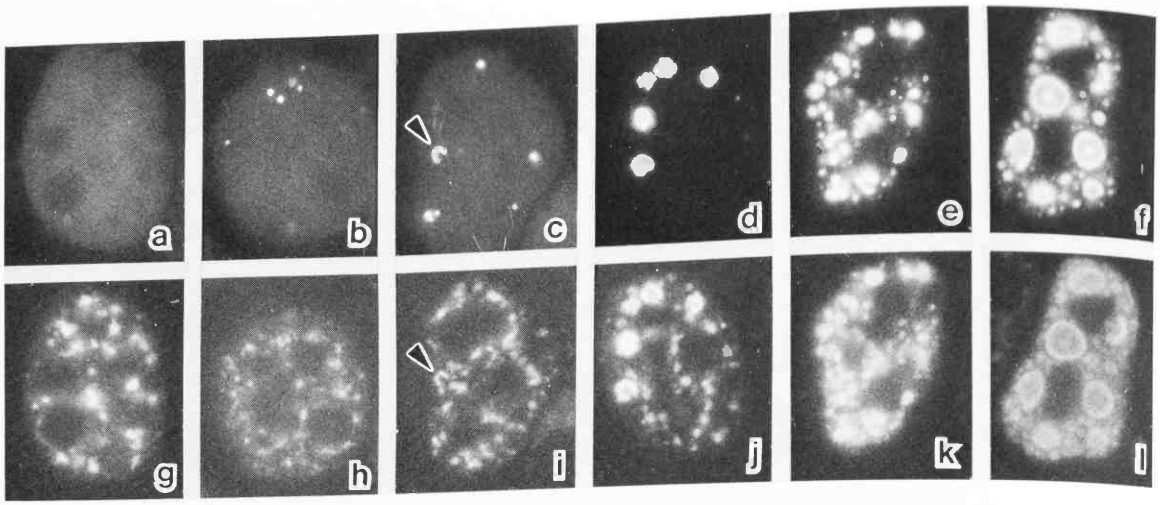

FIGURE 6 HeLa cells were infected with Ad5 at a multiplicity of infection of 30 pfu/cell. Adenovirus transcripts (*a–f*) and the essential non-snRNP splicing factor SC-35 (*g–l*) were localized in infected cells at various time points after infection. See text for details.

Reorganization of the Pre-mRNA Splicing Apparatus upon Adenovirus Infection

L. Jimenez-Garcia, D.L. Spector

We have been interested in understanding the functional significance of the organization of the splicing apparatus in cell nuclei. The adenovirus system provides a unique opportunity to address this goal, since upon adenovirus infection of mammalian cells, the viral transcripts (some of which are extremely abundant) utilize the cellular splicing machinery to process their transcripts. Cells that are not infected with adenovirus 5 (Ad5) do not exhibit a detectable signal after in situ hybridization with an Ad2 biotiny-

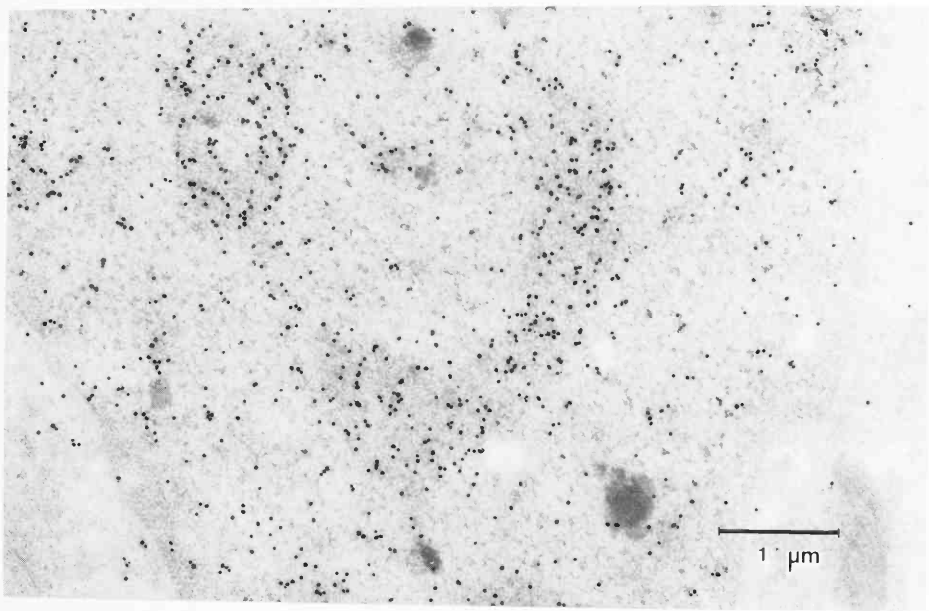

FIGURE 7 At the electron microscopic level, SC-35 appears to concentrate in the doughnut-shaped inclusions, late in infection, as well as being distributed in smaller clusters throughout the nucleoplasm. Colloidal gold particles denote the localization of SC-35.

lated probe (Fig. 6a). In these cells, the non-snRNP splicing factor SC-35 exhibits a typical speckled pattern (Fig. 6g). Cells infected with wild-type Ad5 at a multiplicity of infection of 30 pfu/cell were examined by in situ hybridization at various times postinfection using conditions that would only detect RNA. At 7 hours postinfection, hybridization signal was detected as dots in the nucleoplasm (Fig. 6b). At this early time point, the localization of SC-35 appears to be unchanged (Fig. 6h), although a close association is observed between the viral transcripts and components of the speckled region. Ten hours after infection, viral transcripts appear as larger dots or semicircles (Fig. 6c), and these structures colocalize with regions of SC-35 immunoreactivity (Fig. 6i). The association between viral transcripts and nuclear regions enriched in SC-35 becomes more apparent as the infection process proceeds (compare d and j in Fig. 6; 14 hr postinfection). During the later time points of infection (18–24 hr), the speckled pattern appears to reorganize and splicing factors accumulate in doughnut-shaped nuclear inclusions (Fig. 6k,l), which are characteristic for localization of the virus and viral transcripts (Figs. 6e,f) at these time points. At the electron microscopic level, SC-35 appears to concentrate in the doughnut-shaped inclusions as well as being distributed in smaller clusters throughout the nucleoplasm (Fig. 7). We are currently evaluating the distribution of specific viral mRNAs in order to elucidate the pathways which they take from their sites of synthesis to the nuclear envelope.

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POSTTRANSLATIONAL MODIFICATIONS: REGULATORY AND TARGETING

S.D. Patterson J. Horwitz
Z. Yu

Posttranslational modifications are of critical importance to many proteins for a variety of reasons. The function of many proteins is regulated through the transient addition or removal of specific moieties (posttranslational modification), whereas some posttranslational modifications are permanent and may be involved in targeting proteins to specific compartments within the cell. The studies of this laboratory involve analyses of both of these classes of modifications.

Our basic assay of cellular proteins employs high-resolution two-dimensional gel electrophoresis that allows separation of thousands of proteins at once and the quantitative analysis of these patterns using the QUEST system. We are beginning studies aimed at determining what roles some posttranslational modifications play in cellular processes, such as the cell-division cycle. Much of this work contributes to the human database, being built as part of the mission of the QUEST Protein Database Center, of which I

am affiliated (see QUEST Protein Database Center in this Section). Therefore, as we want to use human cell lines whose normal counterparts can be obtained from the body with relative ease, many of the experiments are conducted using the human T-lymphoblast cell line, Jurkat. Our initial studies are aimed at examining the events associated with the purported G_1 arrest inducible in cells by the antihypercholesterolemic drug, lovastatin (or mevinolin). This is achieved by looking for changes in the two-dimensional gel patterns of antiphosphotyrosine immunoprecipitates, *O*-linked sugar (*N*-acetylglucosamine, GlcNAc) modification, and the expression of cellular proteins including those identified as being isoprenylated (members of this group include the oncoprotein Ras and other related proteins).

Although I joined Cold Spring Harbor Laboratory at the beginning of 1991, I was fortunate to spend the first 3 months of this year in the laboratory of Dr. Ruedi Aebersold at the The Biomedical Research Centre, University of British Columbia, Vancouver, where I used the latest microsequencing techniques, including solid-phase sequence analysis. We also characterized a new experimental protein blotting membrane (see below). Upon my return, I assumed management of the 2D Gel Lab Core Facility (see QUEST Protein Database Center Section) and in July, moved into my laboratory in Demerec where I was joined by my technicians Jill Horwitz and Zailin Yu in August and September, respectively. We have now established an operational protein chemistry laboratory that includes a Hewlett-Packard 1090L LC (funded through a LIBA New Investigator Start-Up Award) and, with Zailin, cell culture support. The instrument has been optimized for reversed-phase high-performance liquid chromatographic separation of peptides and lipids both for micropreparative purposes and for quantitative analyses. As the laboratory has only been functional for a few months, the data presented here will show our interests and direction but will be of a preliminary nature.

A New Protein Blotting Membrane with High Yield Recovery

S.D. Patterson [in collaboration with R.H. Aebersold, The Biomedical Research Centre, Vancouver]

A novel experimental membrane from the Millipore Corporation, based on polyvinylidene-fluoride and derivatized to have a cationic surface, was examined for its ability to bind electroblotted proteins and recover these proteins under relatively mild conditions using solvents compatible with further analyses. The membrane was shown to have a high binding capacity (at least equivalent to standard polyvinylidene-fluoride membrane) and is compatible with both chemical and enzymatic fragmentation of blotted proteins in situ. Both intact electroblotted proteins or fragments thereof (including large cyanogen-bromide-derived peptides) were eluted at high yields, exceeding 70% (Fig. 1). Further structural analysis was demonstrated using reversed-phase high-performance liquid chromatography or gel electrophoresis to separate the cleavage fragments for either pulsed-liquid or solid-phase automated sequence analysis. One drawback of this membrane is that detection of proteins other than by autoradiography is difficult. Much of the work of this laboratory involves micropreparative procedures often employing two-dimensional gel electrophoresis as the final step in the purification. Therefore, the ability to recover proteins electroblotted from gels at high yield will be of enormous benefit to our work.

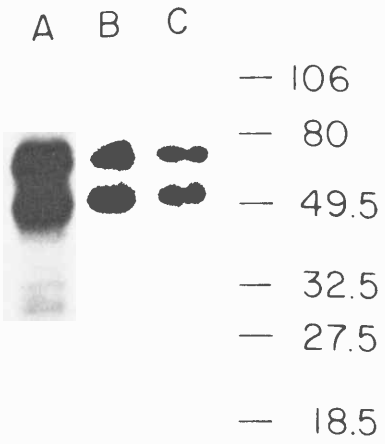

FIGURE 1 Elution of proteins from the Immobilon CD membrane. A monoclonal antibody to phosphotyrosine residues was used to immunoprecipitate proteins from WEHI-231 B lymphoblasts. This precipitate was then phosphorylated in vitro using [γ-^{32}P]ATP. Radioactively labeled proteins were separated by SDS-PAGE and electroblotted onto the Immobilon CD membrane, detected by autoradiography (*A*), and eluted from the membrane using 4 M GuHCl/0.1% Triton X-100 (*B*) or 4 M urea/4 M GuHCl/10% acetonitrile/0.1% TFA/0.05% zwittergent (*C*). Panels *B* and *C* show the membrane strips after protein elution exposed under the same conditions and for the same length of time as the sample shown in panel *A*. Numbers to the right indicate the estimated molecular mass in kilodaltons.

Lovastatin and Isoprenylation of HeLa and CHO Cells

S.D. Patterson [in collaboration with M. Sinensky and R. Lutz, Eleanor Roosevelt Institute]

The drug lovastatin (or mevinolin) is a potent competitive inhibitor of the rate-limiting enzyme of cholesterolgenesis, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), and is used clinically to lower serum cholesterol levels. Another consequence of this competitive inhibition is that post-translational lipid modification (isoprenylation) of cellular proteins is blocked. This block can be overcome by the addition of the product of HMG-CoA reductase, mevalonic acid. If the mevalonic acid is radioactively labeled, then proteins carrying this modification can be identified. In some cell lines, this drug has also been shown to block cell division. Thus, our initial collaboration with Drs. Sinensky and Lutz was to determine which proteins in HeLa and CHO cells were labeled using this method and also to look at the alteration of the whole-cell pattern as a result of the lovastatin block. The results of the HeLa cell experiments are shown in Figure 2, which reveals that most of the label is incorporated into protein in the 20–30-kD mass range. A similar pattern was observed for CHO cells. These low-molecular-mass proteins are likely to be the small GTP-binding proteins that are members of the *ras* superfamily. Many of the protein shifts in the [^{35}S]methionine-labeled patterns correlate with the block of isoprenylation. This is due to carboxymethylation, the next processing step following isoprenylation of proteins, not taking place. Therefore, no loss of negative charge occurs and the protein is not basic-shifted (shifted to the right), as is normally the case. Identification of the prenylated proteins and those proteins that exhibit a charge shift but do not appear to be isoprenylated is under way.

Lovastatin-induced G_1 Arrest of Jurkat Cells (?) and the Anticarcinogen, Limonene

S.D. Patterson, Z. Yu, J. Horwitz

Lovastatin is known to block the S-phase burst of DNA synthesis during the late G_1 phase of the cell cycle in a number of cell lines. Our interest in cell

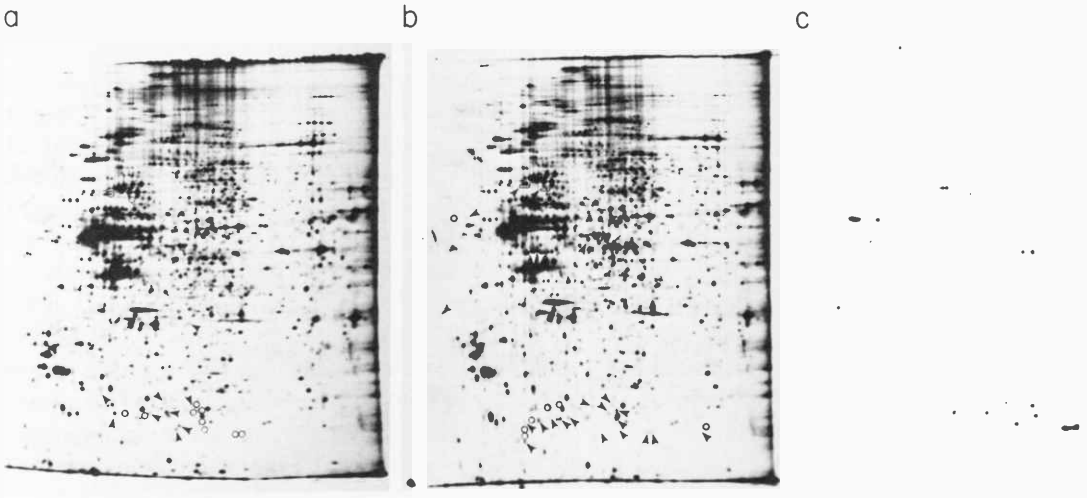

FIGURE 2 Isoprenylated proteins from HeLa cells and the effects of lovastatin treatment. HeLa cells were incubated overnight (~17 hr) with (*a*) or without (*b*) 12.5 μM lovastatin and [^{35}S]methionine or with 12.5 μM lovastatin and [^{3}H]mevalonolactone ([^{3}H]MVA) (*c*). Whole-cell lysates were then subjected to two-dimensional electrophoresis using pH 3–10 ampholytes in the first dimension IEF gel and a 10% T SDS-PAGE gel in the second dimension. The gels are oriented with the anode of the first dimension (acid) to the left and at the bottom for the second dimension. The proteins labeled by [^{3}H]MVA in panel *c* are either arrowed or circled in panel *b* depending on whether or not they are labeled by [^{35}S]methionine, respectively. Arrowed proteins in panel *a* represent new protein species that have appeared following lovastatin treatment, and circles show the position of proteins that have disappeared upon treatment.

cycle control prompted a detailed study of the effects of lovastatin on Jurkat T lymphoblasts for three reasons: (1) to provide a means of labeling isoprenylated proteins in vivo, (2) to arrest cells at a specific stage of the cell cycle (G_1) for subsequent release and synchronization to enable cell-cycle-dependent effects to be observed in these cells, and (3) to attempt to identify the nonsterol product of mevalonate that is necessary for cellular proliferation and determine the pathway responsible for this G_1 arrest. The first of these has been achieved, and preliminary data showing those proteins that are isoprenylated are shown in Figure 3. Initial results indicate that a number of proteins in the 20–30-kD region and others at 40–60 kD are isoprenylated. We are currently determining the type of isoprenylation (C_{15}-farnesyl or C_{20}-geranylgeranyl) and the identity of some of these proteins. This will incorporate the use of an in situ binding assay for the determination of GTP-binding proteins, as it would appear that some of those in the 20–30-kD region may be members of the family of small GTP-binding proteins that includes the products of the genes *ras*, *rab*, and *rho*. The second and third aspects are being examined using both cell biological and high-resolution electrophoretic techniques. We are examining the effects of this drug on protein expression by metabolically labeling with [^{35}S]methionine for either short or long periods and also looking at alterations in the posttranslational modifications of *O*-linked sugar (*O*-GlcNAc) and phosphotyrosine (and associated proteins, as detected by immunoprecipitation). Cells treated with lovastatin show differences by all of these criteria, and we are now attempting to identify the proteins that show these alterations.

Isoprenylation of some 21–26-kD proteins, including *ras*, has also recently been shown to be inhibited by the monoterpene anticarcinogen, *d*-limonene (Crowell et al., *J. Biol. Chem. 266:* 17679 [1991]). The reported selectivity of this effect is currently being examined, and from initial data (see Fig. 3), there does appear to be a selective decrease in isoprenylation of some, but not all, proteins in the 20–30-kD region.

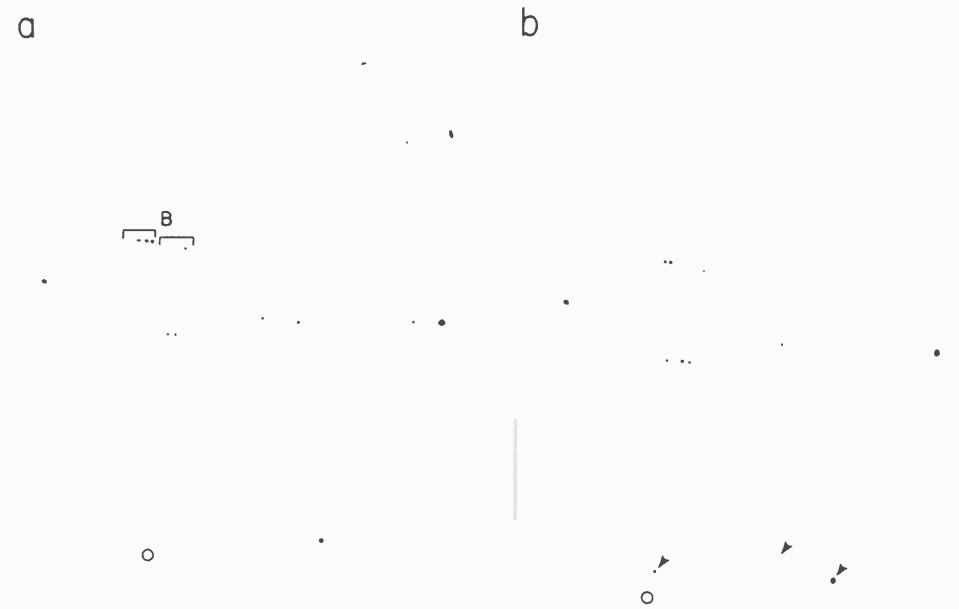

FIGURE 3 Isoprenylated proteins of Jurkat T lympho-blasts and the effect of limonene on this posttranslational modification. Jurkat cells were incubated overnight (~18 hr) with 12.5 μM lovastatin and then concentrated and labeled with [^{3}H]mevalonolactone in the presence (*a*) or absence (*b*) of 5 μM *d*-limonene for 3 hr. The arrowed low-mass proteins in panel *a* show decreased labeling upon limonene treatment. No other proteins, including the low-mass protein circled in both panels, show decreased labeling upon limonene treatment. Putative lamin B isoforms are identified by B in panel *a*. The gels were run and are oriented as described in Fig. 2.

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QUANTITATIVE REGULATORY BIOLOGY

B.R. Franza G. Mak, J. Ross, M. Neumann, A. Giordano, X. Grana, A. Calzolari, J.A. Scheppler, Y. Li, H.R. Tan, G. Niu

We study cellular proteins involved in the growth control of mammalian cells. Lymphoblasts and hematopoietic progenitor cells are the normal human tissues that we use to study the molecular mechanisms regulating cell proliferation and differentiation. So far, the mammalian cells we have studied most comprehensively are human T-lymphoblast cell lines. During the past several years, we have focused on three processes. One is the effect expression of oncogenes has on the entire complement of proteins present in a particular cell type. The second is the induction of protein alterations when quiescent cells are stimulated to proliferate. These studies are directed at determining the molecules involved in conveying signals to the genome and the earliest responses of the genome to these signals. The third process is the control of gene expression at the level of regulation of transcription of mRNA encoding genes. The QUEST protein database system is the tool on which we rely heavily for performing such a comprehensive qualitative and quantitative analysis of regulatory proteins. The goal, implicit in all of our studies, is to construct a molecular description of the networks involved in regulating transcription and other genome responses to a change in the growth state of the cell.

Highlights of the past year include the demonstration of cell-division-cycle-dependent change in the kinase activity of growth control protein affinity complexes, of interactions between c-Rel and a Rel-related gene product, and the elucidation of specific proteins that associate with the "E2F" DNA control element. Some details of each study are presented below. These studies provide further evidence for the need to characterize the temporal and spatial (subcellular) fluctuation in amounts of specific gene products and their intermolecular interactions. Much to the credit of our colleagues Jim Garrels, Jerry Latter, and Pat Monardo, 1992 appears to be the year in which we will have a database software system sufficiently powerful to support the extensive comparative analysis of the quantitative and/or qualitative change among members of different sets of regulatory cellular proteins isolated from cells at different stages of growth. To take full advantage of this system, we are constantly expanding the molecular probes necessary for the identification and characterization of regulatory proteins. The individuals listed above work as a group, with those contributing most to a particular project being recognized by first authorship of published reports (see Publications list).

RETINOBLASTOMA-ASSOCIATED GENE PRODUCT IS PHOSPHORYLATED IN VITRO BY GROWTH-CONTROL PROTEIN COMPLEXES

We previously demonstrated that an immune complex containing cellular proteins associated with E1A phosphorylates histone H1 in a cell-cycle-dependent manner and that association of pRB with E1A apparently was not required for this activity. The pRB

protein is a nuclear phosphoprotein suspected of playing a role in cell cycle regulation. RB can form complexes with at least three DNA tumor virus proteins; papovavirus T, human papillomavirus (HPV) E7, and transforming adenovirus protein E1A.

We have now demonstrated in vitro biochemical evidence for the phosphorylation of pRB by kinases contained in complexes between E1A and cellular proteins. Using mutated forms of E1A, it was determined that p60/cyclin A and cdk2, a member of the cdc2 family, must both be present to phosphorylate pRB in vitro. Phosphorylation of pRB by the E1A immuno-complexes has also been shown to fluctuate during the cell division cycle. We demonstrated in vitro biochemical evidence for the phosphorylation of pRB by other complexes containing growth regulatory proteins. Experiments aimed at understanding the molecular basis of these associations, in particular, how E1A targets these growth regulatory complexes, are now in progress. The results so far are consistent with a mechanism in which E1A expression, independent of physical association with pRB, could abrogate pRB arrest of cell division by altering its phosphorylation status. These studies were accomplished with reagents provided through a collaboration with R. Hollingsworth, Jr., W.M. Yang, and W.H. Lee of the Center for Molecular Medicine, University of Texas, San Antonio.

MOLECULAR INTERACTIONS BETWEEN INDUCIBLE MEMBERS OF THE REL FAMILY OF GENE PRODUCTS

During our initial characterization of the cellular proteins that interact specifically with the κB DNA control element, we identified an inducible set of protein isoforms that appeared to be related. We called these 2D-gel-resolved isoforms HIVEN86A. Subsequently, we demonstrated that HIVEN86A was the product of the c-*rel* gene. High-resolution 2D-gel studies of immune complexes of c-Rel from human cells have revealed that all of the isoforms of Rel we detected using our DNA affinity precipitation (DNAP) assay do indeed react directly with anti-Rel antibodies. We have identified numerous cellular proteins that coprecipitate with Rel, as have other investigators. These proteins are not recognized directly by the anti-Rel antibody. Among these proteins is a p40 protein that we have observed to be associated with Rel in immune complexes isolated from cytoplasmic but not nuclear extracts of Rel. We also have not found conditions in our DNAP assay in which this protein is recovered with Rel using the κB site as the target DNA.

Several groups have reported genes whose products contain regions of structural similarity with those of Rel. One of these genes encodes a protein of approximately 105 kD (p105). It has been demonstrated that an approximately 50-kD amino-terminal portion of the p105 protein is the p50 component of NF-κB. NF-κB is composed of p50 associated with another gene product, p65. p65 is also structurally related to Rel. Using antibodies we have developed to p105 and to Rel, we have found that Rel immune complexes contain p105 and that p105 immune complexes contain Rel. We have not found conditions in which p105 is found with Rel in κB DNA affinity complexes. However, we have found that the synthesis of new p105 is inducible by mitogenic lectins and tumor promoters, as is Rel. We have also found that Rel-p105 complexes can be isolated from nuclei as well as cytoplasm. Our p105 antibody does not immunologically cross-react with Rel or p50. We are raising antibodies to the p50 protein and the p65 protein in order to have sufficient reagents to characterize the temporal change in quantity of each gene product in different stages of cellular growth and to characterize their inter-molecular interactions simultaneously.

PROTEIN INTERACTIONS AT THE E2F CONTROL ELEMENT

The E2F transcription factor demonstrates DNA-binding activity to specific sequences in the adenovirus early region 2 (E2) promoter and apparently similar interactions occur in promoters for cellular genes such as c-*fos*, c-*myc*, and dihydrofolate reductase. Interactions between E2F and the products of the retinoblastoma susceptibility (pRB) gene and the cyclin A (p60/cyclin A) gene have been described recently. Exactly how many proteins, i.e., different gene products, bind the E2F DNA element either independently or cooperatively has not been elucidated. Because of our interest in describing molecular mechanisms that regulate expression of regulatory gene products like Fos and the effect oncogenes like E1A may have in altering these mechanisms, we have begun to define the proteins that interact with the E2F site.

We have used the DNAP assay to enrich proteins that bind to a functional E2F site and not to a mutant form of the E2F site. At this point, cellular proteins

from asynchronously dividing cells have been resolved that associate with the normal site. They are either significantly reduced or absent from the mutant DNAP assays. We appear to have detected pRB in the functional E2F site DNAP complex by immunoblot analysis. We are beginning to study the effect(s) of E1A and the association of each protein with the E2F site, the changes in interaction when cells enriched at different stages of the cell division cycle are used as sources of extracts for binding assays, and the response of these proteins to stimuli known to activate immediate-early gene expression (e.g., c-*fos*).

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MOLECULAR CELL BIOLOGY

D.M. Helfman

W. Guo
J. Kazzaz
J.P. Lees-Miller
G. Mulligan
M. Pittenger
S. Stamm
T. Tsukahara
S. Wormsley

The research in our laboratory continues to focus on two fundamental problems in molecular and cell biology: (1) the mechanisms responsible for tissue-specific and developmentally regulated patterns of gene expression and (2) the functional significance of cell-type-specific protein isoform expression. We

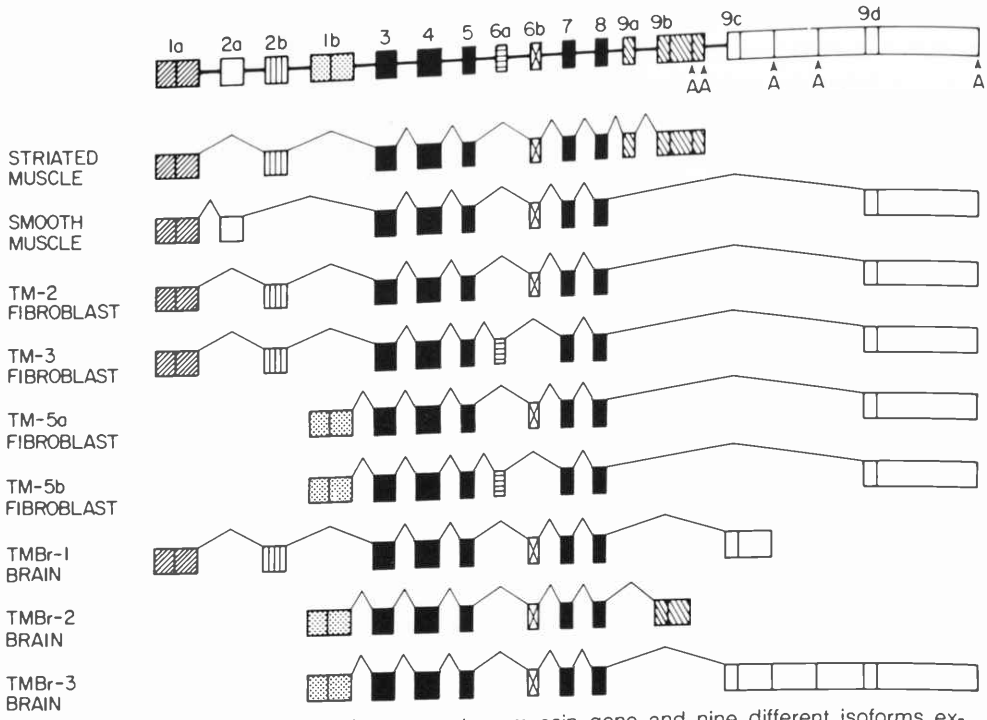

FIGURE 1 Schematic diagram of the rat α-tropomyosin gene and nine different isoforms expressed from this gene. Boxes represent exons and horizontal lines represent introns; they are not drawn to scale. The gene contains two alternative promoters that result in expression of two different amino-terminal coding regions (exons 1a and 1b), two internal mutually exclusive exon cassettes (exons 2a and 2b and 6a and 6b), and four alternatively spliced 3′ exons that encode four different carboxy-terminal coding regions (exons 9a, 9b, 9c, and 9d). The different polyadenylation signals are also indicated (A).

have been interested in understanding the regulation and function of tropomyosin gene expression in muscle and nonmuscle cells. Tropomyosins are among the major components of the thin filaments of skeletal and cardiac muscle and the microfilaments of nonmuscle cells. These filaments are involved in a number of cellular processes, including muscle contraction, cell movement, and the generation of cell shape. Of interest to our laboratory is the observation that different forms of tropomyosin are expressed in different cell types and tissues. Distinct isoforms are present in striated muscle (skeletal and cardiac), smooth muscle, and nonmuscle cells. We now know that at least 12 different tropomyosin isoforms are expressed from three separate genes in rat. The α-tropomyosin (α-TM) gene encodes nine isoforms (Fig. 1), the β-tropomyosin (β-TM) gene encodes two isoforms (Fig. 2), and the tropomyosin-4 (TM-4) gene encodes a single isoform. We have been studying the expression of these genes with particular at-

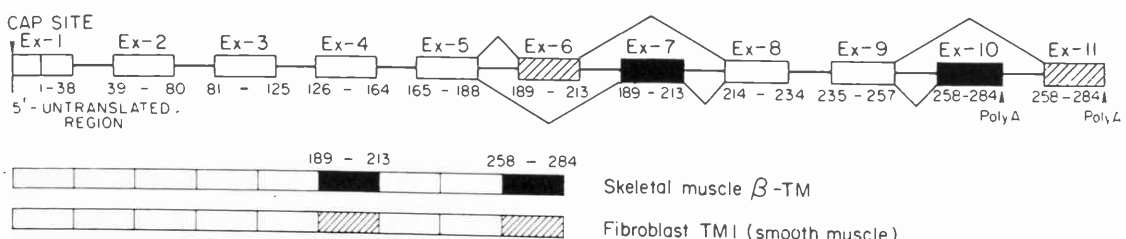

FIGURE 2 Schematic diagram of the rat β-tropomyosin gene and the two different isoforms expressed from this gene. The amino acids encoded by each exon are indicated. The cap site and polyadenylation sites are also indicated.

tention to understanding the mechanisms of their regulation at the posttranscriptional level, i.e., tissue-specific alternative RNA splicing. In addition, we have continued to study the promoter elements in the β-TM gene. We have also begun to study alternative splicing of the clathrin light chain B gene. The expression of a diverse group of tropomyosin isoforms in a highly tissue-specific manner via alternative RNA processing strongly suggests that each isoform is required to carry out specific functions in conjunction with the actin-based filaments of various muscle and nonmuscle cells. The function of these different isoforms is not known and is under study. We have recently identified a gene from the fission yeast *Schizosaccharomyces pombe* that encodes a distantly related member of the actin family and have begun to study the function of this actin-like protein. Below is a summary of our present studies.

Alternative Splicing of β-TM Pre-mRNA: *cis*-Acting Elements and Cellular Factors That Block the Use of a Skeletal Muscle-specific Exon in Nonmuscle Cells

W. Guo, G. Mulligan, S. Wormsley, D. Helfman

We are using the rat β-TM gene as a model system to investigate the molecular basis for developmental and tissue-specific alternative RNA splicing. The β-TM gene expresses both skeletal muscle β-TM and fibroblast TM-1 by an alternative RNA splicing mechanism (Fig. 2). Previous studies from our laboratory have shown that the intron sequences upstream of the 3′-splice site of exon 7 (skeletal muscle-specific splice) are important for alternative splice site selection, because deletion of these sequences resulted in the use of the skeletal muscle-specific exon in nonmuscle cells (Helfman et al., *Genes Dev. 4:* 98 [1990]). We have carried out an extensive mutational analysis to identify *cis*-acting elements that block the use of the skeletal muscle-specific exon 7 in nonmuscle cells. These studies have localized the critical elements for regulated alternative splicing to sequences within exon 7 and part of the adjacent upstream intron. In addition, mutations that inactivate the 5′- or 3′-splice sites of exon 6 do not result in the use of the skeletal muscle-specific exon 7 in nonmuscle cells, suggesting that splice site selection in vivo is not regulated by a simple *cis*-acting competition mechanism but rather by a mechanism that inhibits the use of exon 7 in certain cellular environments. In support of this hypothesis, we have identified sequence-specific RNA-binding proteins in HeLa cell nuclear extracts using native gel electrophoresis and binding competition assays. Mutations in the pre-mRNA that result in the use of the skeletal muscle exon in vivo also disrupt the binding of these proteins to the RNA in vitro. We propose that the binding of these proteins to the pre-mRNA is involved in regulated alternative splicing and that this interaction is required for blocking the use of the skeletal muscle exon in nonmuscle cells.

Purification and Characterization of an RNA-binding Protein That Interacts with Sequences Involved in Alternative Splicing of β-TM Pre-mRNA: Identity with the Polypyrimidine Tract Binding Protein

G. Mulligan, W. Guo, S. Wormsley, D. Helfman

As described above, we have found that the regulatory sequences in the intron upstream of exon 7 interact with sequence-specific RNA-binding proteins in HeLa nuclear extracts (Guo et al. 1991). This has led us to propose that nonmuscle cells contain factors that interact with specific regulatory sequences in the pre-mRNA to block the use of the skeletal muscle exon. To study further the precise nature of the factors that interact with sequences in the β-TM pre-mRNA, we have purified to apparent homogeneity a protein that interacts specifically with the intron regulatory element upstream of the skeletal muscle-specific exon 7. This protein is identical to the polypyrimidine tract binding protein (PTB) which other studies have shown to be involved in the recognition and use of 3′-splice sites (Garcia-Blanco et al., *Genes Dev. 3:* 1874 [1989]). Our analysis of the introns between exons 5 through 8 in the tropomyosin pre-mRNA demonstrates that PTB binds only to the intron upstream of the skeletal muscle-specific exon 7. The nature of the interactions in this intron reveals several new details about the binding specificity of PTB and suggests that PTB does not function exclusively in a positive manner in the recognition and use of all 3′-splice sites. Although we cannot rule out the possibility that other factors will also bind to these sequences, our work

demonstrates that the regulatory element in the intron upstream of exon 7 can interact with PTB in a sequence-specific manner. It is also worth noting that our previous analyses of *cis*-acting regulatory elements in β-TM pre-mRNA indicate that sequences in exon 7 also participate in blocking the use of this exon in nonmuscle cells (Guo et al. 1991). However, these exon sequences do not appear to be required for the interaction of PTB binding, and mutations in this exon do not disrupt the binding of PTB (data not shown). It remains to be determined whether these exon sequences interact with other factors.

Although the biological significance of the PTB binding to sequences upstream of exon 7 remains to be established, a strong correlation exists between the ability of PTB to bind to these sequences and our previous results demonstrating that mutations in this region lead to activation of the skeletal muscle-specific exon in vivo (Guo et al. 1991). The same mutations that result in the use of exon 7 in nonmuscle cells in vivo also disrupt the binding of PTB in vitro. Thus, the interaction of PTB with the intron regulatory elements in the β-TM pre-mRNA may account for the regulation of splice site selection. Additional interactions with PTB are observed with the long polypyrimidine tract upstream of the 3′-splice site (−117 to −143 nucleotides), which has been demonstrated to specify the location of the branchpoints used, 144–153 nucleotides upstream of exon 7 (Helfman et al., *Genes Dev. 4:* 98 [1990]). This interaction is likely to be required for branchpoint formation (Mullen et al., *Genes Dev. 5:* 642 [1991]). On the other hand, interaction of PTB with the regulatory sequences downstream from this polypyrimidine tract appears to block the use of this exon in nonmuscle cells. The results suggest both a positive and negative role of PTB in the regulation of alternative splicing of exon 7 in skeletal muscle and nonmuscle cells.

How these dual functions of PTB might be achieved is not clear, but we can offer a few speculations. Since PTB can exist as multiple isoforms via alternative RNA splicing, skeletal muscle and nonmuscle cells could express a different set of PTB isoforms with different binding specificities, which thereby lead to tissue-specific splice site selection. However, we have thus far been unable to detect differences in isoform expression in different cell types or in the binding of RNAs containing the intron regulatory sequences in nuclear extracts obtained from undifferentiated and differentiated myogenic cells. It is also possible that the binding of PTB to both the polypyrimidine tract and regulatory element is constant in all cell types. PTB bound to the RNA could then serve as a platform for the interaction or recruitment of other factors. For example, the distribution or number of PTB binding sites along a region of RNA could provide different recognition sites for the interactions of additional binding factors, which might be expressed in a tissue-specific manner. Thus, PTB bound to the polypyrimidine tract associated with branchpoint use could provide a binding site for factors that are distinct from PTB bound to the downstream regulatory sequences. Alternatively, the binding of PTB might be involved in packaging of the pre-mRNA into a heterogeneous nuclear ribonucleoprotein (hnRNP) particle. The proper assembly of the β-TM pre-mRNA with hnRNP proteins might be essential for regulation of tissue-specific splicing. Alterations in the distribution of specific hnRNP proteins, e.g., PTB, along the pre-mRNA, could, in principle, result in loss of normal splice site regulation.

In summary, having identified that PTB can bind to specific regulatory sequences in the β-TM pre-mRNA, it remains to be determined whether these interactions do in fact contribute to splice site selection. Work is currently in progress to identify precisely the sequences in the RNA that bind to PTB and to develop in vitro splicing systems to test directly the functional role of PTB in alternative splice site selection.

Regulation of Alternative 3′-splice Site Selection in β-TM Pre-mRNA

T. Tsukahara, D. Helfman

We previously reported that the splicing of exon 5 to exon 6 in the rat β-TM gene required that exon 6 first be joined to the downstream common exon (Helfman et al., *Genes Dev. 2:* 1627 [1988]). Pre-mRNA containing exon 5, intron 5, and exon 6 was not spliced at all. We have carried out a mutational analysis to determine which sequences in the pre-mRNA contribute to the inability of this precursor to be spliced in vitro. Interestingly, we find that mutations in two regions of the pre-mRNA lead to activation of the 3′-splice site of exon 6, without first joining exon 6 to exon 8. First, introduction of a nine-nucleotide poly(U) tract near the 3′ end of intron 5 was sufficient to permit the splicing of exon 5 to exon 6, with as little as 35 nucleotides of exon 6. Second, introduction of a consensus 5′-splice site in exon 6 leads

to splicing of exon 5 to exon 6. The result obtained with the latter mutation is in agreement with the exon definition model recently proposed by Sue Berget and colleagues (*Mol. Cell. Biol. 10:* 84 [1990]). Collectively, our studies demonstrate that three distinct elements can act independently to activate the use of the 3′-splice site of exon 6: the sequences in exon 8, a poly(U) tract in intron 5, and the 5′ splice of exon 6. Work is currently in progress to determine whether each of these sequences interacts with distinct factors for 3′-splice site utilization.

In Vitro and In Vivo Characterization of Four Fibroblast Tropomyosins Produced in Bacteria: TM-2, TM-3, TM-5a, and TM-5b Are Colocalized in Interphase Fibroblasts

M. Pittenger, D. Helfman

Most cell types express several tropomyosin isoforms, the individual functions of which are poorly understood. In rat fibroblasts, there are at least six isoforms: TM-1, TM-2, TM-3, TM-4, TM-5a, and TM-5b. TM-1 is the product of the β gene, TM-4 is produced from the TM-4 gene, and TM-2, TM-3, TM-5a, and TM-5b are the products of the α gene. To begin to study the localization and function of the isoforms in fibroblasts, cDNAs for TM isoforms TM-2, TM-3, TM-5a, and TM-5b were placed into bacterial expression vectors and used to produce TM isoforms. The bacterially produced TMs were determined to be full-length by sequencing the amino and carboxyl termini. These TMs were found to bind to F actin in vitro, with properties similar to that of skeletal muscle TM, except that TM-5a had a relatively weaker affinity. To investigate the intracellular localization of these fibroblast isoforms, each was derivatized with a fluorescent chromophore and microinjected into rat fibroblasts (see Fig. 3). TM-2, TM-3, TM-5a, and TM-5b were each found to associate along actin filaments. There was no preferred cellular location or subset of actin filaments for any of these isoforms, although TM-5a appeared to give weaker labeling in vivo. Furthermore, coinjection of two isoforms labeled with different fluorochromes showed identical localization. At the level of the light microscope, these TM isoforms do not appear to achieve different functions by binding to particular subsets of actin filaments or locations in cells, although this needs to be studied further. The results show that bacterially produced TMs can be used to study in vitro and in vivo properties of the isoforms. In the coming year, we plan to study the dynamic distribution of each isoform using video microscopy to follow the fluorescently labeled tropomyosins during cell spreading, mitosis, and cell movement.

Transcriptional Control of β-TM Gene Expression

J. Kazzaz, D. Helfman

The rat β-TM gene expresses two distinct isoforms via an alternative splicing mechanism (see Fig. 2). Although the gene is expressed in different muscle (skeletal, cardiac, and smooth) and nonmuscle cells, a single transcription initiation site is used in the various cell types that express the gene. We have identified three *cis*-acting elements involved in the regulation of transcription of this gene. The first is a region located 200–500 base pairs upstream of the transcription initiation site. This element is responsive to exogenous MyoD when rat or mouse fibroblast cell lines (REF52 and NIH-3T3, respectively) are cotransfected with a tropomyosin promoter-CAT construct and a construct that contains a copy of MyoD cDNA clone (courtesy of A. Lassar). To determine whether this element is responsive to endogenous MyoD, we transfected C_2C_{12} cells with the various tropomyosin-promoter constructs. This cell line, upon induction with low serum, differentiates from myoblasts to form myotubes, and express MyoD. Transfection into differentiated myotubes indicates that this region is sufficient for activation of transcription in differentiated muscle cell lines. Sequence analysis of this region indicates that it contains three copies of a skeletal muscle-specific enhancer element located approximately 304, 352, and 398 base pairs upstream of the transcriptional start site. The consensus sequence of this 14-nucleotide-long element has been found in a number of genes expressed in skeletal muscle, including the muscle creatine kinase, δ-subunit of the acetylcholine receptor, myosin-light-chain 1/3, desmin, and vimentin (Buskin and Hausch-ka, *Mol. Cell. Biol. 9:* 2627 [1989]).

The second element involved in regulation is found within the first intron of the gene. This region contains a 124-base-pair direct repeat within the first intron of the β-TM gene. This repeat has a 45-

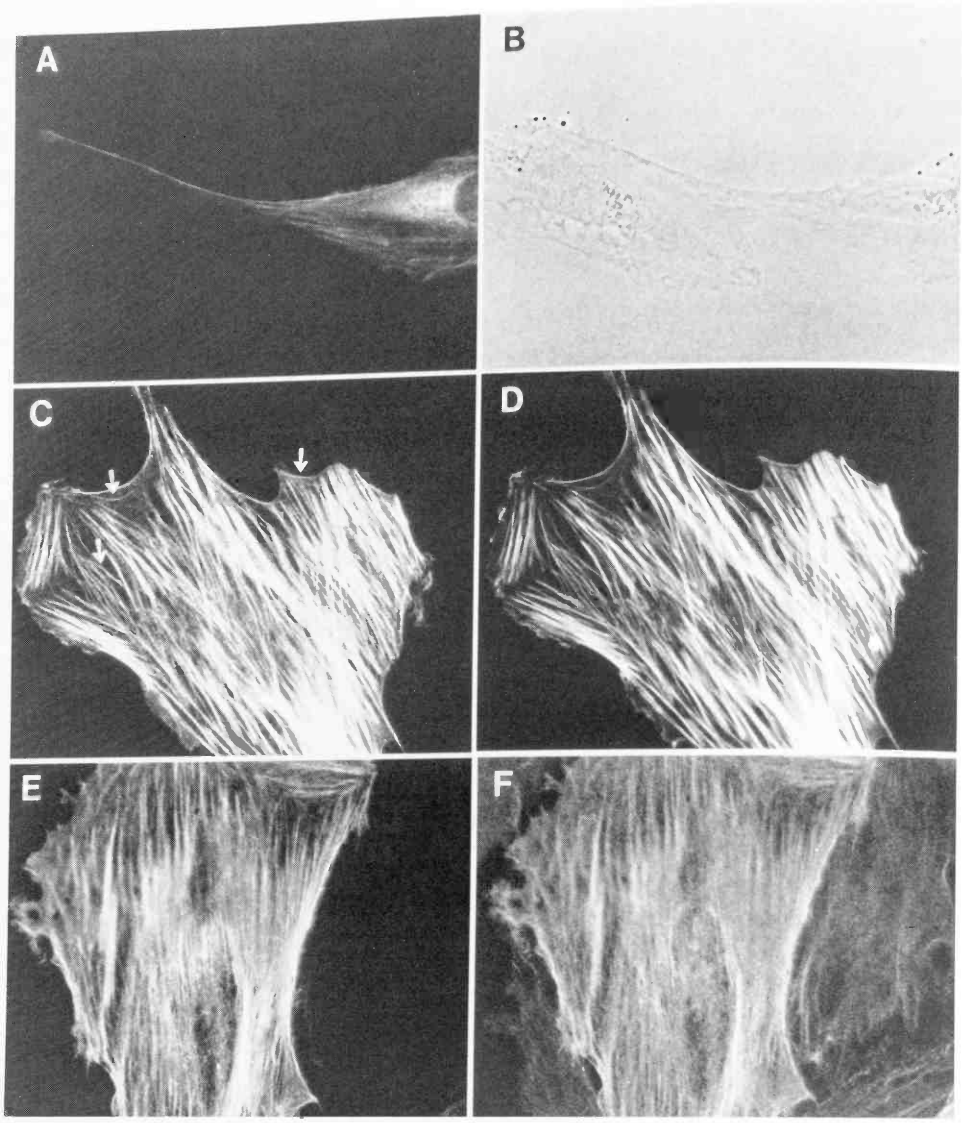

FIGURE 3 Localization of TM-2 in fibroblasts. Bacterially produced fibroblast TM-2 was labeled with lissamine rhodamine B sulfonyl chloride (LRB) and microinjected into rat fibroblasts (REF52 cells). Three hours after injection, the cells were fixed and visualized by fluorescence microscopy. (*A,C,E*) Cells microinjected with LRB-TM-2. In panel *A*, TM-2 can be seen in the long cellular process as well as the cell body. An uninjected neighboring cell is also seen in the phase image in panel *B*. TM striations can be seen in well-spread cells (see arrows in panel *C*), which are not seen with FITC-phalloidin-stained microfilaments (panel *D*). (*E*) Injected TM-2 viewed in the rhodamine channel; (*F*) same field when cells are stained with antibodies to TM and a fluorescein-labeled second antibody.

nucleotide purine/pyrimidine (dA-dC) stretch. A poly(dA-dC) repeat has been shown to have enhancer properties in SV40 (Berg et al., *Mol. Cell. Biol. 9:* 5248 [1989]). To determine whether this element has enhancer-like activity, we have cloned both copies of this element upstream of the promoter elements in the tropomyosin-CAT constructs indicated above. Transfection experiments in muscle and fibroblast cell lines suggest that this element functions as an enhancer in muscle cell lines. Cotransfection with MyoD indicates that the effect of the internal enhancer is additive to that of the muscle regulatory region. Further

investigation will determine whether the enhancer activity resides in the poly(dA-dC) repeat, the flanking regions, or a combination of both.

The third element is found within the second intron. This element activates transcription in nonmuscle cells without exogenous MyoD. Work is in progress to determine whether this element has enhancer-like activity and whether its effect is additive with respect to the *cis*-acting elements described above.

Rat Clathrin Light Chain Gene Structure, Expression, and Regulation

S. Stamm, D. Helfman [in collaboration with Jurgen Brosius and Diana Casper, Mount Sinai School of Medicine, New York]

We are studying the structure, expression, and regulation of the rat clathrin light chain B. Clathrin heavy and light chains form a basket-like structure that constitutes the protein backbone of coated pits and vesicles. Coated pits are found on the surfaces of cells where they participate in the internalization of various molecules, whereas coated vesicles are thought to function in the transport of proteins from the Golgi to the cell surface. There are no tissue-specific forms of the heavy chains of clathrin, whereas the light chains can be expressed in a tissue-specific manner. The rat clathrin light chain B gene expresses two distinct isoforms via alternative RNA splicing. Interestingly, one form is only detected in brain and appears to be specific to neurons. To understand the molecular basis for the tissue-specific splicing, we have isolated and characterized genomic clones for the clathrin light chain B gene. The gene was found to span approximately 11 kb of DNA and contains six exons. The brain-specific exon, termed exon EN, is located between exon 4 and exon 6. Expression of this exon is found in brain, primary neurons from central or peripheral nervous system, but not in fibrobasts, HeLa cells, primary glial cultures, or glial-type cells. Thus, it appears that the brain-specific splicing is found only in neurons. We have begun experiments to study the *cis*-elements and cellular factors required for alternative splice site selection. We have identified a critical sequence in the brain-specific exon that is required for the use of this exon in primary neuronal cell cultures. Further work is planned to identify cellular factors that might bind to these sequences and thereby mediate tissue-specific splicing.

Identification of an Essential Gene Encoding an Actin-related Protein from *Schizosaccharomyces pombe*

J.P. Lees-Miller, D. Helfman

Actins are a family of conserved proteins that have been characterized from a broad range of phyla including vertebrates, invertebrates, fungi, plants, and protozoa. They have between 70% and 90% amino acid sequence identity and are 374 to 376 amino acids in length. We have recently cloned a gene (*act2*) from the yeast *Schizosaccharomyces pombe* that encodes a distantly related member of the actin family. Disruption of the *act2* gene revealed that it is essential for spore germination. In contrast to previously identified actins, the derived amino acid sequence of the act2 protein is only 35% to 40% identical to other actins, including that of *S. pombe*, and is 427 amino acids in length. The crystallographic structure of rabbit skeletal muscle actin is known (Kabsch et al., *Nature 347:* 37 [1990]). The regions of the act2 amino acid sequence that are conserved relative to actin include the predicted ATP and calcium ion contact sites and much of the overall core structure. However, some of the regions in actin that are predicted to be important for actin:actin and actin:actin-binding protein interactions are highly diverged in act2. Recent analyses of actin-related sequences from budding yeast (Schwob and Martin, *Nature 355:* 179 [1992]), *C. elegans*, and human brain indicate that at least three families of actin-related proteins exist and that each may have essential cellular functions distinct from that of actin. Our present efforts are directed at determining the function of the actin-related proteins. This involves expression and purification of proteins, immunofluorescence localization, and the development of temperature-sensitive mutants in *S. pombe*.

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QUEST PROTEIN DATABASE CENTER

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C. Chang, P. Myers, P. Monardo, A. Nikzad
J. Kos, H. Sacco, N. Sareen
K. Duhamel, S. Fang, Z. Yu
J. Horwitz, J. Slott, B. Rose

Activity in the QUEST Protein Database Center in 1991 has included software development, database development, and protein characterization. Scott Patterson joined Cold Spring Harbor Laboratory in January as a Staff Investigator and a member of the QUEST Center. He has taken responsibility for the QUEST 2D gel laboratory, and his efforts for the QUEST Center include protein identification and characterization. Scott spent the first 3 months of 1991 in the laboratory of Dr. Ruedi Aebersold at the Biomedical Research Center in Vancouver learning new techniques relevant to the characterization of proteins from two-dimensional gels. His work is described below and in a separate report (see Posttranslational Modifications: Regulatory and Targeting).

The research of the QUEST Center continues to focus on protein synthesis and modification in mammalian and yeast cells using our quantitative two-dimensional gel technology. Our collaborative project to build a mouse embryo database is highlighted below. The mission of the QUEST Center, as a National Institutes of Health Biomedical Research Technology facility, has always included service and collaboration with the scientific community at large. This part of our mission was redefined in 1991 so that we no longer offer two-dimensional gel analysis services. Instead, our computing group is focused on providing software and support for scientists at Cold Spring Harbor Laboratory and in other institutions who wish to build and access two-dimensional gel protein databases. A new QUEST software system is being readied by the Computing Group of Jerry Latter, and the first application of this software will be a human database. This database will be a major focus of the QUEST group, bringing together many past and future studies by Robert Franza, Scott Patterson, and Jim Garrels and depending heavily on the support of the Computing Group. Reports on the software and database progress are given below.

Cecile Chang, who had served the QUEST Center in many roles both in the laboratory and in the computing group, left in July to accept a management

position in a drug company in Taiwan. Phyllis Myers, who had been our tireless two-dimensional gel analyst since 1986, retired in March. Arman Nikzad, a programmer who was hired in September to replace Jim Kos, was forced by family needs to return to his native Turkey in December. Staffing changes in the two-dimensional gel lab are given below.

QUEST Computing Group

G. Latter, P. Monardo, A. Nikzad, J. Kos, J.I. Garrels

The change of emphasis in the QUEST services, from 2D gel analysis services to software and database support, is consistent with our commitment to offer a high-end software package to members of the scientific community who wish to build and access 2D gel databases. The new software system, described below, will allow the construction of larger databases as well as much better access to data and program modules over the Internet. The primary application for the new software within QUEST will be the construction of a new human database.

As indicated in last year's Annual Report, the QUEST software system is being dramatically restructured using modern software standards to provide a powerful tool for database construction that will serve QUEST and the scientific community throughout the 1990s. The software is adapted to today's networked computer environments where database servers, computational servers, and graphics engines may reside on separate computers linked through a network, and the network may even link computers at different institutions.

The QUEST software is organized in three layers. The *lowest layer*, the database server layer, contains three servers. One (QDB) provides 2D gel spot database access, another (IDB) provides image database access, and the third (MDB) provides access to laboratory records and accounting information. In the *middle layer* are the compute servers. The ISP server performs computational tasks that involve images, such as background subtraction, smoothing, spot detection, and spot fitting. The MSP server carries out computational tasks on spot data, such as matching, editing, and spot set construction. The DSP server is a graphics engine containing plotting functions written using the X Graphics Language (XGL). The *top layer* is the user interface. The Matchset Tool is the main editing interface. A separate tool (ISPTool) controls the image processing functions. All interface layers conform to the OpenWindows standard.

The architecture described above was designed and put in place primarily by Pat Monardo. The modular structure has allowed others to interface particular modules to the system. For example, Dr. Garrels was able to convert the spot detection and fitting algorithms, which he has developed and improved over the years, into a quantitation server that is now a part of ISP.

All six servers are now built and are in a testing phase. The completion of the Matchset Tool will soon allow us to begin construction of the human database. The design and implementation of specialized analysis tools will continue during database construction. After a thorough shakedown by the members of the QUEST group, the software will be released to collaborators for use with their databases and soon thereafter to the general scientific community.

New Scanning Technology: We have completed testing of storage phosphor technology as a replacement for film in the detection of radioactively labeled proteins in 2D gels. Two different systems (Molecular Dynamics and Fuji) were evaluated while on loan to Dr. Beach's laboratory. Both systems produced an image equivalent to a 30-day fluorogram with an exposure of less than 1 week to an imaging plate. Initial examinations indicate that the dynamic range of the phosphorimaging systems is sufficient to replace our present system of multiple film exposures, although a very short exposure (1 hr) may be needed to detect the most intense proteins without saturation. The systems evaluated differ with respect to technical criteria such as resolution, spatial uniformity, erasure of plates, sensitivity, and rate of image capture. The errors of quantitation and spatial resolution are below the level of errors inherent in the process of resolving proteins on 2D gels.

We expect to adopt phosphorimager technology as a replacement to our current fluorographic processing of gels. This will avoid the cost of film, developing chemicals, and fluorographic chemicals. It will also remove the need for calibration strips that have been used with every film exposure. The phosphorimager will be further exploited for sensitive double-label analysis to determine protein amino acid compositions on 2D gels. Our strategy for identification of human and yeast proteins by this method is given in a later section.

2D Gel Laboratory Core Facility

S.D. Patterson, J.I. Garrels, K. Duhamel, S. Fang, H. Sacco, N. Sareen

This Cold Spring Harbor Laboratory Core Facility runs 2D gels for researchers at CSHL as well as for a limited number of outside users. As in past years, a wide range of samples have been handled by the laboratory. Just over 1600 gels were run this year, with 86% of these for Cold Spring Harbor Laboratory staff and the remainder for external users. Although relatively few external gels were run this year, the laboratory continues to provide an important service to the general scientific community. In addition, a large number of samples were also run for quality control and optimization of first-dimension running conditions for the new QUEST human database. In April, Dr. Garrels handed over the management of the facility to Dr. Patterson.

This will be the last full year that the gel lab will be in its original location, the McClintock building. We are presently gearing up to move to a more compact laboratory in Demerec. This move to lesser space requires that we change our method of detection of radioactive samples. Since its inception in 1979, the gel lab has used DMSO/PPO as a fluorographic enhancer to give increased signal to noise (five- to eightfold) over direct autoradiography. With Jerry Latter, we have been involved in evaluating a new technology, storage phosphor plates, with the aim of eliminating the hazardous DMSO/PPO fluorography reagents and the space required for this system. On average, our longest of multiple exposures are often at least 1 month. This new technology allows reduction of that time to 5–8 days with no loss in resolution. We are looking forward to employing this new technology to improve the turnaround time of our service.

By the end of the year, the gel lab staff had turned over completely, with Kris Duhamel leaving for New Jersey in March, Shu-Ling Fang for San Francisco with her husband, upon completion of his Ph.D. in June, and Heidi Sacco in December to have her second child. Only two of the three staff have been replaced in an effort to improve both the efficiency and therefore the cost effectiveness of this core facility. Neena Sareen has been with us since June and Nick Bizios will begin in late January 1992. We look forward to this coming year of change for the gel lab with optimism.

Identification of Some Members of the "PCNA-like" Set from Rat Cells in Human T Lymphoblasts and Prefractionation Procedures

S.D. Patterson, Z. Yu, J. Horwitz

The so-called "PCNA-like" set of proteins, with regulation coordinate with that of the DNA polymerase δ processivity factor, proliferating cell nuclear antigen (PCNA), was identified in REF52 (rat) cells by Garrels and Franza (*J. Biol. Chem. 264:* 5299 [1989]).

We are currently engaged in identifying the human homologs of some of these proteins in Jurkat T lymphoblasts by finding proteins that have similar physical properties. These properties include subcellular location, isoelectric point, relative molecular mass, hydrophobicity (as determined by detergent phase separation), and peptide maps. Subcellular fractionation studies have begun with nuclei and mitochondria being enriched in Jurkat cells, and further studies are planned for plasma membrane, nuclear matrix, microsomes, Golgi apparatus, and endoplasmic reticulum.

Phase separation using the detergent Triton X-114 (TX-114) has been performed on both WT2 (a REF52-related cell line) and Jurkat cells. Prefractionation such as this allows higher loads of protein to be loaded onto a 2D gel for preparative purposes. This procedure also provides information as to whether the protein is lipophilic or not, i.e., whether it is possibly membrane-associated. The detergent phase of whole-cell lysates has already shown enrichment of proteins in the 20–30-kD region that may prove to be the small GTP-binding proteins (members of the *ras* superfamily).

Another protein shown to be enriched in the detergent phase of WT2 whole-cell lysates and nuclei is the protein labeled MG20 from the REF52 database. This protein is one of the "PCNA-like" set of proteins. A protein with similar physical properties (as defined above) has been located in Jurkat cells, and this is currently being enriched for comparative peptide mapping to confirm the identity of this protein with the REF52 MG20 protein and to provide sufficient material for subsequent amino acid sequence analysis. These prefractionation studies contribute a "preparative" aspect to the human database and another means of linking the rat and human databases.

Mouse Embryo Database

J.I. Garrels, C. Chang, P. Myers [in collaboration with Keith Latham, Wistar Institute, and Davor Solter, Max Planck Institute for Immunology]

The mouse embryo database has grown considerably and has been applied to several specific questions of mouse early development. Dr. Latham and Dr. Solter have designed the database to reveal detailed changes of protein synthesis in mouse embryos from fertilization to postimplantation stages, and they have used the database for experimental studies utilizing protein kinase inhibitors and nuclear transplantation, as described below. Our role at QUEST has been to couple our 2D gel technology to this experimental expertise of the collaborators, to solve new problems in database construction, and to provide critical evaluation of the results. The analysis of the mouse database has required cluster analysis and has required linking dissimilar matchsets to a degree not faced in constructing the REF52 database.

The cornerstone of the mouse embryo database is the series of embryos labeled each 3 hours from fertilization through the four-cell stage. This study, published in 1991, has revealed five significant patterns of protein synthesis during the two-cell stage of development, which is the critical stage when protein synthesis switches from using maternal mRNA to using newly synthesized embryonic mRNA. The patterns for the two-cell stage are summarized in Figure 1. About 37% of the proteins detected are synthesized for the first time at this stage. Another 36% of the proteins (clusters 2 and 3) are turned off or are significantly repressed at this stage. The final two clusters of proteins show transient induction or repression during the two-cell stage. It is significant that the changes among the transient expression profiles begin a few hours before the first appearance of the long-term changes.

These studies have been extended through the preimplantation period. Profiles (fertilization through morula) for some representative proteins are shown in Figure 2. Protein EL60 is one of the proteins that disappears during the two-cell stage. Protein DE90 is one that appears transiently during the two-cell stage and is not seen later. Proteins KJ20 and LD30 first appear in the two-cell stage and continue to be synthesized beyond the morula phase. Note that the transient expression of protein DE90 begins 3–6 hours earlier than the synchronous wave of induction represented by proteins KJ20 and LD30.

Among the known cytoskeletal proteins, tubulin is synthesized in substantial quantity, and actin and tropomyosin-5 (TM5) are synthesized at low levels in the zygote. Significant increases of all three proteins occur at the two-cell stage, probably reflecting a switch from maternal to embryonic mRNA. Tubulin synthesis then declines by 5–10-fold after the two-cell stage, whereas actin shows another increase in synthesis (10–20-fold) in the eight-cell and morula stages.

The database is a valuable standard against which to compare abnormal patterns of protein synthesis. Drs. Latham and Solter use nuclear transplantation to construct embryos containing only male pronuclei (androgenones) or only female pronuclei (gynogenones). Such embryos become blocked in later development, and 2D gel experiments were carried out to determine when the first differences in protein expression are apparent. In one mouse strain (C57 BL/6), no significant differences were seen between androgenetic and gynogenetic embryos from among 1200 proteins examined at the eight-cell stage. However, in another mouse strain (DBA/2), 11 changes were observed. Expression profiles from the database reveal that eight of these proteins are normally induced during the eight-cell/morula stage but are not induced in the DBA/2 androgenones. The other three proteins are normally synthesized at declining rates during the eight-cell/morula stage, and the synthesis of these proteins does not decline in the DBA/2 androgenones.

One further use of the database has been to examine the effects of protein kinase inhibitors on development. It has been known that zygotic gene activation is at least partially sensitive to protein kinase A inhibitors but is little affected by inhibitors of protein kinase C. Cluster analysis was used to detect the patterns of protein expression in cells treated with α-amanitin (inhibitors of RNA polymerase), H8 (inhibitors of PKA), or H7 (inhibitor of PKC). One large cluster was found representing the proteins that normally increase in the two-cell stage. The members of this large cluster (36% of detected proteins) are not induced in the presence of α-amanitin or H8. Two other clusters (18% of detected proteins) contain proteins normally repressed in the two-cell stage, but which are not repressed in the presence of H8. The PKC inhibitor H7 did not have a significant effect on the members of these clusters. Proteins induced transiently in the two-cell stage are likewise not induced in the presence of H8 but are induced in the presence of H7.

Major patterns of synthesis from the 2-cell stage mouse embryo.

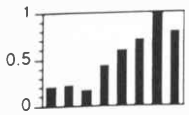
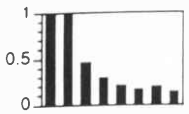
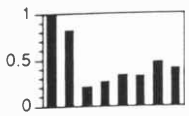
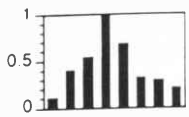
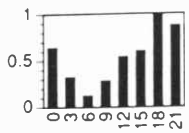
description	fraction of analyzed (%)	profile
increases	37	
decreases	20	
transient decreases, high early	16	
transient increases	6	
transient decreases, low early	4	
Total	77	

FIGURE 1 Cluster analysis shows the major protein expression profiles during the two-cell stage of mouse development. Proteins with similar expression patterns are automatically grouped into "clusters" and plotted as a composite profile. This analysis reveals the major patterns of regulation inherent within a critical 21-hr period of mouse development.

Yeast Database

J.I. Garrels [in collaboration with C. McLaughlin, University of California, Irvine, and Jonathan Warner, Albert Einstein College of Medicine]

The yeast database includes studies of heat shock, mRNA splicing, and amino-terminal acetylation. During 1991, we analyzed an experiment in collaboration with John Smith and Ulrich Teichert of Massachusetts General Hospital to characterize a methionine aminopeptidase (MAP1) mutant of budding yeast. A knockout of the *MAP1* gene is not lethal but might be expected to affect many of the same cellular functions as a knockout of the *NAT1/ARD1* genes. The latter genes code for subunits of an *N*-acetyltransferase that recognizes proteins only after removal of amino-terminal methionine.

Some of the results of this study are shown in Figure 3. On these gels, 64 of the known acetylated (*NAT1/ARD1*-modified) proteins can be scored. These proteins are normally present in their fully acetylated state, and the unacetylated precursors are never detected on 2D gels. In the *MAP1* mutant, 16 of the 64 acetylated proteins are only partially acetylated, and the remainder are fully acetylated. This result indicates that other methionine aminopep-

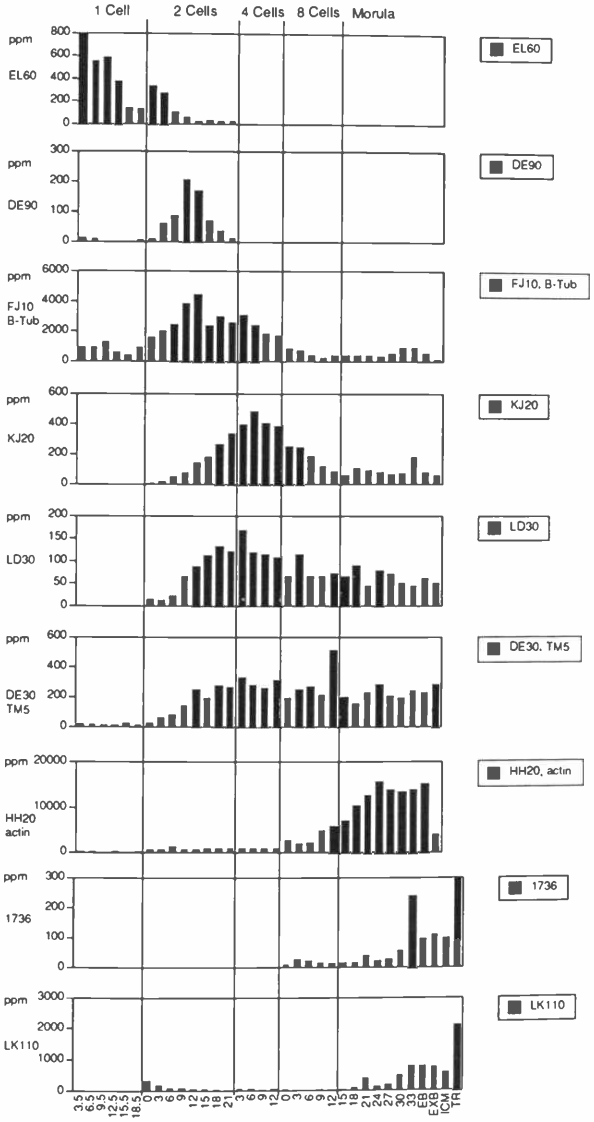


FIGURE 2 Some representative expression profiles for individual proteins during the fertilization to blastocyst stages of mouse development. Each protein intensity is expressed as parts per million (ppm) of total incorporation of the radioactive label. The legends give spot numbers and names when known. The samples are labeled by hours relative to each stage of development, except for EB (early blastocyst), EXB (expanded blastocyst), ICM (inner cell mass), and TR (trophoblast).

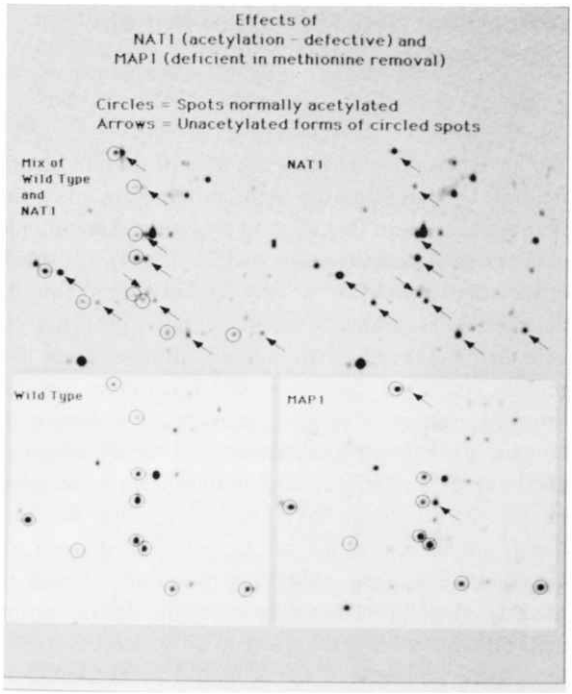


FIGURE 3 Yeast gels from wild-type, *NAT1* (*N*-acetyltransferase), and *MAP1* (methionine aminopeptidase) mutants. Only a small region of the gels is shown. These results show many of the spots affected by amino-terminal acetylation and reveal that a fraction of the acetylated spots are incompletely acetylated in the *MAP1* mutant.

tidases are present in cells but that these other activities are insufficient in activity, or perhaps not properly localized, for the complete methionine removal of all NAT1/ARD1 target proteins. Other than these effects on acetylation, the *MAP1* knockout mutant has very little effect on the protein synthesis pattern of the cell.

Further studies of amino-terminal modification are planned in collaboration with Dr. Smith. Another known acetyltransferase (*NAT2* gene product) is responsible for acetylation of proteins that retain amino-terminal methionine (Met-Asp, Met-Glu, or Met-Gln sequences). Actin is one of the substrates for this transferase, but the fraction of proteins modified by this protein is not yet known. The *N*-myristoyltransferase (*NMT1* gene product) provides a modification essential to the function of certain membrane-associated proteins such as the Gα protein (*GPA1* gene product) of the mating pathway. Because myristoylation occurs only on amino-terminal glycine, this modification also requires the action of a methionine aminopeptidase. It will be of great interest to identify more of the particular proteins modified by each of these enzymes.

A Potential New Method of Protein Identification

J.I. Garrels, G. Latter

We have been interested in the idea of protein identification by partial amino acid composition since the first application of this method by Latter, Leavitt, and colleagues (*Electrophoresis 4:* 122 [1983]). If the relative composition of a few amino acids can be determined accurately enough, proteins from the 2D gels can be matched to known proteins from the protein sequence databases. We have done simulations for proteins of budding yeast because these cells contain a relatively small number (5–6000) of genes and because a relatively high (and rapidly increasing) number of the genes have been sequenced. We find that if amino acid ratios can be determined to an accuracy of 3%, then only four ratios are needed to identify most yeast proteins uniquely. If the amino acid ratios can be determined to an accuracy of 1%, then three amino acid ratios would be sufficient to identify most yeast proteins.

To obtain highly accurate amino acid ratio data, our strategy is to run gels of double-labeled samples, using one ^{35}S-labeled amino acid and one ^{14}C-labeled amino acid. Because the ^{35}S label decays with a half-life of 87 days, we can measure the relative amounts of the two isotopes in each pixel of the image by plotting the rate of decay. The double-label method should be much more accurate than comparing integrated spot data from gels labeled with single amino acids because no spot integration is necessary. If film detection is used, the method is quite daunting because of calibration and multiple exposure problems, but using the phosphorimager, which gives a linear response over 4–5 logs of intensity, these problems disappear.

Our simulations show that weekly exposures over a period of 2 months should give the required 1–3% accuracy in the amino acid ratios.

This method also relies on the ability to align accurately and compare images from separate exposures, but such algorithms are already a part of the QUEST system. If the simulations are borne out in practice, it should be possible to identify many of the proteins that are both detectable on 2D gels and products of sequenced genes. Although the simulation was carried out for yeast, we also plan to apply the method to the identification of proteins for the human database.

Human Regulatory Protein Sets

B.R. Franza

With the advent of the new QUEST system, we embark on the coalescence of protein data from several different human cell lines. We have accomplished the identification of a number of transcriptional and cell division cycle regulatory proteins in both T-lymphoblast and B-lymphoblast cell lines. This is an ongoing effort to identify and characterize nuclear regulatory proteins as a component of the human protein database. We have also studied the differences in gene expression in matched pairs of lymphoblast cell lines that contain either a normal chromosomal complement or translocations specific for different types of hematologic malignancy. This work continues to be accomplished in collaboration with Dr. Bayard Clarkson and his colleagues at Memorial Sloan-Kettering. We have conducted an extensive characterization of the gene products from different class I major histocompatibility alleles in an extensive family of lymphoblast cell lines derived by mutagenesis of a parent B-lymphoblast cell. These studies are performed with Dr. Robert DeMars at the University of Wisconsin. We are extending our studies with Dr. DeMars to the characterization of the proteosome complexes in human lymphoblast cell lines. We also have a number of studies of normal human peripheral lymphoblasts that will be entered into the database.

We therefore now have the means to bring all of these data into a network-based, computer-accessible system that will permit the study of quantitatively and/or qualitatively analyzed sets of cellular proteins. During the next 12 months, we will begin assembling the sets of oncogene, transcription control, cell division cycle control, MHC I, and other identified proteins into this distributable system. We will use the coordinate system published for REF52 cells (Garrels and Franza, *J. Biol. Chem. 264:* 5283 [1989]) to provide for continuity of assignments of alphanumeric designators to proteins identified to be conserved between the two species, and we will begin making similar assignments to the mouse protein products that we demonstrate to be conserved.

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GENETICS

Since the days of the "phage" group, genetic approaches to biological problems have provided a conceptual and technical cornerstone to the work at Cold Spring Harbor Laboratory. The revolution brought about by recombinant DNA methods has its roots in molecular genetic methodology and now leads us to the dawn of a true era of genetic engineering. Where this will lead is not totally clear, but current developments in DNA fingerprinting of individuals and genetic therapy for disease states give a faint hint of the future. This section describes the work of the geneticists at Cold Spring Harbor.

EUKARYOTIC CELL CYCLE CONTROL

D. Beach

S. Allan
J. Bischoff
M. Caligiuri
D. Casso
T. Connolly
G. Cottarel
S. Davey
D. Demetrick
H. Feilotter
K. Galaktionov
I. Garkavtsev
C. Gawel
J. Hofmann
C. Jessus
N. Kaplan
D. Lombardi
K. Lundgren
T. Matsumoto
T. Mizukami
L. Molz
B. Nefsky
K. Okamoto
A. Tesoro
N. Walworth
Y. Xiong
H. Zhang

During the last year, we have continued our work on the eukaryotic cell cycle. Use was made of genetic models such as the budding and fission yeast, in addition to mammalian tissue-culture cells. Furthermore, 1991 marked the year in which the fission yeast genome project became firmly established at Cold Spring Harbor Laboratory.

During the course of the year, Ulrich Deuschle left to take a position with the Basel Institute for Immunology in Switzerland. We were joined by Doug Demetrick (visiting clinician scientist), Nancy Kaplan (technician), and several postdoctoral fellows, Koji Okamoto, Johannes Hofmann, and Hui Zhang.

Activation of cdc2 at Mitosis

N. Walworth, D. Beach

The regulation of the activity of $p34^{cdc2}$ in fission yeast is dependent on both complex formation of $p34^{cdc2}$ with the product of *cdc13* and phosphorylation of $p34^{cdc2}$ on threonine and tyrosine residues. Tyrosine phosphorylation of $p34^{cdc2}$ prevents entry into mitosis and is dependent on the products of the *mik1* and *wee1* genes. Simultaneous loss of function of mik1 and wee1 results in premature advancement into mitosis, leading to cell death. A genetic screen was set up to identify suppressors of this lethal mitotic phenotype and cold-sensitive alleles of *cdc2* were found. These alleles permit the cell to survive without functional *mik1* and *wee1*, but themselves lead to a cell division cycle defect at low temperature. Multi-copy expression of *cdc13*, a B-type cyclin which is known to associate with $p34^{cdc2}$, suppresses the cold-sensitive growth defect of these *cdc2* alleles. Weak suppression results from overexpression of cdc25, which is thought to encode a tyrosine phosphatase. A genomic library prepared from *Schizosaccharomyces pombe* DNA has been introduced into two of these *cdc2* strains and high-copy suppressors have been identified. Molecular analysis of the suppressors is under way and should elucidate additional mechanisms which regulate $p34^{cdc2}$ activity.

Human D-type Cyclin Gene Family

Y. Xiong, T. Connolly, D. Beach [in collaboration with B. Futcher, Cold Spring Harbor Laboratory]

There have been several major developments in the study of mammalian cell cycle in the past year. Three

new classes of mammalian cyclins, C, D, and E, were isolated using a genetic complementation screening procedure.

Following the isolation of human cyclin D1 gene last year, we have cloned two additional human D-type cyclin genes, cyclin D2 and D3. All three human D-type cyclins encode small (33–34 kD) proteins that share an average of 57% identity over the entire coding region and 78% in the cyclin box. The D-type cyclins are most closely related to cyclin A (39% identity) and cyclin E (36%), followed by cyclin B (29%) and cyclin C (21%). The appearance of cyclin D1 mRNA and protein after serum stimulation is very early (e.g., 2–5 hr in human A431 cells), suggesting that it may function in the G_0/G_1 transition or early G_1 phase of cell cycle. We are currently conducting an experiment to search for the genes whose products interact with D-type cyclins. In a series of Northern analyses, we found that, unlike cyclin A and cyclin B genes, which appear to express at a similar level in different cell types, levels of different cyclin D gene mRNAs could be dramatically different in different cell types. This suggests that individual members of the cyclin D gene family may function in specific tissues. In a separate line of experiments, we have isolated and characterized the genomic structure of three cyclin D genes. All three cyclin D genes are interrupted by an intron at the same position. We also found two pseudogenes corresponding to cyclin D2 and D3, respectively. Sequence analysis of the promoter region suggests several potential regulators of cyclin D gene transcription, some of which are currently being tested. In a collaboration with Dr. Ward of Yale University, we have mapped cyclin D2 to chromosome 12p band p13 and cyclin D3 to chromosome 6p band p21. Ongoing experiments test the possible involvement of cyclin D2 and D3 in those neoplastic transformations that were previously found to be linked to these chromosomal locations.

Cell Cycle Checkpoints

T. Matsumoto, H. Feilotter, D. Beach

Mitosis normally follows the completion of DNA replication. Premature initiation of mitosis without completion of DNA replication will result in lethality as daughter cells lack the normal complement of genetic information.

We have investigated a number of genes involved in regulation of this interdependency of mitosis and DNA synthesis. The *pim1* gene encodes a homolog of the mammalian *RCC1* gene. Loss of function of *RCC1* also causes cells to go into mitosis before the completion of DNA replication, suggesting functional conservation of the gene. A gene known as *spi1*, which can rescue a *pim1* mutant when carried on a multicopy plasmid, encodes a 25-kD GTPase, whose homolog was also found in mammalian and plant systems. The *spi1* GTPase and its human homolog together define a novel class in the *ras*-like GTPase family, whose members have a common molecular structure and may act as molecular switches in signal transduction pathways. It is expected that the fission yeast *pim1* and *spi1* gene products act in a manner similar to their homologs in higher eukaryotes.

At least four other genes whose products appear to play a role on the *pim1/spi1* regulatory pathway have been identified as conditional mutations that cause premature entry into mitosis in the absence of DNA synthesis. One of these has been identified as a mutation in the *spi1* gene itself. Another encodes one of the two type-1 phosphatase genes of fission yeast.

A biochemical approach to study the function of *pim1* and *spi1* has suggested that the *pim1* product converts the *spi1* GTPase into an active form that is GTP-binding and that mutation of the *pim1* gene reduces this activity. This may suggest that the active form of the *spi1* GTPase causes an inhibition of mitotic events until DNA replication is completed. The pathways by which such a signal is received or by which such inhibition is achieved are unknown, but the biochemical interaction of some of the other genes on this pathway (i.e., a type-1 phosphatase) may shed some light on this question.

A Gene Interacting with the cdc2 Protein Kinase of Fission Yeast

L. Molz, D. Beach

MPF (M-phase promoting factor) is an activity regulating entry into mitosis in eukaryotic cells. Homologs of the fission yeast cdc2 protein kinase comprise the catalytic subunit of MPF. Activity of the cdc2 protein kinase is regulated by both phosphorylation and association with regulatory sub-

units termed cyclins. We have characterized mutants from a genetic screen designed to identify new regulators or substrates of the cdc2 protein kinase. We isolated mutations (*mcs* mutations) that suppress the lethality of a strain carrying a dominant *cdc2* mutation. A genetic analysis revealed that the *mcs* mutations define six new genes that are not alleles of previously identified mitotic control genes. Many of the *mcs* mutations interact in novel ways with alleles of the known mitotic control genes. The details of the genetic interactions have been previously published. One of the *mcs* mutations (*mcs2-75*) displays striking allele-specific interactions with various alleles of *cdc2*. These allele-specific interactions suggest that the *mcs2* and *cdc2* gene products may interact.

The genetic analysis suggests that *mcs2* may interact closely with *cdc2*, and therefore we wished to characterize this gene further. *mcs2* was cloned by complementation of the *mcs2-75* mutation. While cloning *mcs2*, we also recovered a gene suppressing the *mcs2-75* defect, but which was unlinked to the *mcs2* locus. Both *mcs2* and the suppressor were sequenced and examined for homologies with proteins in databases. *mcs2* encoded a 37.5-kD protein that displayed weak homologies with cyclins, known regulatory subunits of the cdc2 protein kinase. The *mcs2* suppressor encoded a 34.5-kD putative protein kinase. We further examined the role of *mcs2* and the suppressor in the *S. pombe* cell cycle by creating null mutations using the gene-replacement method. Strains carrying the *mcs2* null mutation formed microcolonies of inviable cells arrested late in mitosis. Cells have multiple division septa and nuclei containing condensed chromosomes. This phenotype is indicative of an arrest point late in mitosis and is very different from the phenotype of null mutations of other cloned cyclins in *S. pombe*. Finally, strains carrying a null allele of the *mcs2* suppressor were viable, displaying no dramatic cell cycle defect.

To investigate the role of these genes in the *S. pombe* cell cycle further, we have produced polyclonal antibodies against *mcs2* and the *mcs2* suppressor proteins expressed in *Escherichia coli*. We have also generated a monoclonal antiserum to these gene products by using the epitope addition method. Western blotting experiments using these reagents reveal that neither *mcs2* nor the *mcs2* suppressor proteins oscillate in abundance during the *S. pombe* cell cycle. We are currently using these reagents to investigate the possibility that *mcs2* may be a subunit or a substrate of either the *cdc2* or the *mcs2* suppressing protein kinases.

Activation of cdc25 Tyrosine Phosphatases by Cyclins

K. Galaktionov, C. Jessus, D. Beach

In eukaryotic cells, mitosis is initiated following the activation of a protein kinase known as MPF, the M-phase-specific histone kinase, or more simply as the M-phase kinase. This kinase consists of at least three subunits; the catalytic subunit (cdc2), a regulatory subunit (cyclin B), and a low-molecular-weight subunit (p13-Suc1). The cyclin B/cdc2 enzyme is subject to multiple levels of control. Among these, the regulation of the catalytic subunit by tyrosine phosphorylation is the best understood. Tyrosine phosphorylation inhibits the cyclin B/cdc2 enzyme, and tyrosine dephosphorylation, which occurs at the onset of mitosis, directly activates the pre-MPF complex. The cdc2 tyrosine kinases are encoded by two genes, initially identified in fission yeast and known as *wee1* and *mik1*. The *cdc25* gene serves as a rate-limiting mitotic activator. The *cdc25* gene is normally essential for cell division, except in the absence of the wee1 and mik1 tyrosine kinases. Increasing evidence suggests that cdc25 serves as the cdc2 tyrosine phosphatase. A human *cdc25* gene has previously been described. We assumed that more than one such gene is likely to exist and therefore designed a polymerase chain reaction (PCR)-based strategy to isolate further potential members of the family. Using this strategy, we isolated two previously undescribed human *cdc25* genes, namely, *cdc25A* and *cdc25B*. Both human cDNAs could efficiently rescue the temperature-sensitive mutation of the *cdc25* gene in the fission yeast. Human cdc25 A protein had an endogenous tyrosine phosphatase activity that was activated by the mitotic cyclins, but not cyclin A or D1. Another observation pointing in the same direction was made during the course of a visual comparison of cdc25 A and B with known tyrosine PTPases and other proteins involved in cell cycle control (Fig. 1). First, the region of cdc25 that is immediately carboxy-terminal to the putative catalytic domain (CA) is not highly related to other known PTPases, such as cytoplasmic PTPases from higher eukaryotes and the vaccinia virus serine-tyrosine phosphatase (VH-1). More interestingly, this region within the PTPases was found to contain sequence similarity to cyclins, particularly of the B-type (Fig. 1). The similarity is detected immediately at the junction of the so-called cyclin box and included some nearly invariable residues among cyclins. In the

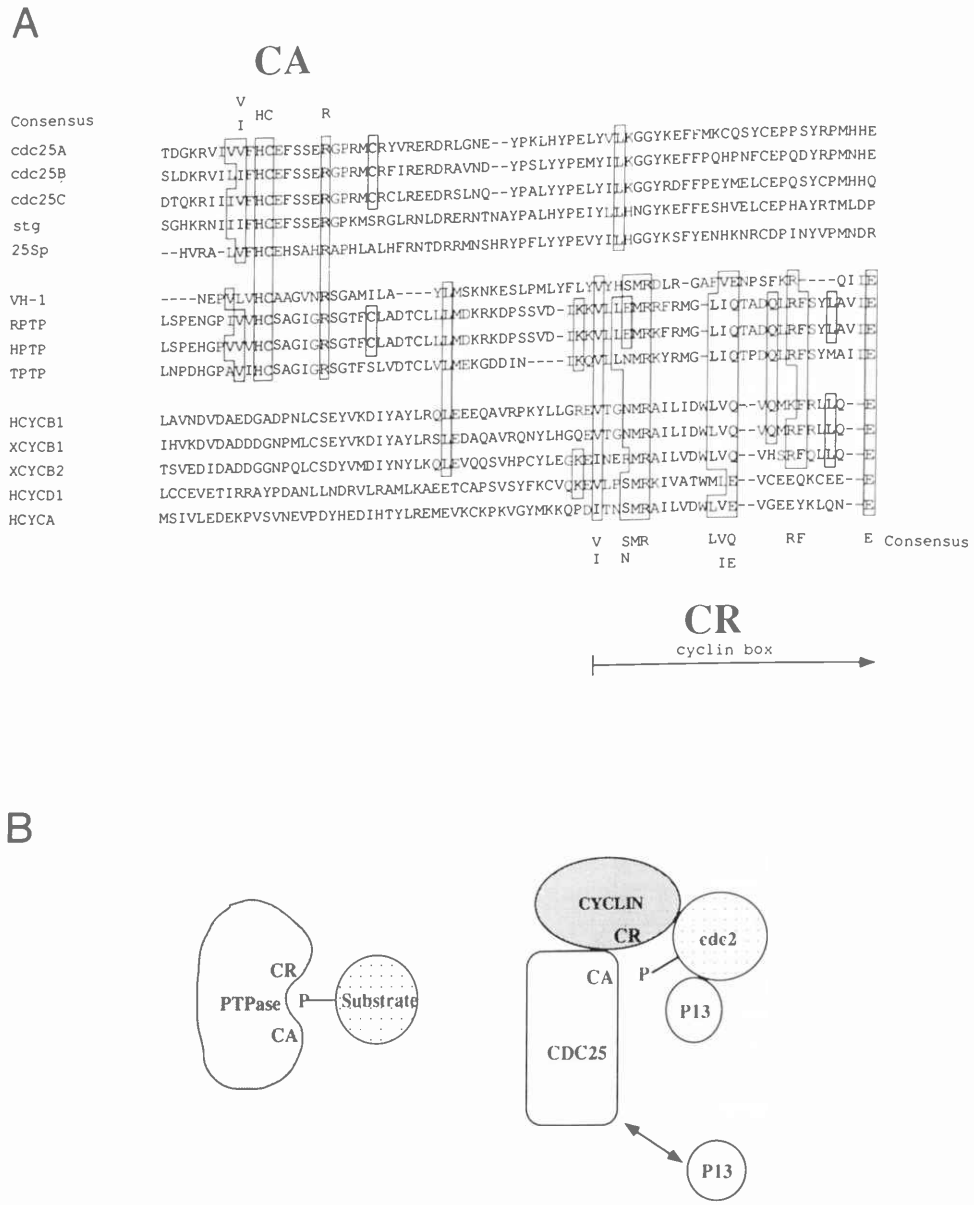

FIGURE 1 (*A*) Alignment of the cdc25 proteins, PTPases, and cyclins. (*B*) Model. (*A*) Tyrosine phosphatases were aligned with each other and with cyclins and cdc25 proteins. The cyclin alignment was done by visual inspection. Only identity or similarity (V or I) within at least three members of one gene family and a minimal of two members of another family is boxed. CA indicates the putative catalytic domain of the cdc25 and cytoplasmic tyrosine phosphatases, and CR indicates the cyclin related domain, present in tyrosine phosphatases but absent in cdc25 proteins. (*B*) Schematic representation of a hypothetical relationship between PTPases, M-phase kinase, and cdc25 phosphatase. The association between cdc2 and p13, and between cyclin and cdc2, is well documented. The interaction of cdc25 and cyclin is proposed here. p13 is proposed to have a low-affinity interaction with cdc25. CA is the catalytic domain of PTPases and CR is a region of similarity between PTPases and cyclins. (Reprinted, with permission, from Galaktionov and Beach 1991, copyright by Cell Press).

region of similarity between PTPases and cyclins that we call the cyclin region (CR), there is no equivalent in the cdc25 proteins.

The newly found motif lies almost immediately adjacent to the domain (V/IXHCXXXXR) that has been directly implicated in the catalytic function of the PTPases and cdc25 protein. The finding allows the following speculation. The catalytic activity of the other PTPases is considerably greater than that of cdc25. cdc25 lacks the motif that is shared by cyclins and other PTPases. This motif may be an activating domain, which is functioning in "*cis*" in most PTPases, but in the case of cdc25, it is provided in "*trans*" by intermolecular interaction with cyclin (Fig. 1).

Genes That Interact with Human *p53*

J. Bischoff, D. Casso, D. Beach

Mutations in the *p53* gene are the most frequent genetic alteration in human cancer. In normal cells, *p53* probably acts as a negative regulator of the cell cycle, since overexpression of the wild-type protein, in some cell types, results in a block in the G_1 phase of the cell cycle.

We have set up a simple system in the fission yeast *S. pombe* to study human *p53*. The overexpression of wild-type human *p53* blocks growth in *S. pombe*. We believe that this is a specific effect, since the overexpression of mutant *p53* polypeptides in *S. pombe* does not block growth.

To identify genes that interact with *p53*, an *S. pombe* strain was constructed that possesses a single chromosomal copy of a wild-type human *p53* cDNA under the control of an inducible promoter. cDNAs could then be introduced into this strain and screened for their ability to suppress the negative growth effect of wild-type *p53*. To determine if this type of screen was feasible, we introduced nine mutant alleles of human *p53* into this strain with the hope of identifying dominant negative mutations in the *p53* gene. Two of the nine *p53* mutants were found to be dominant to the wild-type gene. One had previously been classified as dominant and the other was a newly identified dominant allele of human *p53*. More than 500,000 transformants from a human cDNA library have been screened in this strain. So far, one partial cDNA has been isolated that can relieve the *p53*-induced growth arrest in *S. pombe*. This cDNA has no effect on the level of expression of *p53* and does not appear to physically associate with *p53*. Sequence analysis of the cDNA does not reveal any significant homology with any genes in the usual sequence databases. The putative coding region does, however, have at least five potential calcium-binding pockets (EF hands). We are currently isolating a full-length cDNA of this possible *p53* suppressor.

Another approach we have taken to identify genes that interact with *p53* has been to mutagenize chemically the strain mentioned above in order to isolate extragenic suppressors of *p53*. Several strains have been isolated that can tolerate the overexpression of wild-type *p53*. These strains are now being characterized.

Fission Yeast Genome Mapping

T. Mizukami, I. Garkavtsev, N. Kaplan, T. Matsumoto, D. Lombardi, D. Beach [in collaboration with Tom Marr's group, Cold Spring Harbor Laboratory]

A fivefold representation cosmid library was prepared for the physical mapping of the fission yeast genome. This has been hybridized with multiple DNA probes that allow contiguous clones (contigs) to be assigned. The objective of this work is to create a high-resolution (~10 kb) map of the 14-megabase fission yeast genome. At the time of writing (February, 1992), essentially the entire genome has been assembled into approximately 50 contigs. Second-round strategies are being employed to reduce this figure to three chromosome-sized contigs. In addition to the linking of clones in contigs, we are generating a *Pst*I and *Bam*HI restriction map of the genome that will aid in the eventual nucleotide sequencing of the genome of the fission yeast.

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PRE-mRNA PROCESSING AND SMALL NUCLEAR RNA SYNTHESIS IN FISSION YEAST

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A Gene Encoding an RNase Can Complement a Mutant Defective in snRNA Synthesis

M. Gillespie, D. Frendewey

A few years ago, we isolated a temperature-sensitive (ts^-) mutant that maintained a reduced steady-state content of several small nuclear RNAs (snRNAs), including the spliceosomal RNAs U1, U2, U4, U5, and U6 and the RNA subunit of the tRNA processing enzyme RNase P. We named this mutant *snm1* to indicate a defect in *sn*RNA *m*aintenance. When shifted to the restrictive temperature (37°C), *snm1* also accumulates aberrantly large U2 and U4 RNAs, which are extended at their 3′ ends, and an unspliced U6 RNA precursor. A single recessive mutation is responsible for the ts^- growth and snRNA phenotypes.

To appreciate the nature of the defect in *snm1* fully, it is necessary to understand how a wild-type fission yeast strain responds to a change in growth from 23°C to 37°C. When a normal *Schizosaccharomyces pombe* culture growing at 23°C is shifted to 37°C, the culture quickly assumes a new growth rate that is about twice that at 23°C. Stated another way, the generation time decreases from approximately 5 hours at 23°C to 2.5 hours at 37°C. We showed that the

elevated growth rate is accompanied by a two- to fivefold increase in the steady-state content of the major snRNAs. The *snm1* mutant exhibits a "leaky" temperature-sensitive growth phenotype; when shifted to the restrictive temperature, it continues to grow at approximately the same rate as it was growing under the permissive conditions. Therefore, at 37°C, the growth rate of the *snm1* mutant is about one half that of the wild type. The effect of temperature shift on snRNA content in *snm1* correlates with growth rate; we observe no difference in snRNA levels in *snm1* grown at either 23°C or 37°C.

Two possible explanations are suggested by the *snm1* mutant phenotype: either (1) the inability of *snm1* to increase its net snRNA synthesis in response to a shift to 37°C is the cause of the ts^- growth impairment or (2) the reduced snRNA content in *snm1* relative to the wild type is only a symptom of a more general defect in growth rate control that prevents faster proliferation at 37°C.

To try to learn more about the connection between snRNA synthesis and growth rate control, we attempted to clone the $snm1^+$ gene (the normal version of the gene mutated in the *snm1* mutant) by complementation of the ts^- growth defect in *snm1*. The mutant was transformed with a library of *S. pombe* genomic fragments carried on a multicopy plasmid, and temperature-resistant (ts^+) transformants were selected for their ability to grow well at 37°C. We obtained six ts^+ transformants from two independent transformation experiments, and plasmid DNA was isolated from the ts^+ clones and recovered in *Escherichia coli*. Restriction analysis of the clones showed that five of the six were related; they shared a 3.2-kbp *Hin*dIII fragment. The sixth clone had a unique restriction map, and the ts^+ yeast transformant from which it was derived grew much slower at 37°C than the other five. We have completed the analysis of one of the clones with the common 3.2-kb *Hin*dIII fragment. A 2.2-kb subclone was sufficient to transform *snm1* to rapid growth at 37°C, and its sequence was determined. A single long open reading frame was found. To our surprise, this gene had been cloned by two other groups, one being Mike Wigler's laboratory (Xu et al., *Nucleic Acids Res. 18:* 5304 [1990]; see the 1990 Annual Report) at Cold Spring Harbor Laboratory. The gene was also cloned by Yamamoto's group in Tokyo (Iino et al., *EMBO J. 10:* 221–226 [1991]).

The gene we have all cloned encodes a protein of 363 amino acids that over its carboxy-terminal two thirds shares 25% amino acid identity with *E. coli* RNase III, including a stretch of 11 identical amino acids. RNase III is a ribosomal RNA processing enzyme in *E. coli* that also plays a role in mRNA stability. It is interesting that a mutation that disrupts RNase III function in *E. coli* lies within the region of 11 amino acids shared by the yeast and bacterial enzymes (Nashimoto and Uchida, *Mol. Gen. Genet. 201:* 25–29 [1985]). The Wigler laboratory cloned the putative *S. pombe* RNase III homolog by virtue of its ability to cause sterility when present at high copy number. They named the gene *hcs* for high-copy sterile. Yamamoto and his colleagues cloned the gene as a high-copy suppressor of the *pat1* mutant (also known as *ran1*), which is an inducer of uncontrolled meiosis, and gave it the name $pac1^+$. They also demonstrated that $pac1^+$ has double-strand ribonuclease activity when expressed in *E. coli* and referred to the *S. pombe* protein as Pac 1 RNase. These results indicate a role for the Pac 1 RNase in mating and sporulation.

How can we reconcile our cloning of $pac1^+$ as a gene that can complement the *snm1* mutation? Larger than normal U2 and U4 RNAs accumulate in *snm1*, perhaps indicative of a defect in snRNA processing. The Pac 1 RNase might be an snRNA processing nuclease or it may be able to compensate for loss of the normal enzyme. Alternatively, our results could imply a connection between the control of snRNA synthesis and the switch between vegetative growth and the mating/sporulation pathway.

PLANT GENETICS

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Evolution of an Intron by Transposable Element Insertion

V. Sundaresan, J. Colasanti [in collaboration with Z.-Y. Zhao, Pioneer Hi-Bred International]

Last year, we described the isolation and characterization of a pseudo-revertant allele of the *Bronze1* (*Bz1*) gene of maize. This allele was derived from a mutable *bronze1* allele, *bz Mum9*, that carried an insertion of the *Mu1* transposable element. The derivative allele conferred full purple color to kernels, but molecular analysis showed that it still carried about two thirds of the *Mu1* element. We also found that the mRNA transcribed from the *Bz1* promoter was spliced using donor and acceptor sites within *Mu1* to eliminate most of the *Mu1* sequences from the spliced transcript; the splicing of the *Mu1* sequences maintained the reading frame of the *Bz1* protein. We have now demonstrated, by functional complementation of a *bz1* mutant in a transient expression system using particle bombardment, that the spliced transcript that we had previously identified encodes a functional *Bz1* protein as predicted. Furthermore, we have shown that the new *Bz1* allele is stable, is under the same regulation as the wild-type *Bz1* gene, and is not regulated by the activity or the methylation state of the *Mu* transposable element system, i.e., the new *Bz1* allele is genetically indistinguishable from a wild-type *Bz1* gene, and can only be distinguished molecularly. Therefore, the *Mu1* sequence in this *Bz1* allele behaves like a new intron. Since the discovery of introns, the question of whether they are "old" or "new" has been extensively debated. There is now considerable evidence suggesting that many nuclear introns are ancient and pre-date the divergence of plants and animals. Our observations demonstrate that although most nuclear introns are likely to be old, the evolution of new introns by transposable element insertion can also occur.

Insertional Mutagenesis of *Arabidopsis thaliana* Using Gene Trap and Enhancer Trap Transposons

V. Sundaresan, R.A. Martienssen, P. Weinberg, A. Baron [in collaboration with H. Ma, Cold Spring Harbor Laboratory, and J.D.G. Jones and C. Dean, John Innes Institute, United Kingdom]

To identify and isolate genes that control the development of higher plants, we are developing a technique for efficient insertional mutagenesis of the model plant *Arabidopsis thaliana*, using the maize transposable element *Ac*. Our approach has two features that constitute improvements over existing approaches: (1) the utilization of engineered transposons carrying a reporter gene (*gus*) such that insertions of the transposon into a target gene will result in expression of the reporter gene under the developmental control of the target gene, either directly by gene fusions ("gene traps") or indirectly by enhancer action ("enhancer traps") and (2) the development of a selection scheme that will select for unlinked transpositions.

The flowering plant *A. thaliana* has become a model organism for the study of plant biology for several reasons. *Arabidopsis* is a diploid, with a well-developed genetic map, a short generation time allowing up to six generations a year (vs. two for maize), and small plant size (6 in. vs. 6 ft. for maize), allowing thousands of plants to be grown and screened within a limited space. It also has a small genome (7×10^7 bp vs. 3×10^9 bp for maize) with a low repeated DNA content, and transgenic plants can be generated using *Agrobacterium*. However, one major obstacle has remained in making *Arabidopsis* a versatile model genetic system for plants (e.g., as *Drosophila* is a model system for animals), i.e., the lack of an efficient insertional mutagenesis system to identify and isolate genes of interest.

To address this problem, we are developing a

transposon mutagenesis scheme for *Arabidopsis*. Our scheme utilizes a two-element system, in which transgenic *Arabidopsis* plants carrying an immobilized (or "wings clipped") *Ac* element will be crossed to plants carrying a *Ds* element (a nonautonomous *Ac* element) containing the *gus* reporter gene. The immobilized *Ac* element lacks the *Ac* termini, and therefore cannot transpose, but can supply transposase constitutively. The particular construct we will use (provided by Drs. J. Jones and C. Dean) carries a small deletion of GC-rich sequences in the untranslated leader of the transposase gene, as well as a strong 35S promoter to drive transcription of the transposase gene. These modifications result in increasing the germinal transposition frequency of a *Ds* element in *Arabidopsis* to 10–50%, i.e., transpositions occur in one tenth to one half of the progeny of a plant carrying both this modified *Ac* element and a tester *Ds* element. *Ds* elements contain a deletion of the transposase gene but have intact termini; therefore, they can transpose when transposase is provided by an *Ac* element. In our case, the *Ds* elements are engineered so that they also carry the *gusA* reporter gene and a kanamycin resistance (Kan^R) gene. The *gusA* (β-glucuronidase) gene is an *Escherichia coli* gene widely used in plant systems as a histochemical marker instead of *lac*; most plants show considerable background activity with *lac*, but essentially no background *gus* activity. For the enhancer trap vector, the *gus* gene will have a promoter with a TATA box, but without an enhancer. Therefore, expression of the *gus* gene will depend on the *Ds* element landing near a cellular enhancer. For the gene trap vector, the *gus* gene is preceded by a triple splice acceptor sequence (containing three adjacent plant splice acceptor sequences in different reading frames) inserted immediately upstream of the ATG start codon. Therefore, insertion into an intron will generate an in-frame fusion 50% of the time, leading to a fusion protein expressing *gus* activity. It is known that such *gus* fusions are active and localize intracellularly according to the targeting signals present on the fused gene. In addition, we will utilize endogenous splice-donor sites within *Ds* located at 14, 24, and 28 bp from one end of the *Ds* element, so that insertion into an exon can also generate an in-frame fusion after the intervening 190 bp of *Ds* sequence is spliced out (the length of the *Ds* terminus is approximately 210 bp). All of the elements described above, i.e., the immobilized high-efficiency *Ac* element, and the gene trap and enhancer trap *Ds* elements carrying the suitably engineered *gus* reporter gene as well as the gene for Kan^R, have been constructed. Furthermore, both the enhancer trap and gene trap *Ds* elements have been tested using transient assays with the particle gun to confirm that (1) the enhancer-trap element results in *gus* expression from a nearby enhancer sequence and (2) the gene-trap element results in *gus* expression when inserted into a transcribed DNA sequence. For the latter, we also verified using various constructions that all three splice-acceptor sequences of the synthetic triple splice-acceptor sequence were utilized and that the endogenous *Ds* splice-donor sequences at the element function as expected. The *Ds* elements have been inserted into T-DNA vectors. These vectors are used for delivery of DNA to plants using *Agrobacterium*. In all cases, the T-DNA vectors carry the IAAH (indole acetic acid hydrolase) gene, which will be used as a dominant marker for negative selection. When seedlings carrying the IAAH gene are germinated on medium containing the auxin analog NAM (naphthalene acetamide), they wilt and do not grow due to the conversion of NAM to toxic levels of naphthalene acetic acid. Seedlings not carrying this gene are unaffected. In the case of the immobilized *Ac* element, the T-DNA vector carries the Kan^R gene in addition to the IAAH gene. The T-DNAs carrying the engineered transposons have been introduced into *Arabidopsis* by *Agrobacterium*-mediated root transformation. Kan^R calli were selected, and transgenic plants have been generated and propagated (see report by Hong Ma in this Section). We are currently characterizing the transgenic lines prior to initiating large-scale mutagenesis.

Intracellular Localization of $p34^{cdc2}$ Kinase during the Plant Cell Cycle

J. Colasanti, V. Sundaresan

A significant difference between mitosis in plant cells and animal cells is the determination of the plane of cell division. Plant cells, unlike animal cells, are enclosed in a cell wall structure and cannot migrate. Therefore, the planes of cell division during cell proliferation are extremely important in plant morphogenesis, and organ initiation is often accompanied by a change in the plane of cell division, e.g., from anticlinal to periclinal. What initially determines the plane of cell division of a given cell is not known, but some knowledge of the process of determination is available from extensive studies of the cytoskeleton

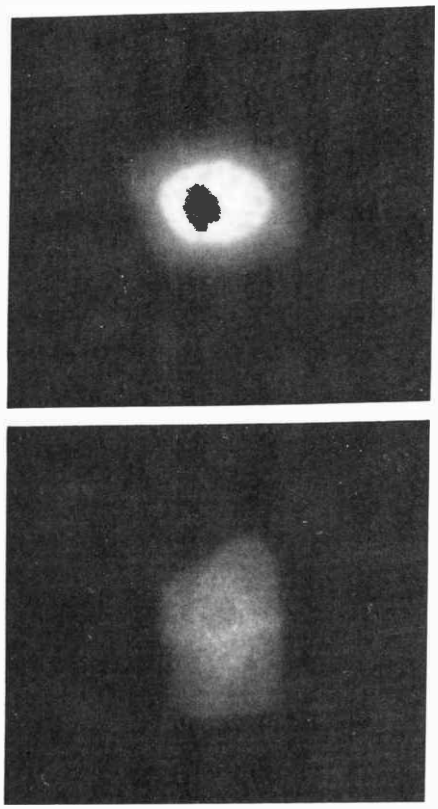

FIGURE 1 A maize root tip cell approaching mitosis, stained with DAPI, showing some chromosome condensation (*top*), and $p34^{cdc2}$ antibody, showing both nuclear and preprophase band localization of $p34^{cdc2}$ (*bottom*). (The dark region within the nucleus is the nucleolus.)

in dividing plant cells. Immediately before entry into M phase (at "preprophase"), there appears a belt of microtubules, called the preprophase band, that anticipates the division plane and marks the site where the new cell plate will fuse with the cell walls. By metaphase, the preprophase band completely disappears. At telophase, however, residual polar microtubules form a structure at the plane of the preprophase band, called the phragmoplast. Golgi vesicles filled with cell-wall precursors accumulate at the center of the phragmoplast to form the beginning cell plate, which expands until it contacts the side walls at the position of the original preprophase band. Thus, the plane of cell division is determined at preprophase, but somehow imprinted until telophase. The mechanism of this "imprinting" has not been established.

We have used an antibody against the functional homolog of the cdc2 kinase from maize to localize the $p34^{cdc2}$ protein within the plant cell through the cell cycle. The cytoskeletal structure of plant cells was visualized concomitantly with monoclonal antibody specific for the α-tubulin subunit. At interphase, antibody staining localized the cdc2 protein to the nucleus of the cell, although it was excluded from the nucleoli. During metaphase and anaphase, the cdc2 protein was generally diffused throughout the cell, although there was a higher concentration of staining in the region surrounding the chromosomes, with the chromosomes themselves excluded from staining. In cells at early prophase, a subpopulation of $p34^{cdc2}$ protein was transiently distributed as a band bisecting the nucleus (Fig. 1). Triple staining with anti-cdc2, anti-tubulin, and DAPI revealed that this band colocalized with the preprophase band of microtubules. The cdc2 protein did not, however, colocalize with other microtubular structures, i.e., neither the spindle nor the phragmoplast. Cells in which the microtubules were disrupted with oryzalin had no band of cdc2 in early prophase, suggesting that the preprophase band may be involved in positioning the cdc2 kinase at this position. Association of $p34^{cdc2}$ protein with the preprophase band at the G_2/M transition of the cell cycle, when cdc2 kinase activity is expected to be maximal, suggests that the cdc2 kinase of higher plants is involved in setting the plane of cell division.

Isolation of New Alleles of *anther ear* and *indeterminate*

J. Colasanti, V. Sundaresan

We have taken advantage of the proximity of a *Ds2* element at the *bz2* locus (*bz2-m2*) on chromosome 1 to isolate new alleles of the closely linked *indeterminate (id)* and *anther ear (an1)* genes by transposon tagging. In the first set of crosses, plants homozygous for the *bz2-m2* allele and carrying an active *Ac* element were crossed to *an1bz2* homozygous plants. These plants carry a large deletion that includes both the *an1* and *bz2* loci. Germinal revertant kernels (i.e., *bz2-m2* to *Bz2*) from the F1 of this cross were planted and screened for semi-dwarf plants typical of the *anther ear* phenotype. One plant, from 650 purple kernels planted, that exhibited this phenotype was selfed and outcrossed. Genetic experiments showed that this mutant is allelic to *an1* and that an active *Ac* element did not segregate with the original mutant. The outcross progeny from this plant were

found to carry a 4.3-kb *Ds2*-hybridizing *Bam*HI band that cosegregates with the semi-dwarf phenotype.

Plants from the original screen of germinal revertants were also selfed and subsequently screened for other potential *Ds2*-induced recessive mutations. From 600 families screened, one family segregated mutant plants with a phenotype typical of the *id* mutation. These plants flowered between 3 and 8 weeks later than their normal siblings and exhibited other characteristics of the *id* phenotype. However, preliminary experiments with different light regimes showed that short-day conditions did not reverse the indeterminate effect. Alleles of *id* that do not respond to day length have also been found by other investigators (Burr and Szabo, *Maize Genetics Newsletter 65:* 110 [1991]). Thus, *id* mutants appear to be defective in the vegetative to inflorescence transition of the meristem. In this respect, it differs from the mutants *floricaula* in snapdragon and *leafy* in *Arabidopsis* which are defective at a later stage, i.e., in the transition from inflorescence to floral meristem. Genetic experiments show that our putative new *id* allele is closely linked to *Bz2*. A search for a *Ds2* element that cosegregates with this phenotype, which would permit us to clone the gene, is in progress.

Mitotic Cyclins in Higher Plants

J.P. Renaudin, V. Sundaresan

Although the key components of the cell division machinery in plants and animals are likely to be similar, because of the different strategies used by plants to control cell division, higher plants offer the chance to study these same components in a very different context. Considerable attention has been paid to cyclins in animals and yeasts, but there is relatively little known about cyclins in higher plants. In this proposal, we have described experiments to characterize further the putative mitotic cyclins that we have identified in higher plants. We have used polymerase chain reaction (PCR) to conserved domains of animal and yeast mitotic cyclins to isolate cDNA sequences encoding putative mitotic cyclins from maize. We have found cDNAs encoding six different mitotic cyclins, which fall into four different classes. We have termed them 1a, 1b, 2a, 2b, 3, and 4. Cyclins 1a and 1b are very closely related (98% identity), as are 2a and 2b. The homologies between cyclins 1, 2, 3, and 4 range from 44% (for 1 and 2) to 65% (for 1 and 3). In the 48-amino-acid region that is encoded by our partial cDNA clones (which excludes the sequences of the PCR primers), none of these cyclins are clearly identifiable as homologs of animal A- or B-type cyclins, although they have homologies with both types. The homologies of these cyclins with cyclin A and cyclin B range from 42% to 56%. Cyclin 2 showed the greatest homology with cyclin A (56% amino acid identity) and least with cyclin B (48%), and cyclin 4 showed the greatest homology with cyclin B (55%) and the least with cyclin A (42%); cyclins 1 and 3 have intermediate values of homology with both A and B. To summarize, we have evidence for four distinct types of mitotic cyclins in higher plants, which are not clearly A or B cyclins, although related to both. Experiments are in progress to distinguish between the four distinct types of plant cyclins that we have identified and to determine if any one or more can be classified as a homolog of the known animal cyclins on the basis of the expression pattern. It is possible that some of the plant cyclins may resemble cyclin A or cyclin B in terms of expression during the cell cycle or have a unique expression pattern different from both, suggesting novel functions distinct from both A- and B-type cyclins.

The Hcf106 Gene Product and Its Role in Thylakoid Membrane Biogenesis

A. Yonetani, A. Baron, R. Martienssen

High-chlorophyll fluorescence mutants of higher plants represent a class of nuclear genes that are required for the development of photosynthetically active chloroplasts. These mutants have near-normal levels of chlorophyll pigments, so that they can transiently absorb light energy but cannot utilize it in photosynthesis. The energy is lost as elevated levels of chlorophyll fluorescence, and *hcf* mutants die as seedlings when seed reserves have been depleted. On the basis of extensive mutagenesis in barley, maize, and *Chlamydomonas*, more than 100 *hcf* genes are thought to exist in higher plants and algae. Their products may include nucleus-encoded components of the photosynthetic apparatus, enzymes required for the biosynthesis of various cofactors, and regulatory genes required for the expression of the plastid genome. Because of their effects on chloroplast morphology, some *hcf* loci are thought to encode proteins required for the correct assembly and organization of

the thylakoid membrane system itself, which is the site of the primary photosynthetic reactions. These gene products might include factors required for protein uptake and assembly, membrane biogenesis within the organelle, and protein modifications required for proper thylakoid function and assembly.

Maize seedlings homozygous for the *hcf106* mutation have altered thylakoid membrane composition and morphology. Specifically, the three major thylakoid electron transport protein complexes (photosytems I and II and the cytochrome f/b_6 complex) are present at severely reduced levels (5–10% wild type), whereas other thylakoid complexes, and several soluble proteins, are unaffected. The thylakoid membranes have lost their characteristic lateral heterogeneity, and form large whorled structures instead. The lipid and pigment composition of mutant membranes is near normal, and nonallelic mutants of maize that have similar lipid and pigment profiles do not have aberrant membrane morphology. This suggests that the gene product is not directly involved in lipid or pigment biosynthesis.

As we reported last year, cDNA encoding the *hcf106* gene product has been cloned using molecular probes from the locus obtained by transposon tagging. The cDNA sequence encodes a 243-amino-acid (27 kD) polypeptide with a single membrane-spanning domain, a potential nucleotide-binding motif, and no other significant homology with known proteins. We have raised antibodies against TrpE fusion proteins, and these detect a 30-kD protein in leaves of wild-type, but not of mutant, plants. Coupled in vitro transcription and translation of the cDNA produces a protein that migrates aberrantly as a 35-kD band on SDS gels and can be specifically immunoprecipitated with the anti-*Hcf106* sera. We conclude that the precursor is processed to a faster migrating form in vivo.

The 30-kD protein is quantitatively recovered (relative to chlorophyll) from purified wild-type chloroplasts and is found predominantly in low-speed thylakoid membrane pellets. However, some of the protein is in a high-speed membrane fraction characteristic of chloroplast envelopes. Immunofluorescence using the same antibody detects a protein in purified chloroplasts that seems to be localized in both peripheral (envelope) and internal (thylakoid) membranes. If the Hcf106 protein is localized in both envelope and thylakoid membranes, this unusual distribution may reflect a role in membrane biogenesis.

Biochemically, attempts to demonstrate ATP binding have so far been unsuccessful, but the aberrant migration on SDS gels might be the result of protein modification. The predicted protein sequence contains at least four possible sites for phosphorylation via the major thylakoid membrane protein kinase, which is responsible for modifying the light harvesting complex chlorophyll-*a*/*b*-binding proteins. Preliminary results suggest that this kinase is highly active in mutant membrane preparations as well as wild type. However, as expected, the major photosystem II phosphoproteins are severely reduced in *hcf106*.

The Molecular Nature of the *iojap* Mutation in Maize

C.-D. Han, R. Martienssen [in collaboration with E.H. Coe, USDA-ARS, University of Missouri]

The maize mutant *iojap* (*ij*) has been a model system in the study of nucleus-plastid interactions in higher plants since the work of Jenkins (1924) and of Rhoades (1943). Plants homozygous for this nuclear mutation have variegated leaves and transmit defective chloroplasts through their female gametes. Maternally inherited defective plastids have suffered an irreversible change that cannot be rescued by the nuclear genotype of the zygote, resulting in albino seedlings whose plastids are incapable of differentiation into chloroplasts. Furthermore, the pattern of leaf variegation observed in homozygous individuals suggests that the gene product is a key component of the regulatory machinery that coordinates cellular and plastid development in higher plants. As we reported last year, a molecular clone of the *iojap* locus has been obtained by transposon tagging using the *ij-mum1* allele, which is caused by the insertion of the maize transposon *Mu1*. During the last year, cDNA clones have been obtained corresponding to the *Iojap* gene product, and the nature of the original reference allele described by Jenkins (*ij-ref*) has been elucidated. We have also initiated genetic studies aimed at determining the developmental parameters involved in the patterns of leaf variegation and maternal inheritance observed in mutant plants.

The phenotype of plants homozygous for *ij-ref* differs markedly in different genetic backgrounds. In many backgrounds, a bold striping pattern with white leaf margins is observed (Fig. 2C). In other backgrounds, *ij/ij* plants have a more uniform "grainy" appearance, often with narrow white margins (Fig. 2B).

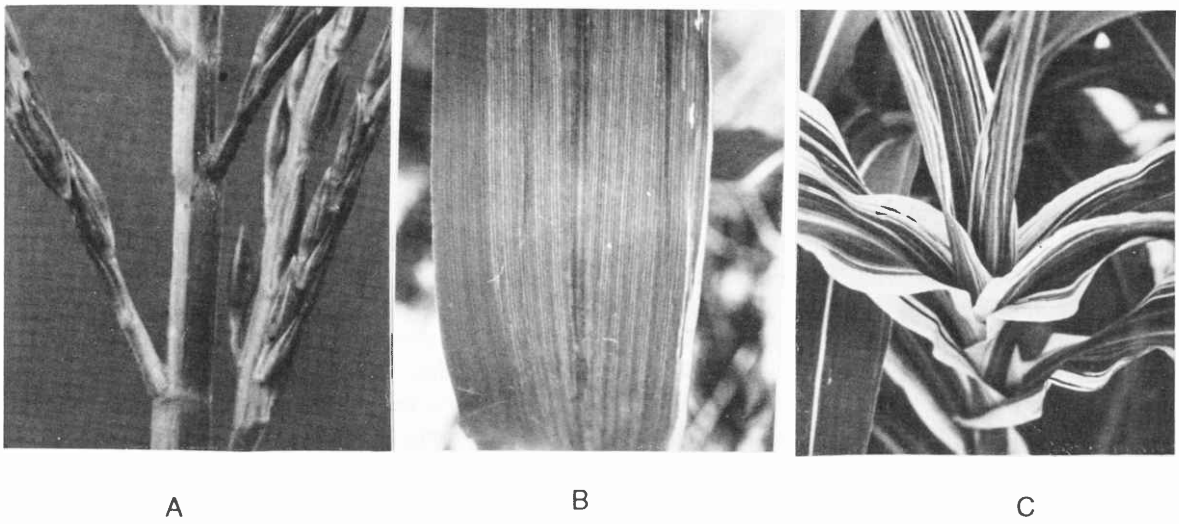

FIGURE 2 Three homozygous *iojap* plants are shown. (*A*) A revertant green sector in the tassel of the first plant gave rise to revertant gametes. (*B*) A revertant green sector in the second plant passed through six leaves, extending to the left margin of the leaf shown. (*C*) The bold striping pattern observed in the third plant is typical of *iojap* homozygotes from several different inbred backgrounds. (Figures *A* and *C* were provided by E.H. Coe, USDA-ARS, University of Missouri.)

In some of these backgrounds, *ij-ref* is unstable and can give rise to revertant sectors and revertant progeny. In Figure 2C, revertant sectors are difficult to distinguish against the bold striping pattern. In Figure 2B, the left margin of the leaf has a revertant sector in which the nuclear genotype has reverted to normal, restoring green pigmentation. This sector was found on six successive leaves and passed from the margin of one leaf to near the middle of the next, suggesting that it comprised a clone of cells derived from a single revertant cell early in shoot development. This is in contrast to the albino sectors in Figure 2C, which occur in nonclonal positions on successive leaves, predominantly at the margins. In Figure 2A, a revertant sector passed into the tassel, and the green tassel branches gave rise to germinal *Ij-Rev* revertant progeny. E.H. Coe, at the University of Missouri, observed two such plants and maintained the mutant and revertant alleles from each by crossing individual tassel branches to tester stocks.

cDNA clones corresponding to the *Iojap* gene and genomic clones corresponding to the original *ij-ref* allele have been obtained using the *ij-mum1* clone as a probe. The cDNA clones were found to encode a 229-amino-acid polypeptide that has no striking homology with known proteins. Molecular cloning of the *ij-ref* allele revealed that it contained a 1.5-kb transposon inserted into the first exon of the *Ij* gene, about 350-bp upstream of the *Mu1* insertion site in *ij-mum1* (see Fig. 3). The ends of the 1.5-kb element closely resemble the ends of the *Ac/Ds* family of transposable elements, sharing 10 of 11 bp at the termini and about 60% homology in the subterminal regions. Insertion of this element was associated with an 8-bp target site duplication in the first exon of the *Iojap* gene. DNA was isolated from two germinal revertants (provided by E.H. Coe) and five independent somatic sectors from leaves such as the one shown in Figure 2B. In each case, Southern blotting and polymerase chain reaction (PCR)-mediated clon-

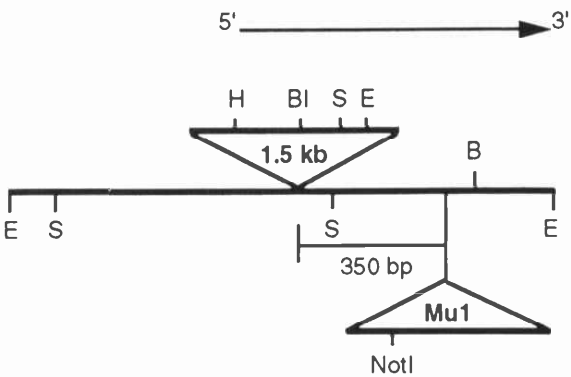

FIGURE 3 A restriction map of the *ij-ref* allele. The 1.5-kb *Ds*-like element is shown as an insertion. The insertion site of the *Mu1* element in the *ij-mum1* allele is also shown. The direction of transcription is shown by an arrow. (E) *Eco*RI; (S) *Sst*I; (H) *Hin*dIII; (Bl) *Bgl*I.

ing showed that phenotypic reversion was accompanied by excision of the 1.5-kb element, leaving in-frame rearrangements at the site of insertion (Fig. 4). We conclude that the insertion of the *Ds*-like element is responsible for the mutant phenotype caused by the *ij-ref* allele. We are currently investigating the possibility that excision of this element is under the control of the autonomous *Ac* transposon. DNA prepared from green and white stripes of the type shown in Figure 2C had no detectable changes at the *ij* locus. Thus, excision of the 1.5-kb element is not responsible for the striping pattern itself in *ij/ij* homozygotes.

In an attempt to elucidate the developmental parameters controlling the pattern of leaf variegation and maternal inheritance in *iojap* mutants, we are constructing a series of double mutants with *ij* and other mutations that affect leaf and flower morphology. In addition, we have begun a clonal analysis of *Ij* gene action using γ-ray-induced chromosome breakage. The *Bronze* gene, which affects anthocyanin pigmentation in many plant tissues including the leaf, will be used as a genetic marker for irradiation-induced clones, by using a reciprocal translocation stock in which the *Bz* gene (on chromosome 9S) is linked to the *Ij* gene on chromosome 7L. In a pilot study, 400 germinating seedlings from the cross T7-9a (*Gl Ij wx Bz*) x *wx bz; gl ij* were irradiated at Brookhaven National Laboratories last summer and grown up in order to look for clonal sectors that had lost the *Ij* allele, resulting in an *ij* phenotype. By examination of the *bz* phenotype of these sectors, we hoped to learn in which cells of the leaf the Ij gene product is required. Numerous *ij* sectors were observed, but there was insufficient leaf color to observe the *bronze* phenotype. Southern blotting revealed that these sectors had lost both the *Ij* and the *Bz* wild-type alleles inherited from the translocation parent, relative to adjacent tissue. Thus, the genes are appropriately positioned on the translocation chromosome for this analysis. We will repeat this experiment next summer using more intensely pigmented stocks.

Inheritance of Somatic Changes in *Mu* Activity

R. Martienssen, A. Baron

As we have described in previous years, the mutant phenotype associated with the *hcf106* mutation is suppressed when homozygous plants lose *Mu* activity. *Mu* activity is lost when *Mu* transposable elements lose the ability to excise or transpose and inactive *Mu1* elements become hypermethylated relative to elements from active lines. Loss of *Mu* activity can occur via loss of the recently identified autonomous regulatory element *MuR*, either by segregation or by transposition. However, *Mu* activity can also be lost in a progressive fashion during development. This can be conveniently observed in plants homozygous for the *hcf106* mutation as sectors of dark green leaf tissue on a mutant pale green background. The dominant *Lesion-mimic* mutation is also phenotypically suppressed by loss of *Mu* activity, and coordinate sectors of phenotypically wild-type tissue arise progressively during development in plants that carry both mutations.

It has been suggested that this progressive loss of *Mu* activity might account for the nonreciprocal loss of *Mu* through male and female gametes. The male inflorescence (the tassel) develops from the apex of the plant, whereas the female inflorescence (the ear) is formed from a lower, axillary bud. Thus, the progressive loss of *Mu* activity in successive leaves might be reflected in the proportion of gametes from each flower that carries *Mu* activity. This phenomenon does not occur with most *Mu* lines but is very striking in some individual plants. Last summer, we

ij-mum1 and cDNA	ccgagccacggcctcgcctg		cgcgcccccg
ij-ref	ccgagccacggcCTCGCCTG	(Ds-like)	CTCGCCTGcgcgcccccg
Ij-Rev #1	ccgagccacggcCTCGCCTG		cgcgcccccg
Ij-Rev #2	ccgagccacggcCTCGCCT		TCGCCTGcgcgcccccg
Ij-Rev #4	ccgagccacggcCTCGCC		GTCGCCTGcgcgcccccg
Ij-Rev #3,5,6,7	ccgagccacggcCTCGCCTCA		GTCGCCTGcgcgcccccg

FIGURE 4 Sequence around the excision site from seven *Ij-Rev* alleles resulting from two germinal and five somatic excisions of the 1.5-kb *Ds*-like element from *ij-ref*. The corresponding sequence from the *ij-mum1* allele, the *Ij* cDNA clone, and the *ij-ref* allele are also shown.

selected individual plants heterozygous for both *Lesion-mimic* and *hcf106*, which were losing the *Lesion-mimic* phenotype in the upper leaves between the ear and the tassel. These plants were crossed reciprocally to homozygous *hcf106* plants that had lost *Mu* activity, and the progeny were scored for both the *Lesion-mimic* and *hcf* phenotypes. Progeny from the ears of the sectored plants were planted in the order they appeared on the ear (an "ear map"), so that spatially distinct clones of kernels that had lost *Mu* activity might be apparent from the seedling phenotype. These clones would occur if *Mu* activity were lost from individual cells during somatic development of the ear.

In four out of seven cases, 98–100% of the progeny from both male and female had lost *Mu* activity, so that no significant difference could be detected. These plants had lost the *Lesion-mimic* phenotype relatively early in development (four or five leaves below the tassel). In another plant, there was no difference in the proportion of active and inactive progeny observed whether used as male or female. This plant expressed the *Lesion-mimic* phenotype even in the topmost leaves. In the remaining two plants, however, a pronounced difference was observed between transmission of *Mu* activity through the male (0%) and through the female (40–43%). Furthermore, large "sectors" of phenotypically suppressed progeny were apparent from both ear maps. DNA made from kernels inside one of these sectors had hypermethylated *Mu* elements relative to those outside the sector. This showed that the sectors were due not to segregation of *MuR*, but rather to the epigenetic loss of *Mu* activity in apparently clonal sectors during ear development.

Somatic Transposition of *Mu*

R. Martienssen, A. Baron

In many Robertson's *mutator* lines, somatic sectors of pale green, yellow, or white tissue, of varying sizes and extents, have been observed. These sectors are typically found in plants that have active *Mu* elements and have been postulated to be due to some type of *Mu*-induced somatic mutation. DNA was prepared from 11 pale green sectors observed on plants heterozygous for *hcf106* and from adjacent tissue on either side of these sectors. Southern blotting showed that 5 out of the 11 sectors had detectable changes at the *Hcf106* locus. Four of these were the result of the insertion of a 1.4-kb element (the same size as *Mu1*) into the wild-type allele at a position within a few hundred base pairs of the *Mu1* insertion site in the mutant allele. This could be either the result of *Mu1* insertion or the result of mitotic crossover between the wild-type and mutant alleles. In the fifth sector, the insertion into the wild-type allele was much larger, and reprobing the Southern blot with other *Mu* probes revealed that the insertion was related to the *MuR* autonomous transposon, which had inserted into the wild-type allele. PCR cloning using specific primers was used to recover the insertion site, and sequencing revealed that the *MuR*-like element had inserted 2 bp upstream of the *Mu1* insertion site in *hcf106*. As we reported last year, the sequence of this insertion site is identical to the last 5 bp of both *Mu1* and *MuR*. It is possible that it represents a hot spot for *Mu* insertion.

Molecular and Genetic Analysis of the Maize *P* Locus

P. Athma, E. Grotewold, T. Peterson

We are studying the maize *P* gene as a model for regulation of gene expression during development. The *P* gene is involved in the production of a red flavonoid pigment in certain maize floral organs including the pericarp, cob glumes, and tassel glumes. The *P* gene is well-suited for genetic analysis, because its pattern of expression is immediately apparent from the localization and intensity of the red pigment.

STRUCTURE AND FUNCTION OF THE *P* GENE

The *P* gene produces several RNA transcripts, and we cloned two of these by conventional and polymerase chain reaction (PCR)-based cDNA cloning. The two transcripts share the same two 5′ exons but are alternatively spliced to alternate third exons. The amino termini of the encoded proteins are homologous to the DNA-binding domain of the *Myb* class of transcriptional activators. We have shown by Northern blot experiments that the *P* gene regulates in the pericarp the accumulation of RNA from three maize genes involved in flavonoid biosynthesis: *C2*, *CHI*, and *A1*, which encode chalcone synthase, chalcone isomerase, and flavonol reductase, respectively (Grotewold et al. 1991; E. Grotewold and T. Peter-

son, unpubl.). In collaboration with Ben Bowen and colleagues at Pioneer Hi-Bred, we used microprojectile bombardment to test the transcriptional activation properties of the *P*-encoded products. The *P* cDNAs were fused to the constitutive cauliflower mosaic virus 35S (CaMV35S) promoter and bombarded together with a reporter gene (luciferase) linked to the promoter of the maize *A1* gene, which is positively regulated by *P* in vivo. The results indicate that the larger *P*-encoded protein alone activates transcription of the *A1* promoter; the smaller *P*-encoded protein has no detectable activating or inhibiting effects (E. Grotewold et al., in prep.).

CLONING OF A *P*-REGULATED CHALCONE-FLAVONONE ISOMERASE GENE FROM MAIZE

To extend our analysis of *P* function, we decided to clone the maize *CHI* gene, which encodes chalcone/flavonone isomerase. The chalcone-flavonone isomerase enzyme should act between the *C2*- and *A1*-encoded products, which opened the possibility that its expression would be regulated by the *P* gene in the pericarp, as are *C2* and *A1* (see above). However, *CHI* activity has not been reported in maize, nor has any mutant in this step been isolated. To clone a maize *CHI* gene, we used highly degenerate primers made from sequences conserved between *CHI* proteins from several plants (we appreciate very much the suggestions provided by Dr. Arjen van Tunen regarding the sequences of the primers). We obtained a 210-bp cDNA by PCR, which was used as a probe for screening a cDNA library from pericarps carrying a functional *P* gene (*P-rr*). Full-length cDNA clones were obtained by extending cDNA clones using the RACE technique, and genomic clones were isolated by using the cDNA clones as probes on a genomic library. The translated sequence of the cDNA clones showed about 65% identity with the sequence of two chalcone-flavonone isomerase proteins described in petunia. However, the intron/exon structure of the maize *CHI* gene is identical to the structure of one of the petunia *CHI* genes, giving some clues on the evolution of the *CHI* gene. Northern blot hybridizations of pericarp poly(A) RNA showed a single transcript of about 1 kb hybridizing with these cDNA clones. No transcript could be detected in RNA from pericarps lacking a functional *P* gene, suggesting that the maize *CHI* gene is regulated by *P* in the tissues in which *P* is expressed (E. Grotewold and T. Peterson, unpubl.). We will test whether *P* regulates the *CHI* promoter by using the particle gun to deliver a *CHI* promoter/luciferase reporter gene construct together with *P-rr* cDNA into embryogenic callus cells. If the results are positive, then the *CHI* promoter will be useful as an additional *P*-regulated promoter for comparisons with the *C2* and *A1* promoters.

Ac INDUCES RECOMBINATION AT THE *P* LOCUS

We previously demonstrated that the maize transposable element *Ac* induces recombination between the long (5.2-kb) direct repeats that flank the *P* gene. Even though the *Ac* element has been subject to intense molecular and genetic analyses, this documents a new activity of *Ac*: the destabilization of flanking direct repeat sequences (Athma and Peterson 1991). To determine the mechanism by which *Ac* induces recombination, we are extending our analysis to cases in which *Ac* is located at different positions within the *P* locus; for example, will *Ac* insertions within the flanking direct repeats give enhanced or reduced recombination frequencies? In addition, we are looking at the effect on recombination of three *Ds* insertions derived by internal deletions from a progenitor *Ac* insertion in *P*. Preliminary results indicate that enhancement of recombination requires an active *Ac*, in addition to the *Ds* insertion in the *P* locus (P. Athma and T. Peterson, in prep.).

INSERTIONAL MUTAGENESIS OF THE MAIZE *P* GENE BY TRANSPOSON *Ac*

A striking feature of *P* is the organ-specific expression specified by the *P-wr* and *P-rw* alleles, which condition colorless pericarp/red cob and red pericarp/colorless cob, respectively. One of our central objectives was to determine whether *P-rr*, which pigments both pericarp and cob glumes, comprises two closely linked organ-specific genes. We reasoned that insertional mutagenesis with *Ac*, which is known to have a preference for localized transposition, could disclose the presence of multiple functional units at *P-rr* if they in fact exist. Therefore, we isolated and mapped the *Ac* insertion sites in 20 alleles derived by intragenic transposition of *Ac* (Fig. 5). In all of these alleles, the pericarp and cob glume pigmentation was affected similarly, and these alleles carried *Ac* insertions within, or near, the limits of the *P-rr* transcript previously mapped by conventional molecular methods. We conclude that *P-rr* contains a single gene required for pigmentation of both pericarp and cob glumes. Our results also demonstrated that *Ac* transpositions can occur in either direction in the *P* gene, with no apparent minimum distance: In one

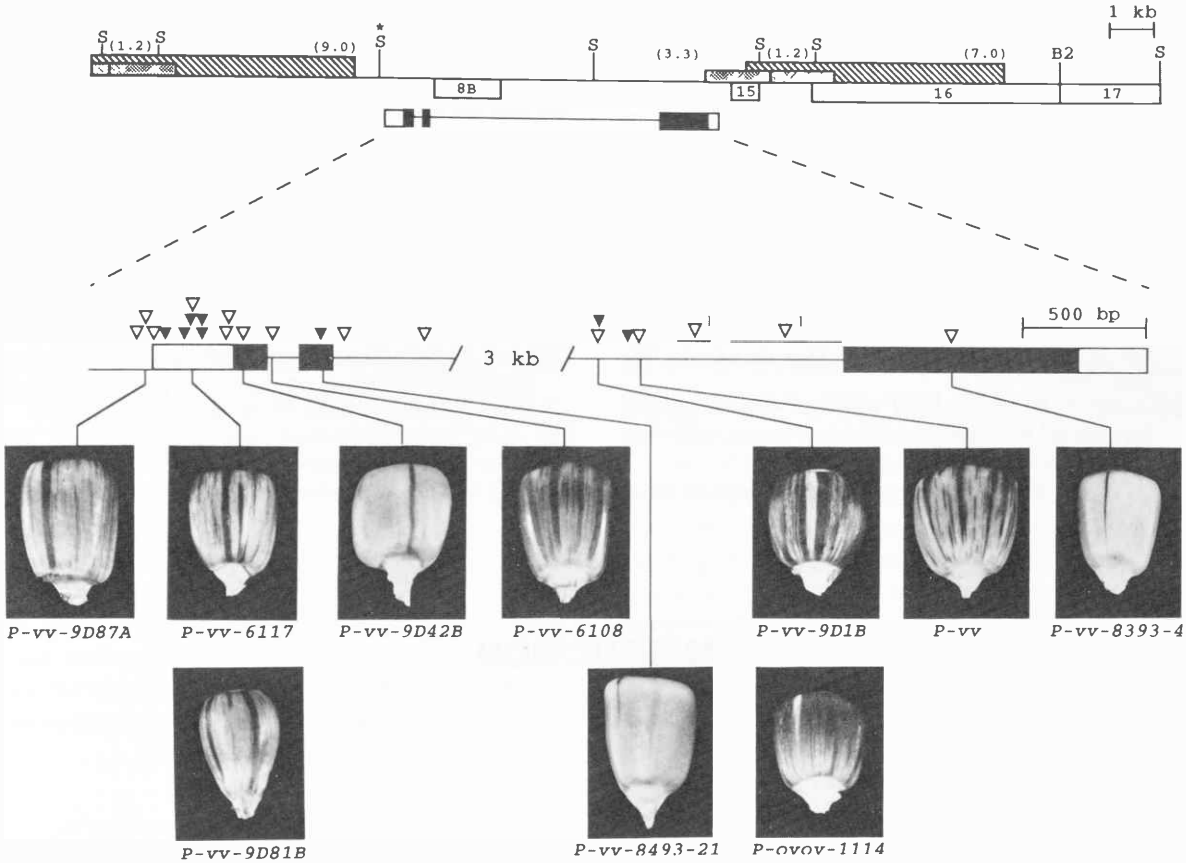

FIGURE 5 (*Top*) Restriction map of the *P* locus. The 5.2-kb direct repeats (*hatched boxes*) flanking the *P* gene and the 1.2-kb direct repeat sequences (*stippled boxes*) are indicated. Open boxes indicate the positions of restriction fragment probes 8B, 15, 16, and 17. Numbers in parentheses indicate the fragment sizes obtained upon digestion of genomic DNA with *Sal*I. Restriction sites for *Sal*I (S) and *Bgl*II (B2) are indicated; not all *Bgl*II sites are shown. The asterisk above the third *Sal*I site from the left indicates that this site is not digested by *Sal*I in genomic DNA, possibly because of methylation. (*Bottom*) Location of *Ac* insertions. An enlarged map of the 1.8-kb *P-rr* mRNA is shown. Open boxes correspond to 5′- and 3′-untranslated regions, closed boxes correspond to protein-coding sequences, and the lines between them correspond to intron sequences. Open triangles represent *Ac* elements in the same orientation with respect to *P* as in *P-vv*; closed triangles represent insertions in the opposite orientation (i.e., same as *P-ovov-1114*). Photographs at bottom show the phenotypes resulting from insertion of *Ac* at representative sites. The upper and lower panels of photos are of kernels from plants with *Ac* insertions in the *P-vv* and *P-ovov-1114* orientations, respectively. Plants producing these kernels were heterozygous with an allele specifying colorless pericarp (either *P-ww* or *P-wr*). (*1*) *Ac* insertion sites mapped by Southern blotting but not sequenced. Horizontal lines indicate approximate locations.

case, *Ac* transposed just 6 bp from its original insertion site. However, the distribution of transposed *Ac* elements was markedly nonrandom: Of 19 transposed *Ac* elements derived from a single *Ac* donor, 15 were inserted in a 1.1-kb region at the 5′ end of *P*, whereas none had inserted in an adjacent 3.2-kb intronic region (P. Athma et al., in press). In a related study, we identified a possible hot spot for *Ac* insertion within the *P* gene (Grotewold et al. 1991).

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ARABIDOPSIS SIGNAL TRANSDUCTION AND FLOWER DEVELOPMENT

H. Ma C. Flanagan, H. Huang, R. Lavi, Y. Mizukami, A. Oates, A. Tagle, C. Weiss

The research in our laboratory is focused mainly in two areas: (1) characterization of G protein function in plant signal transduction pathways and (2) analysis of genes involved in flower development. Plant cells are known to respond to a large number of external and internal signals; however, little is known about the molecular mechanisms of the plant signal transduction pathways mediating these responses. To understand plant signaling processes, we have taken the approach of studying plant G proteins. G proteins are known to play important roles in signal transduction in animals and simple eukaryotes. We believe that G proteins also play important roles in the signal transduction processes of plants. Using the polymerase chain reaction (PCR) technique and degenerate oligonucleotides, we have previously isolated a gene (*GPA1*) from *Arabidopsis thaliana* that encodes a G protein α subunit (Ma et al., *Proc. Natl. Acad. Sci. 87:* 3821 [1990]). We are currently characterizing *GPA1* in several ways, and we are attempting to establish association of *GPA1* with particular plant signaling pathway(s). In addition, we are continuing to look for other G protein genes in *Arabidopsis*.

Flower morphogenesis is a complex developmental process. In recent years, *A. thaliana* has been increasingly used for plant molecular and genetic studies, and a number of *Arabidopsis* floral homeotic mutants have been characterized, including one called *agamous* (Bowman et al., *Plant Cell 1:* 37 [1989]). Wild-type *Arabidopsis* flowers have four whorls (sets) of organs. From the periphery to the center, there are four sepals, four petals, six stamens, and an ovary of two fused carpels. The *ag-1* mutant plants have flowers in which the sepals and petals are normal, but the stamens are replaced by petals and the ovary is replaced by another *ag* flower with sepals, petals, petals and another *ag* flower. Therefore, the *ag-1* flowers have only sepals and petals and no stamens or carpels. The *AGAMOUS* (*AG*) gene has been recently cloned (Yanofsky et al., *Nature 346:* 35 [1990]), and DNA sequence analysis indicates that it encodes a protein that shares striking similarity in its amino-terminal region with the DNA-binding domains of transcription factors from humans (SRF) and yeast (MCM1), suggesting that the AG protein is a transcription factor. This domain is

also found in another floral homeotic gene, *DEFA* from *Antirrhinum majus* (Sommer et al., *EMBO J. 9:* 605 [1990]), and has been referred to as the MADS-box, for *M*CM1-*A*G-*D*EFA-*S*RF (Schwarz-Sommer et al., *Science 250:* 931 [1990]). We reasoned that the process of flower development is likely to require more regulatory genes, some of which may also contain a MADS box. Using low-stringency hybridization strategies, we isolated six new genes, designated *AGL1* through *AGL6* for *AG-L*ike, which share substantial sequence similarity with *AG* in the DNA-binding motif as well as in additional amino acid sequences (the K box; see Ma et al., *Genes Dev. 5:* 484 [1991]). Five of the new genes are preferentially expressed in flowers, and one of them is also expressed in stems and leaves.

In the past year, we have begun the characterization of *GPA1* as well as *AG* and *AGLs*. In the following sections, we summarize the projects in these two areas and report some preliminary results. In addition, we describe a collaborative effort to establish an enhancer/gene trap transposon system in *A. thaliana*.

Analysis of *GPA1* Function Using Transgenic Plants

H. Huang, H. Ma

We have taken two approaches to probe the function of *GPA1*. Many G protein genes are known to be expressed in specific cell types. Thus, the first approach is to analyze the effect of altered *GPA1* expression. The second approach is to characterize the phenotypes of transgenic plants with potential dominant missense *gpa1* mutations (see below). Although *GPA1* is expressed in several organs (flowers, stems, and leaves), it is still possible the *GPA1* is expressed only in a subset of cell types in these organs. We are testing this directly by performing more detailed analyses of the *GPA1* expression pattern (see below), but we would also like to alter the expression of *GPA1* by putting it under the control of heterologous promoters, for example, the cauliflower mosaic virus (CaMV) 35S and heat-shock promoters, to obtain clues about its function. The constitutive CaMV 35S promoter will direct an increased *GPA1* mRNA level in many types of cells. This misexpression of the *GPA1* gene product may produce detectable phenotypes. To achieve inducible expression, we would like to use a heat-shock promoter (HSP). A number of constructs fusing the 35S and HSP promoters to a *GPA1* cDNA have been made.

Mutations are valuable for the analysis of in vivo functions of a gene. It is technically impractical in *Arabidopsis* to obtain chromosomal recessive mutations starting with a cloned gene. However, in the G protein α subunit, conserved residues exist that can be altered in vitro and may lead to dominant mutations in vivo. Using PCR procedures with mutant oligonucleotides and the *GPA1* cDNA, we have generated four missense mutations. These mutant *gpa1* alleles have now been fused to the 35S and HSP promoters, as well as to the native *GPA1* promoter.

The constructs for altered *GPA1* expression and for expressing *gpa1* mutant alleles have been introduced into the *Arabidopsis* genome by using an improved procedure (H. Huang and H. Ma, unpubl.) for *Agrobacterium*-mediated transformation. Multiple independent transformants were obtained for each construct. We are currently characterizing the transgenic lines. Although no gross visible phenotypes have been observed so far, we are following a few lines of investigation. We will compare the response of wild-type and transgenic plants to environmental signals. For example, the blue light response has been shown to require a G protein in pea. We will test the blue light response in the transgenic plants. Other responses are also being considered. We are also in the process of studying transgene expression using Northern and Western procedures. We are hopeful that some of these constructs will cause phenotypic changes in transgenic plants and will provide us with clues about *GPA1* functions.

In Search of New G Protein Genes

H. Huang, H. Ma

We believe that *Arabidopsis*, like most other characterized eukaryotes, has more than one gene coding for G protein α subunits. We have considered a few different approaches to isolate additional G protein genes. PCR was chosen as the major approach to isolate as diverse a group of sequences as possible. We have performed several PCR experiments starting from sterilely grown plants and have found a number of new G-protein-like sequences. However, most of these do not hybridize to *Arabidopsis* genomic sequences, suggesting that they are from non-*Arabidopsis* origin(s). We are also using low-

stringency hybridization to isolate sequences similar to *GPA1*.

Isolation and Analysis of the Tomato *GPA1* Homolog, *TGA1*

H. Ma, H. Huang [in collaboration with M. Yanofsky, University of California, San Diego]

The *Arabidopsis GPA1* gene was the first published plant gene encoding a G protein α subunit. In addition to amino acid sequence similarity to known G proteins, it also has some differences from other G proteins in the conserved consensus regions. We are interested in determining whether these amino acid changes are shared by other plant G proteins and thus may reflect functional properties of plant G proteins. Toward this end, we have isolated cDNAs for a gene encoding a G protein α subunit from tomato (*Lycopersicon esculentum*, cv VF36). This gene, named *TGA1*, was isolated using a cDNA of the *A. thaliana* G protein α-subunit-encoding gene, *GPA1*, as a DNA probe. The sequences of four cDNA clones indicate that the deduced amino acid sequence of the gene product (TGα1) has 384 amino acids (44,906 daltons). The predicted TGα1 protein exhibits similarity to all known G protein α subunits. The amino acid sequences are 84.6% identical and 93% similar (identical residues and conservative changes) to that of *A. thaliana* GPα1 and 34% identical and 59% similar to that of the mammalian transducins. Furthermore, it shares with GPα1 several noticeable changes in the consensus regions for a GTP-binding protein. Analysis of silent codon changes between *GPA1* and *TGA1* supports the conclusion that *GPA1* and *TGA1* are homologs. Finally, hybridizations of tomato genomic DNA indicate that *TGA1* is a single-copy gene.

Analysis of *GPA1* Expression Pattern

H. Huang, C. Weiss, H. Ma

As discussed above, it is of interest to characterize the time and spatial expression pattern of the *GPA1* gene. The expression of many G proteins is known to be regulated; for example, rod and cone transducins are expressed specifically in rod and cone photoreceptors, respectively, and the olfactory G protein is expressed only in the olfactory epithelium. The timing and location of *GPA1* expression may provide clues about its functions. We are taking a number of approaches to analyze the *GPA1* expression pattern. First, RNA dot-blot and Northern blot experiments have been and will be performed to determine *GPA1* expression in different organs at different developmental stages. Second, in situ RNA hybridization experiments will be done to analyze in more detail the spatial pattern of *GPA1* expression. Third, transgenic plants containing the *GPA1* promoter fused to a reporter gene, *GUS*, have been generated, and these will be analyzed at different stages both enzymatically and histochemically. Finally, when we obtain antisera against the GPα1 protein, we will examine the GPα1 protein distribution (see below).

Regulation of *GPA1* Expression

C. Weiss, R. Lavi, H. Ma

It is likely that *GPA1* is not expressed in all cell types at all times. In addition, *GPA1* expression may change in response to environmental signals. Furthermore, mutations affecting *Arabidopsis* signal responses may also alter *GPA1* expression. These possibilities will be investigated using techniques similar to those described above. RNA blots will also be made with RNA from plants grown under different environmental conditions, such as dark and light grown plants. When a particular response is found to affect *GPA1* expression, mutants in such a response will be analyzed for *GPA1* expression. Plants with *GPA1-GUS* fusions will be crossed to the mutants, and further analysis will be done using the progeny that have both the mutant and the *GUS* fusion.

To facilitate future use of the *GPA1* promoter, a limited number of *GUS* fusions will be constructed and analyzed in transgenic plants to identify a relatively small fragment that is sufficient to drive a wild-type level and pattern of expression. In addition, there is a potential regulatory function of two short open reading frames (ORFs) in the 5′ leader region of the *GPA1* cDNA. Short ORFs in the 5′ region are known to affect translation in yeast and, more recently, in maize cell culture. This possibility will be investigated by comparing protein levels to mRNA levels in different organs and at different times. Additional studies will be carried out using *GUS* translational fusions. Mutant constructs with one or both ATGs removed will be tested and compared to the wild-type construct.

Isolation and Analysis of Complete *AGL3* Genomic and cDNA Sequences

H. Ma, H. Huang, C. Weiss

AGL3 is unique among the seven reported *Arabidopsis* MADS-box genes (*AG* and *AGL1-AGL6*) in that it is expressed in both floral and vegetative tissues. To understand its function as a more general transcriptional regulator, we would like to further characterize *AGL3* at a molecular level. Using previously identified partial cDNA and genomic sequences, we have isolated complete *AGL3* cDNA clones, as well as the entire *AGL3* genomic sequence. These clones were subcloned and analyzed by DNA sequencing. Comparison of the genomic and cDNA sequences indicates that *AGL3* has eight exons and seven introns. The intron positions are conserved when compared to those of other *Arabidopsis* MADS-box genes. The predicted *AGL3* amino acid sequence shows that *AGL3* has 258 amino acid residues, with a calculated molecular weight of 29,684. In addition to the conserved MADS-box and K-box, *AGL3* has a glutamine-rich region (10 out of 11 residues) in the carboxy-terminal domain. Because glutamine-rich regions have been shown to be involved in transcriptional activation, the presence of a glutamine-rich region in *AGL3* suggests that *AGL3* may be directly involved in gene activation. Amino acid sequence comparison with other deduced MADS-box proteins indicates that *AGL3* is more similar to *AGL2* and *AGL4* than to any other MADS-box proteins; this conclusion is also supported by the exon-intron structures of these genes. The 5′ leader region of the *AGL3* cDNA has three additional ATGs, followed by 1, 1, and 13 sense codons, respectively, and then a stop codon. Northern blots with an *AGL3*-specific probe show that the *AGL3* mRNA is expressed in several tissues, including rosette leaf, cauline leaf, floral stem, pedicel, and pistil, but not detected in root or seedling cotyledon.

Expression of the *AGL* Genes in Wild-type and Mutant Flowers

H. Ma, C. Flanagan, A. Tagle [in collaboration with M. Yanofsky, University of California, San Diego]

Five of the *AGL* genes are expressed specifically in flowers. Our previous results indicate that *AGL1* is expressed in carpels. Furthermore, during stages 11 and 12 of flower development, *AGL1* expression is concentrated in the ovules. *AGL2* is also expressed in carpels, as well as in stamens. We have performed experiments to determine the *AGL* expression pattern in more detail. Using in situ hybridization analysis, we have found that *AGL1* is expressed in specific cells within the carpels. *AGL1* expression is concentrated in developing ovules and in the regions of the carpel walls where two carpels fuse to form the ovary. Additional in situ RNA hybridization experiments with *AGL4*- and *AGL5*-specific probes were carried out. Preliminary results indicate that *AGL5* is expressed in carpels, similar to *AGL1*. Further analysis will be done with *AGL1*, *AGL2*, *AGL4*, and *AGL5* probes. *AGL6* expression will also be analyzed.

We believe that the *Arabidopsis* MADS-box genes encode transcription factors that function in a regulatory hierarchy controlling flower development. To test this hypothesis, we have started a collaborative effort with Martin Yanofsky to analyze the effect of the *ag-1* mutation on the expression of *AG* and *AGL* genes. Northern analysis of wild-type and *ag-1* mutant floral RNA with *AG* and *AGL* probes indicates that *AG*, *AGL2*, *AGL4*, and *AGL6* are expressed in the *ag-1* mutant flowers, and *AGL1* and *AGL5* are not. We have begun a more detailed analysis of *AG* and *AGL2* expression in *ag-1* mutant flowers using in situ RNA hybridizations.

Functional Analysis of the AG Domains in Transgenic *Arabidopsis*

Y. Mizukami, H. Ma

The deduced AG protein has a region amino-terminal to the MADS-box; this amino-terminal region is much reduced in AGL1 and AGL5 and completely missing in the other AGL proteins and DEFA. To test the function of this region, *AG* cDNAs lacking part or all of it were constructed in vitro using PCR and fused to the CaMV 35S promoter. These constructs are now being introduced into the *Arabidopsis* genome using *Agrobacterium*-mediated transformation. Wild-type plants were used as the recipient of the recombinant constructs because of the difficulty of obtaining enough material for direct transformation into the sterile *ag* mutant plants. Our preliminary results show that the transgenic plants with the full-length *AG* cDNA have flowers similar to those in an *ap2* mutant. Previous genetic results have led to the

model that *AG* and *AP2* mutually antagonize each other (Bowman et al., *Development 112:* 1 [1991]). Because *AG* is likely a transcriptional regulator, it is plausible to propose that AG represses *AP2* expression. The *ap2*-like phenotype of the transgenic plants can then be explained by repression of *AP2* due to the constitutively expressed AG. This hypothesis can be tested in the future by examining *AP2* expression in the *AG* transgenic plants.

Transgenic plants containing constructs with part or all of the amino-terminal region deleted also have *ap2*-like flowers, indicating that the amino-terminal region is not required for repression of *AP2*. However, we do not know whether the amino-terminal region is required for other AG functions, such as carpel and stamen morphogenesis. Transgenic plants with either full-length or amino-terminal-deleted *AG* constructs will be crossed to the *ag* background to test whether these constructs can complement the *ag* defects.

Effects of Altered Expression of *AGL1* and *AGL2*

Y. Mizukami, H. Ma

AGL1 and *AGL2* are specifically expressed in flowers. As a part of the effort to determine the roles of *AGL1* and *AGL2*, we are studying the effects of sense and antisense *AGL1* and *AGL2* cDNAs, under the control of the CaMV 35S promoter, in transgenic plants. The *AGL1* and *AGL2* constructs have been made, and transformations to introduce them into the wild-type *Arabidopsis* genome are well under way. Our preliminary results show that an initial transformant with the *AGL1* sense construct has flowers with an elongated pistil, which extrude outside the floral bud prematurely. These same flowers also have stigmatic tissues at the tips of the sepals and shorter petals. The fact that the *AGL1* transgenic flowers have elongated carpels suggests that *AGL1* is indeed involved in carpel development. We have also obtained some preliminary results from analyzing transgenic plants carrying the *AGL1* antisense construct which show that the carpels have failed to fuse. This result is in agreement with the observation that *AGL1* is expressed in those regions of carpels where fusion occurs. Further analysis of these and other transformants will be performed to characterize in more detail the effects of these constructs. Northern and a limited number of in situ hybridization experiments will allow us to determine the expression patterns of the transgenes, related *AGL* genes, and known floral regulatory genes.

Establishing a Yeast In Vivo System for Functional Analysis of *AG*

C. Flanagan, A. Oates, H. Ma

The yeast *Saccharomyces cerevisiae* has a well-characterized MADS-box gene, *MCM1*. *MCM1* is required for cell-type-specific gene expression in yeast. We are setting up a system to test the ability of *AG* to replace *MCM1* functionally. Two assays will be used initially: the ability of *AG* to complement an *mcm1* mutant functionally and the ability of the AG protein to activate an MCM1 target gene. If *AG* cannot functionally replace MCM1, then chimeras of *AG* and *MCM1* will be constructed and tested in the above-mentioned tests. When a functional AG construct is obtained, it will be further analyzed using molecular genetics and biochemistry. This assay system will allow us to analyze the structure and function of *AG*, and its functional relationship to *MCM1*, a well-characterized protein. In particular, we can use this assay system to screen and characterize mutations in *AG* (deletions, directed point mutations, or random point mutations). Mutations of interest can then be introduced into and analyzed in plants.

Generation of Antisera against *AGL1, AGL2,* and *AGL3*

C. Flanagan, H. Ma

Although we have some preliminary results on the mRNA distribution of the *AGL* genes, we are more interested in the distribution of the AGL proteins. In addition, *AGL2* and *AGL3* both have short ORFs in the 5′ leader region of the cDNAs, suggesting that they might be translationally regulated. By comparing the mRNA levels to the protein levels, we may obtain evidence for such a regulation. Finally, a number of in vitro studies of proteins can be done using antibodies. Therefore, to characterize the tissue distribution of the AGL proteins and to analyze these proteins in vitro, we would like to generate antibodies against these proteins. Fusion constructs for expression in *Escherichia coli* have been made for *AGL1*,

AGL2, and *AGL3*, using the respective cDNAs. AGL1 and AGL3 fusion proteins have been purified from *E. coli* extracts and used to immunize rabbits. We are in the process of increasing the titers by boost injections and analyzing the antisera.

Toward Establishing an Enhancer/Gene Trap Transposon System in *A. thaliana*

H. Ma [in collaboration with V. Sundaresan and R. Martienssen, Cold Spring Harbor Laboratory]

In an effort to augment the available tools for molecular analysis of the plant *A. thaliana*, we have initiated experiments to establish an enhancer/gene trap transposon system, based on the maize *Ac/Ds* transposon system. Enhancer/gene trap systems have been used successfully to identify genes with interesting expression patterns and/or phenotypes in *Drosophila* and mice. We have started experiments to establish an enhancer/gene trap system in *Arabidopsis*, using the maize *Ac/Ds* transposon system. The constructs carrying either a stabilized active *Ac*, an enhancer trap *Ds*, or a gene trap *Ds* have been made by Rob Martienssen and V. Sundaresan here at Cold Spring Harbor Laboratory (see their reports), using plasmids kindly provided by Jonathan Jones (Sainsbury Laboratory) and Caroline Dean (Cambridge Laboratory) at Norwich, England. I have now introduced these constructs into wild-type *Arabidopsis* plants and have obtained seeds from several independent transformants for each of these constructs. We are in the process of testing the marker genes on the constructs, and we are preparing to test the transposition properties of the transgenic lines by crossing them to test lines with known active *Ac* and *Ds* elements.

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CELL CYCLE CONTROL IN *SACCHAROMYCES CEREVISIAE*

B. Futcher B. Elliott, I. Fitch, M. Linskens, L. Meng, R. Nash, G. Tokiwa, M. Tyers, C. Dahmann

Our main interest continues to be the regulation of START in *Saccharomyces cerevisiae*. We are most interested in the tethering of division to cell growth and in the generation of the basic cell cycle oscillation. The G_1 cyclin genes *CLN1*, *CLN2*, and *CLN3* encode unstable proteins that form a central part of the machines that sense growth and generate oscillations. In the past year, we have compared the properties of the Cln1, Cln2, and Cln3 proteins and have found large differences among them. We propose that there is a cascade of Cln-associated kinases such that Cln3 regulates Cln1 and Cln2, and

Cln1 and Cln2 regulate START, perhaps with help from other cyclins.

Our interest in G_1 cyclins has led us to examine the role of various other cyclins. We have partially characterized the roles of four B-type mitotic cyclins of *S. cerevisiae* and have found that they have specialized roles in both mitosis and meiosis.

Comparison of Cln1, Cln2, and Cln3

M. Tyers, G. Tokiwa

We tagged each Cln protein at its carboxyl terminus with a triple copy of a nine-amino-acid epitope derived from influenza virus. Stringent genetic tests showed that each tagged protein had essentially wild-type function. Each protein and its associated protein kinase activity (which was due to the Cdc28 protein kinase catalytic subunit) could then be immunoprecipitated with the 12CA5 monoclonal antibody, and the Cln proteins could be compared.

This type of experiment led to several interesting findings. First, the Cln1 and Cln2 proteins, although rare, were about 15-fold more abundant than Cln3 (Fig. 1), even though *cln3* mutations have stronger phenotypes than *cln1* or *cln2* mutations. Second, Cln1 and Cln2 had roughly 200-fold more histone H1 kinase activity associated with them than did Cln3

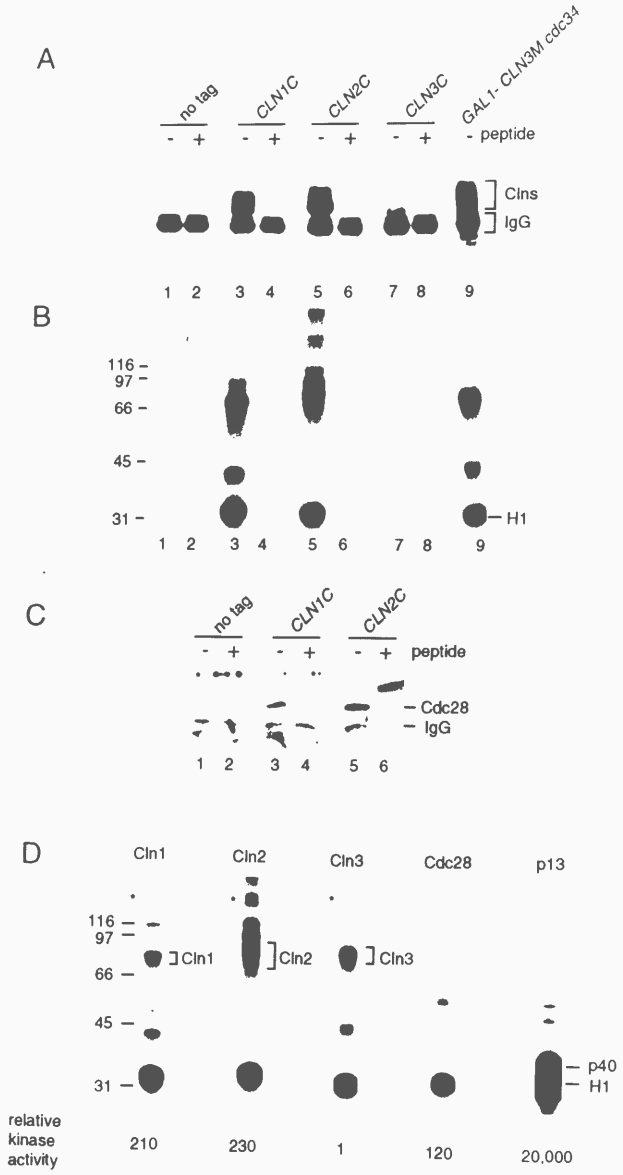

FIGURE 1 Comparison of Cln1, Cln2, and Cln3. (*A*) Cln3 abundance is much lower than that of Cln1 and Cln2. Each Cln was immunoprecipitated with the anti-envelope 12CA5 antibody in either the absence (–) or presence (+) of competing epitope peptide and then detected by Western blot with the same antibody. In lane *7*, a trace of Cln3 is visible just above the heavy-chain IgG band. In lane *9*, Cln3 is overexpressed from the *GAL1* promoter. (*B*) The Cln3-associated kinase activity is very weak. One quarter of the immunoprecipitated material used in *A* was used in histone H1 kinase assays. Various coprecipitated substrates were visualized in addition to exogenously added histone H1. (Lane *7*) Very weak activity associated with wild-type levels of Cln3; (lane *9*) 200-fold elevated levels associated with overexpressed Cln3 in a mutant deficient in a ubiquitin-conjugating enzyme. (*C*) Cdc28 is associated with Cln1 and Cln2. Cdc28 was detected by Western analysis in Cln1 and Cln2 immunoprecipitates. (*D*) Comparison of different Cdc28 kinase complexes. Kinase complexes were precipitated with 12CA5 antibody directed against tagged Cln1, Cln2, or Cln3 (first three lanes), or with a peptide antibody against Cdc28 (fourth lane), or with p13-Sepharose beads (fifth lane). Kinase reactions were exposed to X-ray film for different times to provide equal intensities for each reaction (i.e., in contrast to part *B*, the results shown are NOT a quantitative comparison). Each complex has a characteristic array of coprecipitated endogenous substrates. The relative amount of kinase activity in each immunoprecipitate is shown beneath each lane. The Cdc28 antibody was directed against the amino terminus of Cdc28 and probably only recognizes a subpopulation of all Cdc28 complexes. H1 is exogenously added histone H1; other bands are endogenous coprecipitated substrates.

(Fig. 1). Third, although Cln1 and Cln2 mRNA and protein levels oscillated through the cell cycle, we could find no evidence that any aspect of Cln3 function oscillated. Fourth, α-factor treatment, which arrests cells in G_1, knocked out Cln1- and Cln2-associated histone H1 kinase activities but had no detectable effect on Cln3 abundance or its associated histone H1 kinase activity; i.e., in an α-factor-treated cell, Cln3-associated histone H1 kinase activity was present at unaltered levels but was insufficient for START. This is a paradoxical result, because a *cln1 cln2* deletion strain is quite healthy, suggesting that cells can live on *CLN3* alone.

Because of these and other results, we have formulated a new model for control of START by the Cln proteins (Fig. 2). The main idea behind this model is that Cln3 does not and cannot activate START directly, simply because its associated kinase activity is too weak. Rather, Cln3 functions near the top of a cascade to activate the other Cln proteins, which in turn activate START. In this model, α-factor (or lack of the Swi4 and Swi6 transcription factors; Nasmyth and Dirick, *Cell 66:* 995 [1991]) causes G_1 arrest because it knocks out the activity of Cln1 and Cln2 (and Cln4); normal levels of Cln3 activity remain but are insufficient for START. The model also segregates different functions of cell cycle control to different Cln proteins. Cln3 (which does not oscillate) is most important for tethering division to growth, and Cln1 and Cln2 (and Cln4) are most important for generating oscillations.

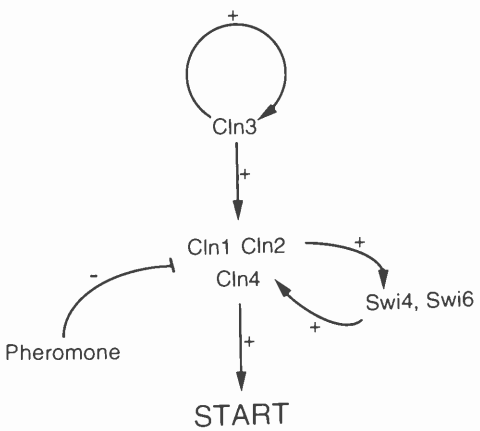

FIGURE 2 A model for activation of START. The weak Cln3-associated kinase activates Cln1 and Cln2 (and Cln4), which activate START. Cln3 may work directly by phosphorylating the Cln1 and 2 kinase complexes, or it may work indirectly. Cln1 and Cln2 are sensitive to α-factor and are part of a positive feedback loop involving the Swi4 and Swi6 transcription factors. Since *CLN3* is not an essential gene, there must be some additional mechanism for activating *CLN1* and *CLN2*; this unknown mechanism is not shown.

There are two difficulties with the model. First, it requires the existence of an additional gene, here named *CLN4*, to explain the fact that a *cln1 cln2* double deletion is viable. Second, since *cln3* deletions are viable, there must be some *CLN3*-independent method of activating Cln1 and Cln2.

CLN4: Fishing for Complements

L. Meng (URP Program), B. Futcher

As described above, we predict a fourth *CLN* gene. To find this hypothetical gene, we used a conditional *CLN* strain (*cln1 cln2 GAL-CLN3*) that is alive on galactose medium and dead on glucose medium because of a Cln deficiency. Into this strain, we transformed a high-copy-number yeast genomic library and selected for colonies on glucose plates. As expected, we re-cloned *CLN1*, *CLN2*, and *CLN3*. In addition, there were four independent clones of one new gene. This gene may be *CLN4*. Sequence analysis is under way.

The Signal for Cln3 Degradation

M. Linskens

The Cln3 protein is very unstable, with a half-life of about 10 minutes. However, a truncated version of the protein, Cln3-1, which makes cells small and α-factor-resistant, has a half-life of about 150 minutes. The stabilized, truncated version lacks the carboxy-terminal third of Cln3. This third contains regions extremely rich in proline, serine, and threonine. These have been called PEST regions and are thought on the basis of their presence in a variety of unstable proteins to be signals for proteolysis. Furthermore, the Cln1 and Cln2 proteins, which are also unstable, also have PEST regions.

To determine whether the PEST regions are important for the instability of Cln3 and whether protein stability causes the Cln3-1 mutant phenotype, we made a set of nested deletions from the 3′ end of the gene. Almost all of the PEST regions could be removed without any phenotypic effect. We are in the process of examining protein stability. The change from the Cln3 phenotype to the Cln3-1 mutant

phenotype occurs quite suddenly when an extra 14 amino acids are removed from a shortened but phenotypically wild-type protein. This region contains PEST residues. However, it also contains a perfect consensus site for cAMP-dependent protein kinase phosphorylation. We are making site-directed mutations in the critical region to determine whether it is the PEST region or the phosphorylation site that is important for the Cln3-1 phenotype.

Characterization of Four Mitotic Cyclins of *S. cerevisiae*

I. Fitch, C. Dahmann [in collaboration with U. Surana and K. Nasmyth, IMP, Vienna]

Last year, we identified four B-type cyclins in *S. cerevisiae*; in other organisms, B-type cyclins have been involved in mitosis. This year, the genes were partially characterized. None of the single *clb* deletions is lethal. All possible combinations of *clb* deletions have been constructed (Table 1). Genetically, *CLB2* is the most important gene. Clb1 and Clb2 form a pair of closely related homologs, whereas Clb3 and Clb4 form a second such pair; however, the functional significance of this is not clear, since the *clb3 clb4* double deletion has little if any phenotype. We do not know whether there are additional B-type cyclins.

All four transcripts oscillate in abundance (Fig. 3). *CLB1* and *CLB2* are expressed during a fairly narrow window around the time of nuclear division, whereas *CLB3* and *CLB4* start being expressed in S phase and continue until the end of nuclear division.

A *clb1 clb2 clb3 clb4 GAL-CLB2* strain has been constructed. This strain is alive on galactose but dead on glucose because of a *clb* deficiency. Preliminary analysis suggests that cells arrest with buds, and with a 2N DNA content (i.e., after S phase), but without any mitotic spindle. At face value, this suggests that the *CLB*s are needed for spindle formation but not for any earlier event. In *S. cerevisiae*, the mitotic spindle starts to form at about the beginning of S phase, perhaps explaining why *CLB3* and *CLB4* are expressed so early. As expected, then, these B-type cyclins seem to be mitotic cyclins.

TABLE 1 Viability of *clb* Mutants

Spore clones				
Are **Viable**				
	clb1			
		clb2		
			clb3	
				clb4
	clb1		*clb3*	*clb4*
	clb1	*clb2*		*clb4*
			clb3	*clb4*
	clb1		*clb3*	*clb4*
Eventually form **Microcolonies**				
	clb1	*clb2*		
		clb2	*clb3*	
	clb1	*clb2*		*clb4*
Are **Inviable**				
	clb1	*clb2*	*clb3*	
		clb2	*clb3*	*clb4*
	clb1	*clb2*	*clb3*	*clb4*

Each *CLB* gene was deleted and replaced with a selectable marker. Double mutants were made by crossing single mutants, and so on. Mutant combinations scored as "Viable" grew at least moderately well, although in some cases with morphological defects. Combination scored as forming "Microcolonies" varied from combinations that caused death after a few cell divisions to combinations that produced visible microcolonies after prolonged incubation. Combinations scored as "Inviable" produced spores that germinated but did not go through even one cell division.

Meiotic Defects of *clb* Mutants

C. Dahmann

Meiotic divisions, like mitotic divisions, are likely to require cyclins. Perhaps one or more of the four known B-type cyclins are specialized for meiotic functions. We have therefore tested all *clb* single mutants and all viable combinations of multiple homozygous *clb* mutants for their ability to undergo meiosis.

Quantitative and qualitative effects were seen. Most of the *clb* mutations did not significantly affect the efficiency of sporulation, but for the *clb1 clb3 clb4* homozygous triple mutant, the frequency of ascus formation dropped to about 20% of the wild-type level. Many lysed cells appeared in this culture, which may have been cells that lysed while attempting sporulation. Most strikingly, however, several combinations of mutants caused meiosis to yield two viable diploid spores, instead of the normal four haploid spores (Fig. 4). A *clb1 clb4* strain with three centromere-linked markers was constructed, and the genotypes of the resulting dyads showed that meiosis I had been completed and that meiosis II had not. We think the most likely interpretation is that the mutants exit the program of meiotic cell division after just one

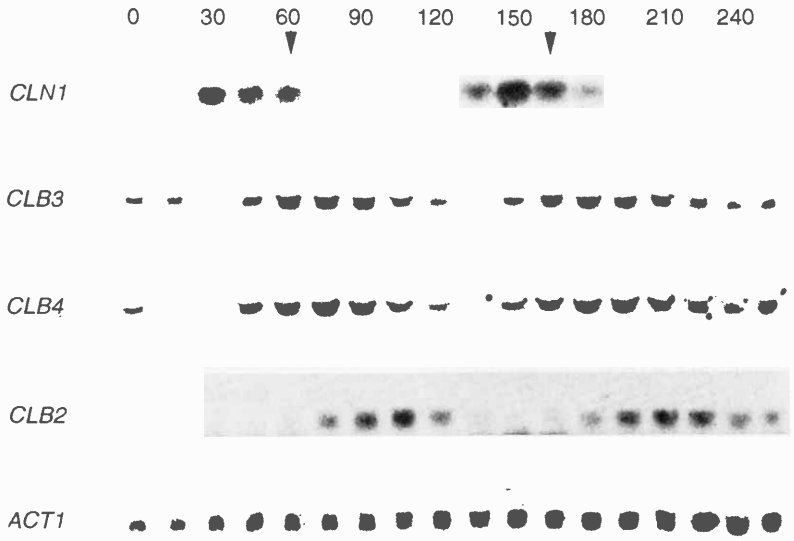

FIGURE 3 *CLB* expression is cell-cycle-regulated. Cells were arrested in late anaphase using a temperature-sensitive *cdc15* mutation. Cells were synchronously released from the block at 0 time, and samples were taken for 255 minutes. A Northern blot of the extracted RNA was probed sequentially with *CLN1*, the *CLB*s, and the actin gene (as a loading control). The arrows show the appearance of new buds, which in these cells occurs at S phase. *CLN1* is expressed in G_1 phase, *CLB3* and *CLB4* appear to be expressed from early S phase until the end of nuclear division, and *CLB1* (not shown) and *CLB2* are expressed only around the time of nuclear division.

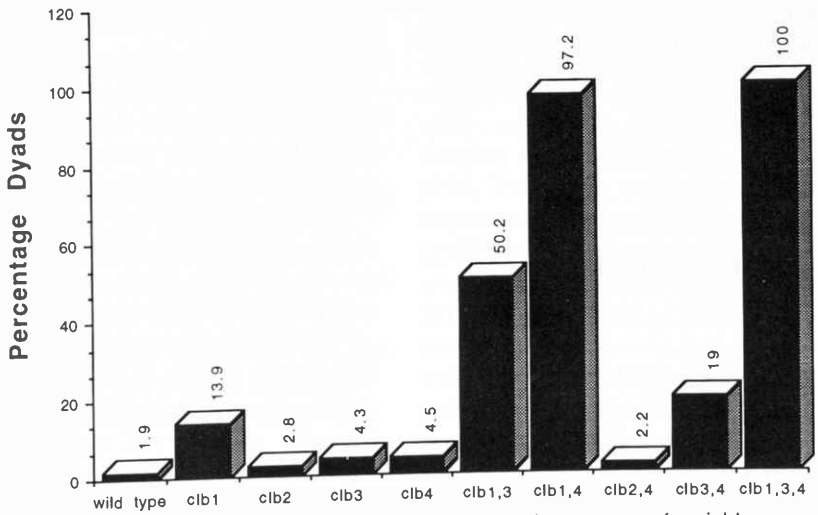

FIGURE 4 A Meiotic defect in *clb* mutants. Diploids homozygous for viable combinations of *clb* mutations (see Table 1) were sporulated. The ratio of dyads (two spored asci) to total asci (mainly four spored asci) is shown. The dyads are diploid and viable and apparently arise because meiosis II does not occur. Even in the *clb1 clb4* double mutant that gives over 95% dyads, the rare tetrads seem to be entirely normal.

division, instead of after two. Thus, *CLB1*, *CLB3*, and *CLB4* help to regulate meiosis.

WHI3, a New Regulator of START

R. Nash

Like *CLN3-1*, *whi3* causes cells to divide at abnormally small sizes. Unlike *CLN3-1*, *whi3* is recessive, suggesting that the wild-type *WHI3* gene inhibits or delays commitment to division. Genetic analysis suggests that *WHI3* acts independently of the *CLN* pathway. Sequencing showed that *WHI3* includes a domain found in a family of RNA-binding proteins.

Whereas the null mutation causes a small cell size, multiple copies of *WHI3* cause a large cell size, and the size is correlated with the number of copies. Thus, *WHI3* is a dose-dependent inhibitor of START. When induced, a *GAL-WHI3* construct causes a lethal, first-cycle, G_1 START arrest. This is the same phenotype as caused by *cdc28* temperature-sensitive mutations or by *cln* deficiency.

Although *WHI3* seems to act independently of the *CLN*s, it shows at least one striking interaction. A *CLN3-1 whi3* double mutant is nearly sterile and is largely defective for α-factor-induced transcription, whereas the two parental single mutants are nearly normal for both responses.

In summary, *WHI3* seems to work at START as a dose-dependent inhibitor, but it may not work via the *CLN/CDC28* pathway. It may define an independent requirement for START.

Stress Resistance in *S. cerevisiae*

B. Elliott

The RAS/cAMP pathway can regulate cell physiology in response to nutritional cues. However, when this signaling pathway is destroyed, cells still respond appropriately to nutritional cues and acquire stress resistance in stationary phase (Cameron et al., *Cell 53:* 555 [1988]). Thus, there is at least one signaling pathway in addition to the RAS/cAMP pathway. We have found two mutations that prevent the cAMP-independent response. The genes have been cloned, and sequence analysis is under way.

It has been thought that the stress resistance of stationary-phase cells requires that the cells be in a special G_1 noncycling state that has been compared to mammalian G_0. However, we found that slowly growing cells also acquired stress resistance, although not quite as much resistance as stationary-phase cells. Furthermore, cells in S, G_2, or M phase were about as resistant as cells in G_1 phase, suggesting that resistance did not depend on cell cycle phase. We think that although stress resistance and stationary phase are correlated in yeast, neither depends on the other.

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TRANSCRIPTION AND CELL CYCLE REGULATION IN YEAST

K.T. Arndt C. Devlin C. DiComo A. Doseff M.J. Fernandez-Sarabia F. Lin M. Luke A. Sutton T. Zhong

We are using the yeast *Saccharomyces cerevisiae* to investigate two basic cellular processes: regulation of transcription by RNA polymerase II and regulation of commitment for entry into the cell cycle.

Genetic Selection Schemes for General Transcription Factors

C. Devlin

In the yeast *Saccharomyces cerevisiae*, three *trans*-acting proteins, BAS1, BAS2, and GCN4, bind to the *HIS4* promoter to activate transcription of the *HIS4* gene. A strain containing deletions of *BAS1*, *BAS2*, and *GCN4* has a His^- phenotype due to insufficient transcription of *HIS4*. An additional protein, RAP1, also binds to the *HIS4* promoter but is not by itself able to activate transcription of *HIS4*. At *HIS4*, RAP1 functions to phase nucleosomes away from the GCN4-, BAS1-, and BAS2-binding sites so that these sites are accessible when present within chromatin. Our research objectives with *HIS4* can be divided into two main areas: One area studies the details of how BAS1, BAS2, GCN4, and RAP1 function for activation of *HIS4* transcription, and the second area of research uses the *HIS4* promoter for genetic selection schemes to isolate mutations in genes whose products function in transcription. To isolate mutations in genes encoding general transcription factors, we previously isolated His^+ revertants of a strain containing deletions of *BAS1*, *BAS2*, and *GCN4*. These studies generated the *sit1* through *sit5* mutations. So far, the *sit* mutations have altered either general transcription factors (*sit1* or *sit2*), nucleosomes (*sit5* and possibly *sit3*), or a factor (*sit4*) that may regulate a general transcription factor. Since the selection scheme is performing as expected, we isolated further *sit* mutations to identify other factors involved in transcription. Most of these new *sit* mutations were in the previously identified *sit1* through *sit5* complementation groups. From a large-scale selection, we have identified seven new *sit* complementation groups: *sit6* through *sit12*. Like *sit1* through *sit5*, the new *sit* mutations cause a strong growth defect and therefore occur in genes required for normal cellular growth. We are further analyzing these additional *sit* mutations for their global transcriptional defects and are in the process of cloning the corresponding wild-type genes.

Role of the SIT4 Protein Phosphatase in the Cell Cycle

M.J. Fernandez-Sarabia, A. Sutton

The *SIT4* gene predicts a protein that is 55% identical to the mammalian type-2A and 43% identical to the mammalian type-1 protein phosphatase catalytic subunits. However, the SIT4 protein phosphatase is a unique phosphatase that is distinct from the *S. cerevisiae* type-1 and type-2A phosphatases. The SIT4 protein phosphatase (PPase) is required in late G_1 (at or very close to the time CDC28 is required) for passage through START. SIT4 coimmunoprecipitates with two high-molecular-weight proteins, termed p155 and p190. The association of SIT4 with p155 and p190 is cell-cycle-dependent. In G_1 daughter cells, SIT4 is not associated with p155 and p190. Very close to the G_1/S phase transition, SIT4 associates (in separate complexes) with p155 and p190. SIT4 remains associated with p155 and p190 until about the middle or end of mitosis. Therefore, SIT4 associates with p155 and p190 very close to the time that SIT4 executes its function. For this reason, we postulate that the p155 and p190 proteins are probably regulatory subunits of SIT4 that physically target SIT4 to the proper substrates or regulate the activity and/or the substrate specificity of SIT4.

Both p155 and p190 are phosphorylated in vivo. In contrast, SIT4 is not detectably phosphorylated in vivo. Interestingly, SIT4 coimmunoprecipitates with a kinase activity that phosphorylates p155 and p190 in vitro. Therefore, the phosphorylation state of p155 and p190 may regulate their association with SIT4.

Since SIT4 is required for transcription of *CLN1* and *CLN2* (which encode G_1 cyclins; see below) and may in fact regulate this process, the regulation of SIT4 by p155 and p190 would be of central importance for the control of START. To investigate p155 and p190 directly, we need to isolate their corresponding genes. We hope to isolate these genes within the next year.

During the last year, we have been focusing on the G_1 role of SIT4. This research indicates that SIT4 is required for at least two processes: (1) transcription of the G_1 cyclins *CLN1*, *CLN2*, and *HCS26* and (2) some other essential function(s).

TRANSCRIPTION OF *CLN1*, *CLN2*, AND *HCS26*

When a *sit4-102 ssd1-d* strain (*sit4-102* is a temperature-sensitive allele of *SIT4*; see below for an explanation of *SSD1*) is arrested in G_1 after 4 hours at the nonpermissive temperature, the levels of *CLN1*, *CLN2*, and *HCS26* RNAs are much lower than in wild-type G_1 cells. In contrast, the levels of *CLN3* and other control RNAs are similar in G_1 cells and *sit4-102*-arrested cells. To examine the requirement of SIT4 for transcription of *CLN1*, *CLN2*, and *HCS26* more closely, we looked at the levels of the *CLN* transcripts after α-factor release. When *MAT***a** cells are treated with α-factor, the levels of *CLN1* and *CLN2* RNAs are repressed, whereas the levels of *CLN3* RNA are slightly induced. After release from the α-factor arrest, the levels of *CLN1*, *CLN2*, and *HCS26* RNAs increase dramatically. We investigated the requirement of SIT4 for this increase using two different *sit4* mutant strains: a *sit4-102 ssd1-d1* strain arrested with α-factor at 24°C and then shifted to 37.5°C after α-factor release or a Δ*sit4 SSD1-v1* strain arrested with α-factor at 30°C and maintained at 30°C after α-factor release. Each of these strains was compared to an isogenic wild-type *SIT4* strain. Both sets of experiments show that SIT4 is required for the normal increase in the levels of *CLN1*, *CLN2*, and *HCS26* RNAs when the cells are released from α-factor arrest. In contrast, the levels of *CLN3* and other control RNAs do not require SIT4. These experiments also show that SIT4 function is required not only for the normal accumulation of *CLN1*, *CLN2*, and *HSP26* RNAs, but also for the normal disappearance of *CLN1* and *CLN2* RNAs (but not *HCS26* RNA).

In support of the Northern data, Δ*sit4 SSD1-v1* cells are sensitive to partial loss of CLN activity, such as the absence of one of the *CLN* genes. Normally, wild-type *SIT4* cells that contain a single deletion of either *CLN1*, *CLN2*, or *CLN3* have a doubling time that is the same as, or very close to, that of wild-type cells. In contrast, Δ*sit4 SSD1-v1* Δ*cln2*::*TRP1* strains grow *much* slower than Δ*sit4 SSD1-v1* strains. In addition, Δ*sit4 SSD1-v1* Δ*cln3*::*URA3* strains are almost inviable. The gene interactions between *SIT4* and *CLN3* provide further support for the model (Fig. 1) that *SIT4* is required for normal transcription of *CLN1* and *CLN2*. Normally, wild-type *SIT4* cells can survive with only one of the three *CLN* genes. However, at least one of the three *CLN* genes is required for viability. In the absence of SIT4 (a Δ*sit4 SSD1-v1* strain), the levels of *CLN1* and *CLN2* RNAs are much lower than normal. Therefore, the model predicts that Δ*sit4 SSD1-v1* cells would be more dependent on the function of *CLN3*, whose transcription does not depend on *SIT4*. That Δ*sit4 SSD1-v1* Δ*cln3*::*URA3* cells are almost inviable supports the model (Fig. 1). A further prediction of the model is that if the *CLN2* open reading frame is transcribed from a promoter that does not depend on *SIT4*, then *CLN3* should be dispensable in a Δ*sit4 SSD1-v1* strain. The experiments show that the requirement of a Δ*sit4 SSD1-v1* strain for *CLN3* is completely eliminated if *CLN2* is provided from a promoter that is not *SIT4*-dependent. Additional experiments show that SIT4 and CLN3 provide parallel pathways for transcription of *CLN1*, *CLN2*, and *HCS26*. The requirement of *SIT4* for transcription of *CLN1*, *CLN2*, and *HCS26* may be via SWI4. The *SWI4* and *SWI6* genes encode proteins that probably function as direct DNA-binding factors for *CLN1*, *CLN2*, and *HCS26* (but not *CLN3*) transcription. The

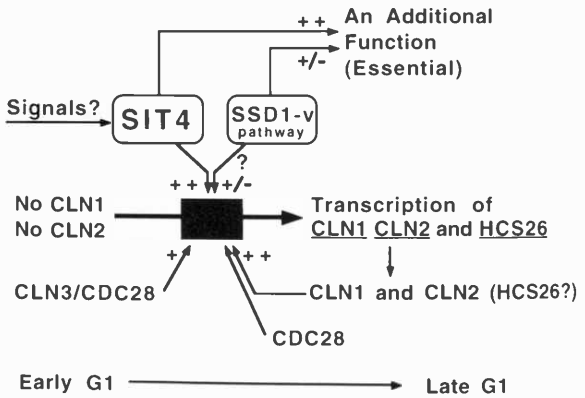

FIGURE 1 Model for the role of *SIT4* in late G_1.

levels of *SWI4* RNA (but not *SWI6* RNA) are cell-cycle-dependent and reach their maximal levels just prior to that of *CLN1*, *CLN2*, and *HCS26* RNAs. Northern analysis shows that the increases in the levels of *SWI4* RNA are *SIT4*-dependent, whereas the levels of *SWI6* RNA are not *SIT4*-dependent. Therefore, the requirement of SIT4 for normal transcription of *CLN1*, *CLN2* and *HCS26* could be due to the requirement of *SIT4* for transcription of *SWI4*.

OTHER ESSENTIAL FUNCTIONS

In addition to the role of *SIT4* for transcription of *CLN1*, *CLN2*, and *HCS26*, the *SIT4* and *SSD1-v* genes together provide an essential function in late G_1. Much evidence indicates that an additional role of SIT4 is for turning *off* the CLN2-associated kinase (and probably the CLN1-associated kinase). Strains containing *sit4* mutations (both *sit4-102 ssd1-d* strains at the nonpermissive temperature and Δ*sit4 SSD1-v1* strains at any temperature) have slower mobility forms of CLN2 on SDS-PAGE gels. These slower mobility forms of CLN2 are probably hyperphosphorylated CLN2, because these forms are eliminated if the CLN2 immunoprecipitates are treated with potato acid or alkaline phosphatase. Interestingly, the CLN2 isolated from *sit4* mutants has a very high associated kinase activity. Presumably, the very high kinase activity is due to the hyperphosphorylation of CLN2. In vitro, the SIT4 PPase not only can dephosphorylate CLN2, but SIT4 actually causes the disappearance of CLN2 from the CLN2 immunoprecipitates (as assayed by Western analysis). Presently, we do not know if the CLN2 protease activity comes from the added SIT4 or if SIT4 activates a protease that coimmunoprecipitates with CLN2. Our results raise the possibility that SIT4 controls two aspects of CLN2 function. First, SIT4 could regulate the rate of increase of *CLN2* RNA and control the timing of START. Second, SIT4 could regulate the inactivation of the CLN2 kinase. The cells may require inactivation of the CLN2 kinase for normal cell cycle progression, and this process could correspond to a late G_1 checkpoint. Since SIT4/p155 and SIT4/p190 most likely regulate different processes, future experiments with SIT4 will require reagents so that the specific functions of SIT4/p155 and SIT4/p190 can be separated and identified. One form of SIT4 could regulate transcription of *CLN2* and the other form of SIT4 could regulate inactivation of the CLN2 kinase.

SSD1: A Polymorphic Locus Where Some Alleles Can Allow Strains to Grow in the Absence of *SIT4*

A. Sutton

In certain strain backgrounds, deletion of *SIT4* is lethal, and temperature-sensitive *sit4* strains (such as *sit4-102*) arrest in late G_1. However, in other strain backgrounds, deletion of *SIT4* results in viability, but with very slow growth. In addition, in the strain backgrounds where deletion of *SIT4* is viable, the *sit4-102* mutation does not result in a temperature-sensitive phenotype. The viability or inviability of a strain containing a deletion of *SIT4* is due to a single unlinked locus, termed *SSD1*. *SSD1* alleles that result in slow growth but viability in combination with a deletion of *SIT4* are called *SSD1-v* alleles (v for *v*iable). *SSD1* alleles that result in lethality in combination with a deletion of *SIT4* are called *ssd1-d* alleles (d for *d*ead). In a survey of laboratory strains, we found that about half of the laboratory strains have an *ssd1-d* allele, and the other half of the laboratory strains have an *SSD1-v* allele. Deletion of *SSD1* by itself results in only subtle phenotypic effects, such as a slight effect on cell size and increased sensitivity to caffeine.

The effect of *SSD1* on *sit4* mutations suggests two possibilities: (1) Either the SSD1-v pathway can partially substitute for the essential functions of the SIT4 phosphatase or (2) SSD1-v functions downstream from SIT4. In the second case, SSD1-v provides some function so that the cells can survive without the proper regulation of some substrate's phosphorylation state by SIT4. Presently, we cannot distinguish between these two possibilities. Many lines of evidence implicate *SSD1* in G_1 regulation. First, as described above, *SSD1* is a modifier of *sit4* mutations that are known to function in late G_1. Second, deletion of *SSD1* alters the arrest morphology of temperature-sensitive *cdc28* strains that arrest in late G_1. Third, disruption of the *BCY1* gene (which encodes the regulatory subunit of the cAMP-dependent protein kinases) in an *ssd1-d* background results in heat-shock sensitivity, sensitivity to nutrient limitation, and temperature-sensitive growth. Both our laboratory and Kelly Tachell's laboratory (who independently isolated *SSD1-v* as a suppressor of a *pde2* cAMP phosphodiesterase mutation) found that the *SSD1-v* gene will partially suppress these three phenotypic defects due to disruption of *BCY1* in a *ssd1* background. Fourth, Kim Nasmyth's laboratory

found that *SSD1-v* is required in cells that have only one of the three *CLN* genes, which encode G_1 cyclins. Since *SIT4* is required for *SWI4/SWI6*-dependent transcription of *CLN1*, *CLN2*, and *HCS26*, these results suggest that *SSD1-v* may provide a SIT4-independent route for transcription of the *CLNS*. Alternatively, *SSD1-v* may function downstream and allow the cells to enter the cell cycle at lower than normal levels of *CLN* function.

The DNA sequence of the *SSD1-v1* gene predicts a protein of 1250 amino acids and a molecular mass of 140 kD. On an SDS-polyacrylamide gel, the SSD1 protein has a mobility of about 180 kD. The SSD1 protein has significant similarity to the *S. pombe dis3* gene isolated in M. Yanagida's laboratory. The conditional *dis3* strain arrests very similarly to the conditional *dis2* strain. Interestingly, the dis2 protein encodes the catalytic subunit of a type-1 phosphatase. In addition, mutations in *dis2* and *dis3* interact (a *dis2 dis3* strain is inviable). Therefore, the homologous dis3 and SSD1 proteins may function in a similar fashion relative to the catalytic subunits of type 1/type 2-related protein phosphatases (dis2 and SIT4, respectively). Unfortunately, the activities provided by dis3 and SSD1 are not known.

To date, we have not been able to detect phosphatase activity in SSD1-v immunoprecipitates. However, such an activity may be very weak. The levels of *SSD1* transcript and SSD1 protein are very low. In fact, expression of the *SSD1-v1*-coding sequences from the *GAL10* promoter in cells grown on glucose medium (which represses the *GAL10* promoter) provides complete *SSD1-v* function and gives transcript levels equivalent to those from the normal *SSD1-v1* gene. Further analysis will be required to understand the function of the *SSD1-v* gene.

A Genetic Search for the SIT4 Regulatory Subunits

A. Doseff

In an otherwise wild-type cell, the *SSD1* gene is dispensable. Cells containing a deletion of *SSD1* have a growth rate that is very similar to that of wild-type cells. However, in cells containing a deletion of *SSD1*, the *SIT4* gene is essential. Put another way, Δ*sit4* strains require *SSD1-v*. Therefore, to obtain mutations in genes functioning in the *SIT4* pathway, we isolated mutants that require the *SSD1-v1* gene for viability. We are most interested in obtaining the genes encoding p190 and p155, two proteins that coimmunoprecipitate with SIT4.

From a screen of 160,000 colonies resulting from EMS-mutagenized cells, we obtained about 80 mutants that are inviable or extremely slow growing in the absence of *SSD1-v1*. As expected, some of these mutants contain a mutation in the *SIT4* gene itself. The other non-*SIT4* mutants were placed into complementation groups. This analysis shows two main complementation groups and possibly six additional complementation groups. We are using further genetic analysis to demonstrate that these mutations are in the *SIT4* pathway. Meanwhile, we are cloning the genes for the two main complementation groups. Hopefully, this analysis will provide us with every gene whose product is required for the *SIT4* pathway. The identification of the genes for p155 and p190 will greatly aid in the biochemical analysis of SIT4.

The SIS2 Protein: A Component of the SSD1 Pathway?

C. DiComo

A Δ*sit4 SSD1-v* strain is surviving via the SSD1-v pathway and has a slow-growth defect. The wild-type *SIS2* gene in high copy number dramatically stimulates the growth rate of this strain. This result raises two possibilities: (1) overexpression of the SIS2 protein stimulates the *SSD1-v* pathway or (2) SIS2 functions downstream from SIT4 but that overexpression of SIS2 compensates for the lack of the normal SIT4-dependent regulation of some substrate's phosphorylation state. We favor the first possibility for a number of reasons. First, like *SSD1*, the *SIS2* gene is not essential. Deletion of *SIS2* causes only subtle phenotypic alterations. Second, deletion of both *SSD1* and *SIS2* causes no additional phenotypic effects. Such a result would be expected from genes functioning in the same pathway. If deletion of *SSD1* causes a nonfunctional pathway, then the additional deletion of *SIS2* should cause no additional effect. Third, deletion of *SIS2* (like *ssd1-d* alleles or deletion of *SSD1*) is lethal in combination with deletion of *SIT4*. Put another way, a Δ*sit4* strain is surviving via the *SSD1-v* pathway and the function of this *SSD1-v* pathway requires *SIS2*. To further support the contention that *SIS2* and *SSD1-v* function in the same pathway, we are determining if deletion of *SIS2* causes an effect similar to that of *ssd1-d* alleles or deletion of

SSD1 for every gene and phenotype for which *SSD1* is known to affect.

Like *SSD1*, the *SIS2* gene is expressed at very low levels. The *SIS2* gene predicts a protein with a molecular mass of 64 kD. The predicted SIS2 protein has a very unusual carboxyl terminus. Within a sequence of 58 amino acid residues, 53 of these residues are glutamate or aspartate. Therefore, the carboxyl terminus of SIS2 is extremely acidic. The remainder of the SIS2 protein does not have significant homology with any sequences in the current databases. In our ongoing biochemical and genetic analyses of *SIS2*, we will determine (1) if SIS2 and SSD1-v coimmunoprecipitate, (2) if SIS2 immunoprecipitates have phosphatase activity, and (3) the biochemical defects of strains containing deletions of *SIS2*.

The SIS1 Protein: An Essential dnaJ Homolog

M. Luke, T. Zhong

The wild-type *SIS1* gene in high copy number also dramatically stimulates the growth rate of a Δ*sit4 SSD1-v* strain. The predicted SIS1 protein is similar to bacterial dnaJ homologs in the amino-terminal third and carboxy-terminal third of the proteins. Bacterial dnaJ proteins are required for bacteriophage λ, P1 phage, and host DNA replication. Although it is believed that dnaJ mediates protein/protein dissociation and is also involved in proteolysis, the precise cellular function provided by dnaJ is not known. The middle third of SIS1 is not similar to dnaJ proteins. This middle third region of SIS1 contains a glycine/methionine-rich region that is required for SIS1 to associate with a protein with an apparent molecular mass of 40 kD. The SIS1/p40 complex elutes as a very large-sized complex (in the exclusion volume of a Sepharose CL-6B column). This complex appears to contain many p40 molecules for each SIS1 molecule. We have developed a rapid method to purify p40. Initial peptide sequence analysis indicates that p40 does not correspond to any proteins in the current databases.

The *SIS1* gene is essential. Strains limited for the SIS1 protein accumulate cells that are blocked for migration of the nucleus from the mother cell into the daughter cell. Certain mutations in SIS1 increase, and other mutations in SIS1 decrease, the stability of a centromere plasmid. The nuclear migration block and the plasmid stability effects may be a result of the same defective process in the *sis1* mutants. The SIS1 protein is localized throughout the cell but is more concentrated at the nucleus. The daughter cells do not stain for SIS1 protein until the nucleus is transferred into the bud. Treatment of a yeast nuclear fraction (which contains most of the SIS1 protein) with RNase releases about one fourth of the SIS1 protein. Treatment with DNase does not release SIS1.

We currently do not know in what cellular process(es) SIS1 functions. In addition, we do not know if the essential function of SIS1 is the function that stimulates the growth rate of a Δ*sit4 SSD1-v1* strain. However, our primary objective is to determine what function SIS1 provides to stimulate the growth rate of Δ*sit4 SSD1-v1* strains. In vivo and in vitro experiments are in progress to determine this function.

The Role of Type-2A Protein Phosphatases in the *S. cerevisiae* Cell Cycle

F. Lin

S. cerevisiae has two genes, termed *PPH2*α and *PPH2*β, that encode good homologs (about 80% identity) of the catalytic subunit of mammalian type-2A protein phosphatases. The predicted proteins, PPH2α and PPH2β, have amino-terminal extensions not seen for other type-2A PPases. Although these amino-terminal extensions are different for PPH2α and PPH2β, the remainder of the proteins are very similar (98% identity over 310 amino acids). Strains containing a deletion of *PPH2*α or *PPH2*β have only subtle growth defects. However, deletion of both *PPH2*α and *PPH2*β results in viability but with a slow-growth phenotype. A third gene, termed *PPH3*, was isolated by Hans Ronne and encodes a predicted protein that is about 60% identical to the mammalian type-2A catalytic subunit. Deletion of *PPH3* by itself causes no readily detectable phenotypic alterations. However, deletion of *PPH2*α, *PPH2*β, and *PPH3* results in lethality. Therefore, PPH3 can partially do some of the functions that PPH2α and PPH2β primarily perform.

One of our goals is to determine the role(s) of the *S. cerevisiae* type-2A phosphatases in the cell cycle. This analysis is greatly simplified with strains containing temperature-sensitive mutations in *PPH2*α or

*PPH2*β. Since we had a very good temperature-sensitive mutation in *SIT4* (i.e., *sit4-102*) and since the residue altered in *sit4-102* is conserved between SIT4 and all known type-2A PPases, we prepared mutant *PPH2*α and *PPH2*β genes that alter this residue. We term these alleles *pph2*α*-102* and *pph2*β*-102*. A Δ*pph2*α Δ*pph2*β Δ*pph3* strain containing either *pph2*α*-102* or *pph2*β*-102* is temperature-sensitive for growth. When an asynchronous culture of either strain is shifted to the nonpermissive temperature, greater than 90% of the cells arrest with a 2n DNA content. The majority of the cells contain a single nucleus with a *single* microtubule-organizing center. These results suggest that type-2A phosphatases are required for spindle pole body duplication or separation, but not for DNA synthesis. We are further characterizing the requirements of type-2A PPases for the *S. cerevisiae* cell cycle.

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INITIATION AND TERMINATION OF TRANSCRIPTION IN HUMAN SnRNA GENES AND HIV-1

N. Hernandez S. Lobo, J. Moreno, F. Pessler, R. Ratnasabapathy, C. Sadowski, M. Sheldon, M.L. Sullivan

SMALL NUCLEAR RNA GENES

Small nuclear RNA (snRNA) genes are members of a family of genes that we refer to as the processor RNA (or pRNA) gene family. pRNA genes encode RNAs involved in the processing of other RNA molecules. In addition, these genes share common promoter elements, even though some, such as the U1 and U2 genes, are transcribed by RNA polymerase II, whereas others, such as the U6 gene, are transcribed by RNA polymerase III. Thus, both the RNA polymerase II and III snRNA promoters contain an enhancer characterized by the presence of an octamer motif and a proximal sequence element (PSE), located around position –50. We have shown previously that the element that defines the U6 promoter as an RNA polymerase III promoter is a TATA box located around position –25, which is absent from the RNA polymerase II snRNA promoters. We are now in the process of characterizing the factors involved in transcription of both the RNA polymerase II and III snRNA genes.

HUMAN IMMUNODEFICIENCY VIRUS TYPE 1

Regulation of expression from the human immunodeficiency virus type-1 (HIV-1) promoter is a complex process that involves cellular and viral proteins. The viral protein Tat is a potent *trans*-activator of HIV-1 gene expression, which functions by activating transcription at the levels of both initiation and elongation. Tat acts through an RNA target, referred to as TAR, which constitutes the upper half of a stable stem-and-loop structure encoded just downstream from the HIV-1 transcriptional start site, between positions +1 and +59.

Intriguingly, in the absence of Tat, transcription from the HIV-1 promoter results mainly in the formation of short, stable transcripts that extend to positions +59 to +65 and therefore encompass the entire stem-and-loop structure including the TAR element. In the presence of Tat, the number of short transcripts is strongly diminished, whereas the number of transcripts that extend through the entire transcription unit is increased. The short transcripts are believed to

result from transcription complexes that are not capable of efficient elongation and therefore terminate at random sites along the template. The resulting prematurely terminated RNAs would then be trimmed back by exonucleases to the base of the stable stem-and-loop structure. In the presence of Tat, the number of elongation-competent transcription complexes initiated at the HIV-1 promoter would be increased at the expense of the transcription complexes incapable of efficient elongation, thus a resulting increase in full-length RNAs and decrease in short transcripts.

We are interested in the mechanisms that govern the formation of short transcripts. Indeed, the short transcripts contain TAR, the target for Tat *trans*-activation, and are therefore likely to play an important role in the regulation of Tat *trans*-activation. In addition, formation of short transcripts in HIV-1 depends only on cellular factors, suggesting the existence of equivalent processes in some cellular genes. In past years, we found that the HIV-1 sequences between positions –5 and +82 relative to the start site of transcription contained an element(s), which we refer to as inducer of short transcripts (IST), that could induce the formation of short transcript from the HIV-1 promoter or any other promoter we tested. IST activates transcription, but the resulting RNAs are all short. Thus, IST may be activating the formation of transcription complexes not capable of efficient elongation. We are now characterizing IST in more detail and searching for factors involved in IST function.

Factors Involved in Transcription of the Human U6 Gene

S. Lobo, J. Moreno, M. Sullivan, N. Hernandez

The factors required for transcription of RNA polymerase III genes with internal promoter elements, such as the 5S genes, the tRNA genes, and the VA genes from adenovirus 2 (Ad2), can be separated into three fractions by chromatography of a crude cellular extract on a phosphocellulose column. The flowthrough fraction (A fraction) contains TFIIIA, a factor required only for transcription of the 5S genes, the 100–350 mM KCl fraction (B fraction) contains TFIIIB, and the 350–600 mM KCl fraction contains TFIIIC. Both TFIIIB and TFIIIC are required for transcription of all RNA polymerase III genes tested to date. In yeast, TFIIIA and TFIIIC have been shown to correspond to assembly factors, whose role is to recruit TFIIIB into the initiation complex. TFIIIB then contacts RNA polymerase III. Although TFIIIA has been cloned and consists of a single polypeptide, little is known about the polypeptide composition of TFIIIB and TFIIIC. TFIIIB may contain a polypeptide of about 60 kD, whereas TFIIIC can be separated into two activities, TFIIIC1 and TFIIIC2. TFIIIC2 activity has been purified to five polypeptides, but TFIIIC1 has not been characterized at all.

Transcription from the human U6 gene can be reproduced in vitro in a combination of three fractions from a phosphocellulose column: the B fraction, the C fraction, and an additional high-salt (600–1000 mM KCl) fraction or D fraction. The D fraction is not required for transcription of other RNA polymerase III genes, but it is known to contain the TFIID complex, which is involved in transcription of mRNA-encoding genes by RNA polymerase II. One of the components of the TFIID complex is the TATA-box-binding protein TBP. We have shown that both the C and D fractions can be replaced by TBP. Indeed, cloned human TBP expressed in *Escherichia coli* binds to the U6 TATA box and selects RNA polymerase III to transcribe the U6 gene. Thus, TBP is not exclusively an RNA polymerase II transcription factor as was widely believed; it can also direct the formation of RNA polymerase III initiation complexes. How, then, does TBP recruit RNA polymerase III to the U6 promoter rather than RNA polymerase II, as it does on TATA boxes of mRNA promoters? A possibility is that TBP interacts with the PSE-binding factor and that the resulting protein surface recruits TFIIIB into the initiation complex. TFIIIB would then in turn recruit RNA polymerase III. An interaction between TBP and the PSE-binding factor is suggested by our observation that the spacing between the TATA box and the PSE is critical for RNA polymerase III transcription of the human U6 gene.

To characterize in more detail the involvement of TBP in transcription of the U6 gene and other RNA polymerase III genes, we have generated a battery of monoclonal antibodies directed against TBP. We have obtained 33 different monoclonal antibodies, which all recognize human TBP in Western blots and in immunoprecipitations. These antibodies will allow us to determine whether TBP involved in RNA

polymerase III transcription is in a complex and whether this complex differs from the TFIID complex involved in RNA polymerase II transcription.

We are also fractionating the B and the C phosphocellulose fractions further to identify additional factors involved in U6 transcription. The B fraction has been fractionated over two additional columns, and the activity required for U6 transcription copurifies with an activity required for transcription of the Ad2 VA gene. This activity therefore most probably corresponds to TFIIIB. The C fraction has been further purified over several columns. Fractions required for U6 transcription contain a factor that binds to the U6 PSE. The activity may therefore correspond to the PSE-binding factor.

Factors Involved in Transcription of the Human U1 and U2 snRNA Genes

C. Sadowski, S. Lobo, N. Hernandez

Transcription of RNA polymerase II snRNA genes has been very difficult to reproduce in vitro. Yet, an efficient in vitro transcription system is a prerequisite for the identification and characterization of transcription factors involved in transcription of these genes. Recently, Gunderson et al. (*Genes Dev. 4:* 2048 [1990]), using a G-less cassette construct, were able to obtain transcription of the human U1 snRNA gene in vitro. We have reproduced these results and can now obtain efficient in vitro transcription of RNA polymerase II snRNA genes. We are in the process of determining whether TBP is required for RNA polymerase II transcription of snRNA genes.

Precise Mapping of the IST Element in the HIV-1 LTR

M. Sheldon, R. Ratnasabapathy, N. Hernandez

From our previous work, the IST element was known to be located between positions –5 and +82 relative to the HIV-1 start site of transcription. Its precise location, however, was not clear, and we did not know whether IST consisted of a DNA or an RNA element, or both. To characterize IST further, we introduced a series of mutations in the HIV-1 region extending from +1 to +65. Because the secondary structure that folds the short RNAs may be required for their stability, all of the mutations we generated maintain the secondary structure. In addition, the effects of the different mutations were tested both by analysis of steady-state RNA and by analysis of nascent RNA in run-on assays.

These analyses showed that the IST element is located at the base of the stable stem-and-loop structure, consistent with the strong conservation of this region in different HIV-1 isolates. As shown in Figure 1A, IST is distinct from TAR, which encompasses the upper half of the secondary structure. Indeed, IST can be inactivated by the six double point mutations shown in Figure 1A, which have little effect on Tat *trans*-activation. In fact, IST activity can be completely separated from TAR activity by deletion of the upper part of the stem-and-loop structure, as shown in Figure 1B. This construct, referred to as pISTBu, produces large amounts of short transcripts (which are shorter than the short transcripts produced by the wild-type construct), but cannot be *trans*-activated by Tat.

Interestingly, the construct pIST shown in Figure 1C, which is identical to that shown in Figure 1B except for the insertion of two nucleotides that provide pairing partners for the two bulged nucleotides, does not produce any short transcripts as determined by analysis of steady-state RNA. However, this construct is active as determined by analysis of nascent RNA in run-on experiments. This observation suggests that this construct generates short RNAs efficiently but that these are too unstable to be detected by analysis of steady-state RNA. Thus, one element required for IST function is an RNA element, whose role is probably to stabilize the short transcripts. The analysis of additional constructs shows that IST also consists of a second element located between positions +1 and +19, which activates transcription of the short RNAs.

Factors Involved in IST Function

R. Ratnasabapathy, F. Pessler, N. Hernandez

To understand further the mechanisms that govern IST function, we need to identify the *trans*-acting

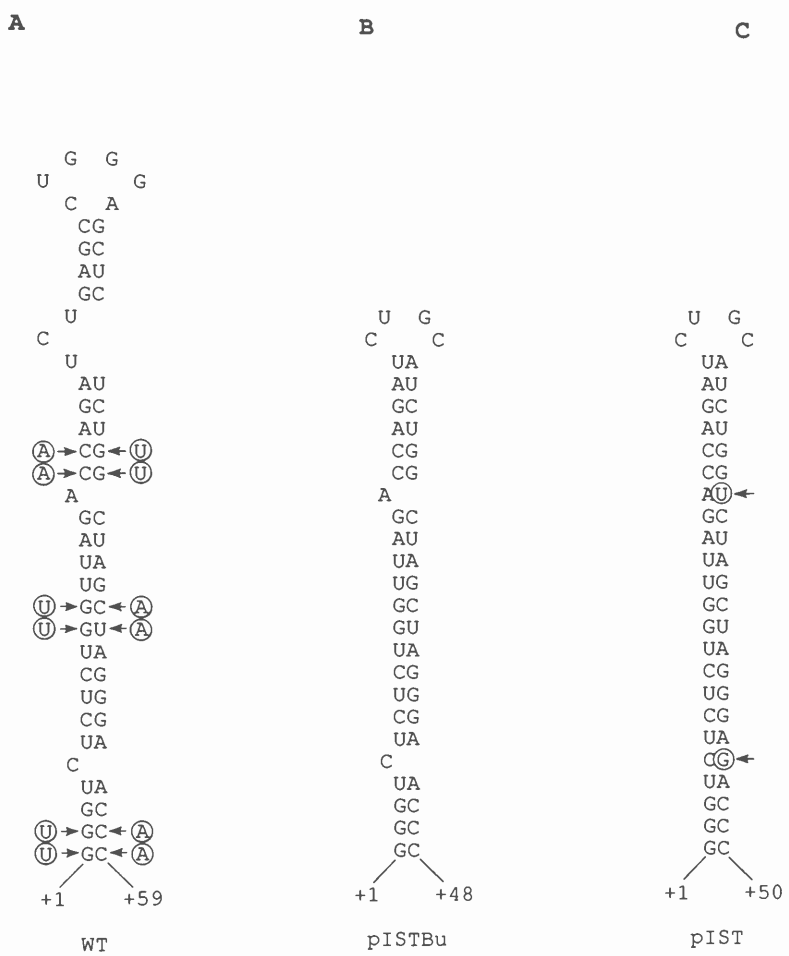

FIGURE 1 (*A*) Secondary structure that folds the first 59 nucleotides of HIV-1 RNAs. The six double point mutations that inactivate the IST element are shown, as well as the locations of the TAR and IST elements. (*B*) Putative secondary structure assumed by the short transcripts encoded by pISTBu. (*C*) Putative secondary structure assumed by the short transcripts encoded by pIST. The two inserted nucleotides are indicated.

factors involved in the process. We are screening crude extracts as well as more purified fractions for factors that will bind to the RNA and DNA components of IST but not to mutated versions of the element that do not direct the formation of short transcripts in transfection experiments.

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TELOMERASE BIOCHEMISTRY AND REGULATION

C.W. Greider C. Autexier, A.A. Avilion, L.A. Harrington, L.L. Mantell, K.R. Prowse, S.K. Smith

Telomerase is a highly specialized DNA polymerase that synthesizes the telomeric sequence repeats found at the ends of eukaryotic chromosomes. The enzyme contains an essential RNA component that is used as a template for the de novo synthesis of telomeric sequences. This de novo synthesis allows maintenance of telomere length during replication and may allow healing of broken chromosomes. Telomerase activity has been found in ciliates and transformed human cell lines. Our long-term goals are to understand both the detailed biochemistry and mechanism of telomerase and its regulation and effects on telomere length in senescent and immortalized mammalian cells. In the past year, our efforts have been concentrated in the following areas: The role of telomerase RNA in primer recognition, the regulation of *Tetrahymena* telomerase RNA in vivo, the structure and dynamics of human telomere shortening, and the role of telomere length and telomerase in cellular senescence and immortalization.

Telomerase Primer Recognition and Chromosome Healing

L.A. Harrington, C.W. Greider

Last year, we described the analysis of *Tetrahymena* telomerase processivity. The essential RNA of telomerase contains within it the sequence 5′-CAACCCCAA-3′, which provides the template for the synthesis of d(TTGGGG) repeats (Greider and Blackburn, *Nature 337:* 331 [1989]; Yu et al., *Nature 344:* 126 [1990]). Characterization of the telomerase mechanism using primer challenge experiments indicated that the enzyme processively synthesized about 500 nucleotides before half of the enzyme dissociated from a given primer. Analysis of the banding pattern suggested that translocation on the RNA template is a slow step in the elongation of the TTGGGG repeat chain.

To define the role of the RNA in primer binding and elongation further, we examined the elongation of chimeric oligonucleotides. Telomeric oligonucleotides are elongated by telomerase, whereas nontelomeric oligonucleotides are not telomerase substrates. To test whether hybridization between the 3′ end of primer oligonucleotides and the telomerase RNA template was an absolute requirement for elongation, a series of chimeric oligonucleotides containing nontelomeric sequence and telomeric d(TTGGGG) repeats at either the 5′ or 3′ end were tested for elongation in vitro. One to three d(TTGGGG) repeats, or d(GGGG), when placed at the 3′ end of the nontelomeric pBR sequence, were sufficient for elongation. Strikingly, two or three repeats of d(TTGGGG) placed at the 5′ end of oligonucleotides with nontelomeric 3′ ends were also telomerase substrates. We tested chimeric oligonucleotides containing two d(TTGGGG) repeats at the 5′ end and from 6 to 36 bases of pBR sequence at the 3′ end; all of these chimeric oligonucleotides were elongated by telomerase. The elongation of all primers was inhibited by preincubation of telomerase with RNase A, indicating that elongation of these chimeric oligonucleotides, like that of telomeric sequences, requires telomerase RNA. To determine the specificity of telomerase for the chimeric oligonucleotides, titration experiments were performed. Chimeric oligonucleotides with two d(TTGGGG) repeats at the 5′ end were elongated at concentrations as low as 24 nM, as was the telomeric substrate $d(TTGGGG)_4$. The nontelomeric oligonucleotide was only a very weak substrate at oligonucleotide concentrations above 3 mM.

Three pieces of evidence showed that the elongation of chimeric oligonucleotides with 5′ d(TTGGGG) repeats was not due to removal of nontelomeric sequences. First, telomerase elongation products began above the position of the input marker oligonucleotide. Second, oligonucleotides incubated with telomerase in the presence of [^{32}P]dTTP migrated with full-length marker oligonucleotides on denaturing acrylamide gels. Finally, these end-labeled chimeric oligonucleotides were subjected to Maxam-Gilbert cleavage at dC residues. Digestion products of the predicted sizes were obtained, providing further evidence that nontelomeric pBR se-

quences were retained during telomerase labeling. Thus, hybridization of the 3′ end of primer oligonucleotides to the 5′-CAACCCCAA-3′ template region is not required for primer elongation.

We envision at least three models for the specific elongation of oligonucleotides with nontelomeric sequence at the 3′ end (Fig. 1). In the first model, telomerase binds to the 5′ end of a chimeric oligonucleotide by RNA hybridization, and "slides" to the 3′ terminus to initiate d(TTGGGG) synthesis. Alternatively, the 3′ nontelomeric sequences are "looped out" to juxtapose directly the 3′ end of the oligonucleotide with the RNA template. A third possibility is that telomerase recognizes telomeric DNA independent of telomerase RNA, for example, by DNA-protein recognition of G-rich sequences. This complex could then translocate to the 3′ terminus of the oligonucleotide, to initiate $d(TTGGGG)_n$ synthesis. In addition to dissecting the telomerase mechanism, these experiments also provided the first biochemical evidence that telomerase can "heal" broken chromosomes by adding telomere repeats onto nontelomeric sequences.

To distinguish between the RNA-dependent and RNA-independent mechanisms for primer recognition, we needed to develop a new assay. Previously, primer binding was measured by assaying telomerase d(TTGGGG) addition. However, since the RNA component is required for elongation, this assay cannot distinguish between RNA-dependent and RNA-independent primer recognition. To distinguish between these models, we have begun using methods that look directly at primer binding, without the requirement for elongation.

Telomerase Proteins and Antibodies

L.A. Harrington

We are continuing our efforts to identify the protein components of telomerase. Our most highly purified preparations are approximately 3000-fold purified and contain about eight polypeptide species. Although purification has been difficult due to the instability of the enzyme, we are pursuing several approaches to identify telomerase subunits. First, we are purifying the enzyme further to identify the polypeptides that copurify with enzyme activity. Second, we are using UV cross-linking and South- and Northwestern blots to identify the polypeptides that bind (TTGGGG) primer and RNA. Finally, we are making monoclonal antibodies against the most highly purified fractions. These approaches should allow the identification of the functional polypeptide(s) in our most pure preparations.

Telomerase RNA Expression

A.A. Avilion, L.A. Harrington

We have carried out a developmental study of telomerase expression to study enzyme regulation and to determine the optimal conditions for enzyme preparation. Preliminary experiments suggested that more telomerase activity was present during macronuclear development in *Tetrahymena* than during vegetative growth (Greider and Blackburn, *Cell 43:* 405 [1985]). During macronuclear development, germ-line chromosomes are fragmented and new telomeres are added onto the ends of each fragmented piece. Since telomerase adds these new telomeres, it was reasonable to believe that more enzyme might be present during this developmental stage.

To test this directly, we estimated the amount of telomerase by determining the level of telomerase RNA present in mating, starved, or vegetative cultures. Using quantitative Northern blots and the Phosphor Imager, we determined that there are approximately 25,000 telomerase RNA molecules per cell. We next compared the amount of telomerase RNA at 0, 3, 6, 9, 12, 15, and 24 hours after mixing of cells for mating. To normalize the Northern signal to the number of cells loaded, equal counts of a radioactive marker were added to equal numbers of cells before RNA extraction. Telomerase RNA levels increased at 9 hours after mixing cells of opposite mating type (Fig. 2). There was only a small increase in the level of telomerase RNA in the mated cultures compared to nonmated (starved vegetative) control cultures. To determine whether the RNA levels reflect the level of telomerase activity, extracts were made from mated or nonmated cultures. Again, only a small increase in the activity of the mated extracts was found. These results indicate that the level of telomerase activity parallels the amount of telomerase RNA. The finding that extracts from both starved and mated cultures had significantly more activity than extracts from cells growing in rich media has allowed us to optimize our enzyme preparation.

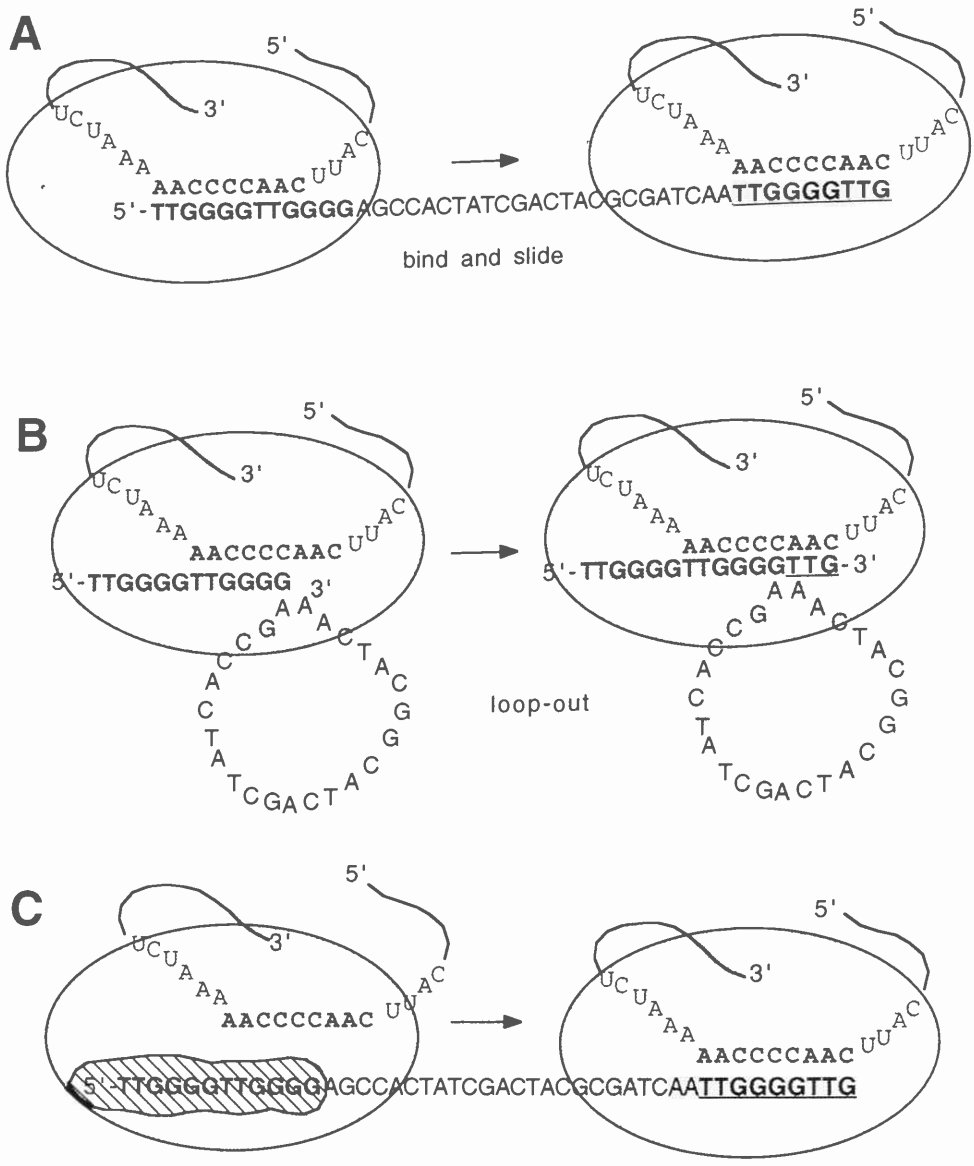

FIGURE 1 Three models for telomerase elongation of nontelomeric oligonucleotide termini. The telomerase RNA template region and complementary telomeric sequence are shown in bold. Newly synthesized d(TTGGGG) is shown in bold and underlined. (*A*) Telomerase binds to the 5′ telomeric or G-rich sequence via hybridization of telomerase RNA. After recognition, the complex tracks to the 3′ end of the oligonucleotide and initiate d(TTGGGG) synthesis. (*B*). Telomerase RNA recognizes 5′ G-rich sequences as in *A*. Nontelomeric sequences are looped out, retaining hybridization of the 5′ end of the oligonucleotide, while bringing the 3′ end close to the RNA template to allow d(TTGGGG) synthesis. (*C*) Telomerase possesses an RNA-independent ability to recognize and bind G-rich structures that positions telomerase RNA at the 3′ terminus of the oligonucleotide to initiate telomere addition.

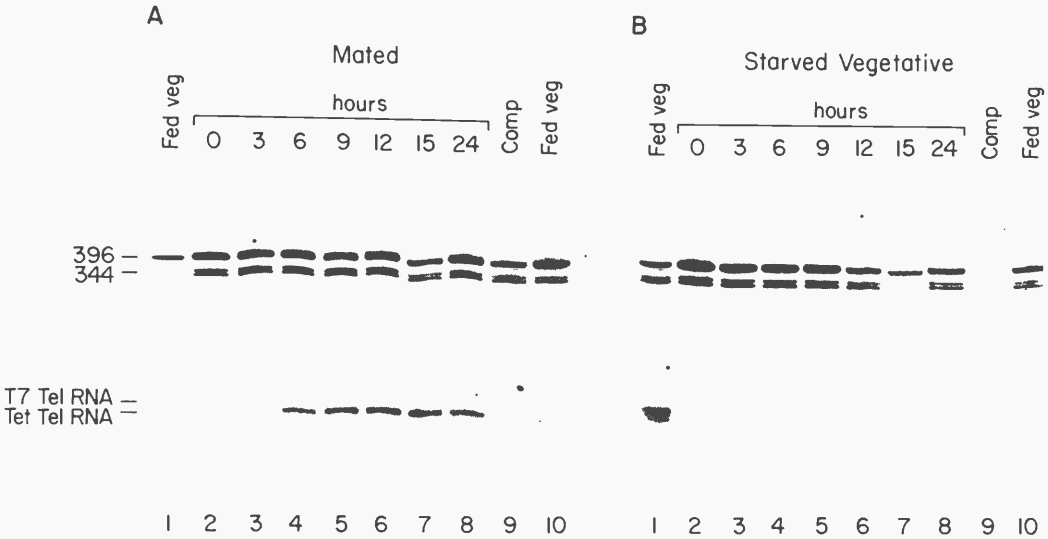

FIGURE 2 Northern blot analysis of *Tetrahymena* telomerase RNA levels in mated and nonmated cells. RNA was extracted from a total cell lysate (4.5 x 10^5 cells per time point) and analyzed by a Northern blot. The radioactively labeled 346 and 396 DNA fragments were used as an internal control. (*A*) Mated cells. (Lane *1*) Telomerase T7 RNA transcript (1 ng) mixed with total cellular RNA from vegetative cells; (lanes *2–8*) cellular telomerase RNA from mated cells at the time points indicated above each lane; (lane *9*); cellular telomerase RNA from mated cells that were transferred to complete media 13 hr after mixing and harvested at 24 hr; (lane *10*) cellular telomerase RNA from vegetative cells grown in complete media (control). (*B*) Same as in *A* but with nonmated (starved vegetative) cell culture.

Structure and Dynamics of Human Telomeres

K.R. Prowse [in collaboration with B. Abella and B. Futcher, Cold Spring Harbor Laboratory, and C. Harley, McMaster University, Canada]

In addition to telomerase biochemistry, we are interested in how telomere length is maintained in vivo. We have found that as primary human fibroblasts divide and undergo senescence in culture, telomeric sequences are lost from chromosome ends (Harley et al., *Nature 345:* 458 [1990]). The limited replicative capacity of primary fibroblasts was well documented by Hayflick and others in the 1960s; however, the mechanism that limits cellular life span is not yet known. We have recently shown that telomere shortening occurs in vivo as well as in vitro. Primary fibroblasts from older individuals have shorter telomeres than those from younger individuals.

To determine whether telomere shortening in vitro was due to the cell's chronological age or to the number of divisions, primary fibroblasts were passaged for 48 mean population doublings (MPD). The culture was then divided into two, and half of the cells were cultured continuously at 1:8 split ratios. The other half were allowed to reach quiescence (day 5) and held quiescent for a further 3, 10, or 17 days. The genomic DNA was prepared from each culture, digested with restriction enzymes, and hybridized with the $(TTAGGG)_3$ probe. Similar experiments were carried out using cells that were passaged continuously for either 57, 66, or 69 MPD: At each of these MPD, several plates were set aside and the cells were held quiescent for up to 30 days. The mean length of the terminal restriction fragment was determined from quantitation of the $(TTAGGG)_3$ hybridization signal in the agarose gels (Fig. 3). No shortening of the terminal restriction fragment was seen while the cells were quiescent in culture. However, those cells that were passaged continuously did show the characteristic telomere shortening. The constant length of the telomere restriction fragments when cells are not allowed to divide suggests that telomere shortening is linked to DNA replication.

Primary cells from different donors show distinct modes of telomere length distributions (Fig. 4A). These multimodal distributions could arise in several

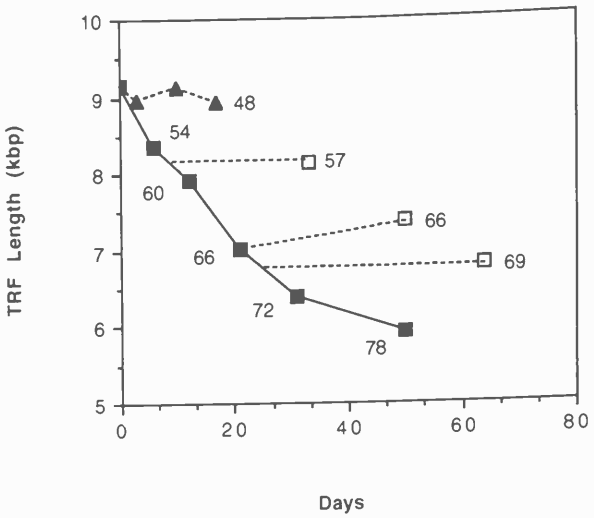

FIGURE 3 Cell division is required for telomere shortening. The mean length of the terminal restriction fragment was determined by quantitating the (TTAGGG) hybridization signal in the gel at 30 contiguous regions ranging from 4 kbp to 30 kbp. Several plates were set aside from a continuously growing culture of HSC172 at four different mean population doublings (MPD) (48, 57, 66, and 69). The cells were allowed to reach confluence and held quiescent for the number of days indicated. The TRF length is plotted versus the total number of days in culture for both the passaged and the quiescent cells. The solid line connects cells that were continuously passaged. Dashed lines represent cells that were held quiescent in culture. The numbers at each point refer to the number of MPD the cells attained before the culture was either passaged or held quiescent.

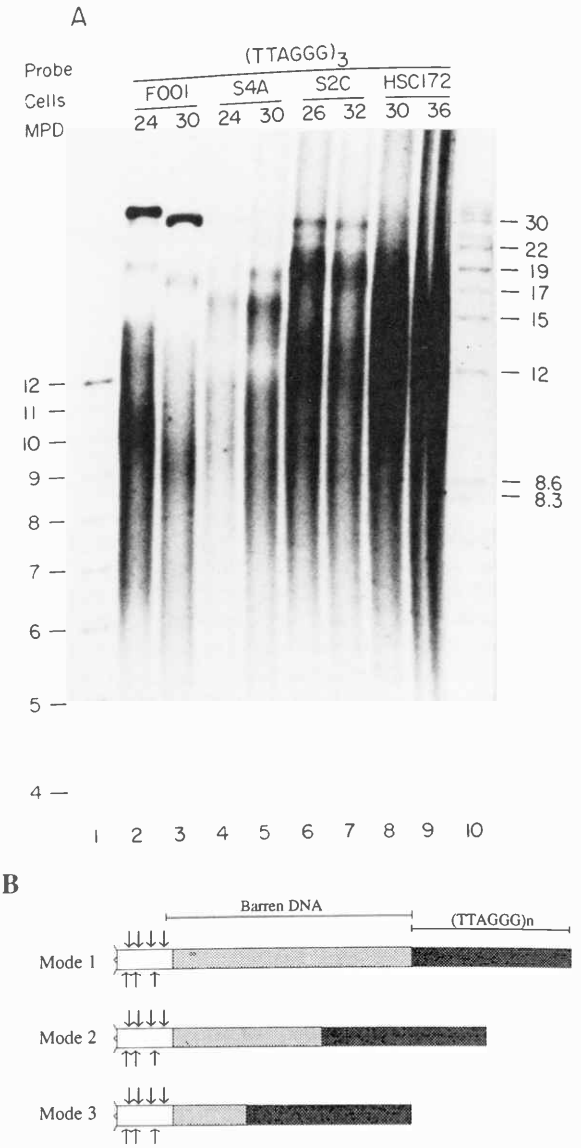

FIGURE 4 Fibroblast DNA samples from different individuals show multimodal telomere distributions. (*A*) Fibroblast DNA from FOO1, S4A, S2C, or HSC172 cells at the indicated MPD were digested with *Msp*I and *Rsa*I and separated in a 0.5% agarose gel, and the gel was hybridized to end-labeled $(TTAGGG)_3$ oligonucleotide. (Lane *1*) End-labeled 1-kbp ladder marker DNA; (lanes *2,3*) FOO1 DNA; (lanes *4,5*) S4A DNA; (lanes *6,7*) S2C DNA; (lanes *8,9*) HSC172 DNA; (lane *10*) end-labeled HMW(BRL) markers. (*B*) Multiple modes are due primarily to difference in the non-TTAGGG "barren" DNA on the terminal restriction fragment.

ways. They may reflect the different position of the most terminal restriction site on different chromosomes or sets of chromosomes. Alternatively, if the terminal restriction site is a fixed distance from the beginning of the d(TTAGGG) repeats, the multimodal distribution could be due to different numbers of d(TTAGGG) repeats on different chromosomes. If the amount of d(TTAGGG) on the end of human chromosomes determines the number of replications the cells can undergo, it is important to know whether there is one or several distinct tract lengths of d(TTAGGG) on all of the chromosome ends. Quantitative analysis of several different multimodal samples suggests that most of the length differences in the different modes are attributable to different amounts of subtelomeric DNA. There is only a small amount of variation in the length of d(TTAGGG) between the different modes (Fig. 4B). This is consistent with what has been found for telomeres in single-cell eukaryotes, where the number of telomeric repeats is similar on different chromosomes.

Human Telomeres and Telomerase in Cellular Senescence and Immortalization

A.A. Avilion [in collaboration with C. Counter, C. Harley, and S. Bacchetti, McMaster University, Canada]

Human sperm telomeres are several kilobases longer than somatic cell telomeres. This, together with the telomere reduction observed during replication of primary fibroblasts, suggests a simple model for telomere shortening: Human telomerase is active in the germ line where telomeres are lengthened, and then telomerase is inactive in somatic cells. The net loss of telomeric sequences may then occur in somatic tissue through incomplete replication at each round of division. We have begun experiments to look for the presence of telomerase activity in the primary fibroblasts where telomere shortening has been documented. In three separate experiments, we found that activity was detected in transformed human 293 cells but was not detectable in extracts from primary cells. As a control, primary fibroblasts were mixed with 293 cells; telomerase activity was detected, indicating that a diffusible inhibitor is not present in the fibroblasts. These experiments support the model that telomerase may not normally be active in somatic cells.

Telomerase activity is found in some immortal human cell lines such as HeLa and 293, where telomere length is maintained at a constant, but shorter, size than that of the tissue of origin. In collaboration with Calvin Harley and Silvia Bacchetti at McMaster University, we have looked at the effect of SV40 transformation on telomere length and telomerase activity in primary human embryonic kidney (HEK) cells. HEK cells undergo only 10–12 doublings in vitro. Clones of HEK cells transformed with SV40 have an extended life span; after about 100 doublings, the clones undergo "crisis." Most clones die at crisis; however, the few that survive are immortalized. Telomere length decreased as the primary HEK cells were passaged and continued to decrease in the extended life span clones. However, in the one immortal clone obtained to date, telomere length was short but stable after many rounds of division. Telomerase activity was absent in the three extended life span clones tested but was present in the immortalized clone. These results suggest that one of the events which occurs during immortalization is the reactivation of telomerase activity.

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MOLECULAR CHROMOSOME STUDIES OF *ARABIDOPSIS THALIANA*

E.J. Richards A. Vongs
S. Chao
J. Yang

We are currently pursuing three related projects investigating different aspects of molecular chromosome structure and genome organization using the flowering plant *Arabidopsis thaliana* as a model system. Two projects concern the characterization of genomic DNA organization of centromeric and telo-

meric regions. We are also continuing our characterization of *A. thaliana* mutants that contain markedly reduced levels of cytosine methylation in an effort to understand the function of DNA modification.

CHARACTERIZATION OF TELOMERIC DNA FROM *A. THALIANA*

The ends of linear eukaryotic chromosomes are specialized structures, called telomeres, which allow complete replication of the end of the chromosomal DNA molecule and stabilize otherwise reactive DNA ends. Telomeres in most organisms have an unusual DNA structure, being composed of tandem arrays of short, G-rich repeats. The genomic regions that flank the telomeric repeats, termed telomere-associated sequences, are generally rich in more complicated repetitive elements that exhibit variability in terms of DNA structure and chromosome distribution.

In an effort to complete the molecular description of *A. thaliana* chromosomal termini, we are analyzing *A. thaliana* telomere clones recently isolated by selection for plant DNA fragments that function as telomeres on yeast artificial chromosome (YAC) vectors. The eight telomere clones represent six different genomic locations. We have characterized three of these clones in some detail. All three clones contain a block of telomeric repeats and flanking telomere-associated sequences. Surprisingly, the flanking sequences in two of the clones are composed of single-copy sequences. The region flanking the telomeric repeat block in the third clone contains a repetitive element present at several other *A. thaliana* telomeres. DNA sequence analysis revealed a substructure to the telomeric repeat block in two of the clones: Long arrays of TTTAGGG repeats are found in the distal portions of the clones, whereas the centromere-proximal regions contain variant and degenerate repeat motifs.

In addition to providing a description of the genomic organization of the chromosome ends, we hope that the new telomere clones will allow the ends of the genetic and physical maps of *A. thaliana* chromosomes to be established. Toward this end, we have been collaborating with a number of *A. thaliana* mapping groups. Recently, the DuPont group (Wilmington, Delaware) and the Meyerowitz laboratory (California Institute of Technology) have positioned two of the telomere clones on the restriction-fragment-length polymorphism (RFLP) map, at the end of chromosomes 1 and 5.

CHARACTERIZATION OF CENTROMERIC DNA FROM *A. THALIANA*

We are interested in identifying and characterizing the DNA sequences required for centromere function as a first step toward understanding how centromeres work. At present, functional centromeric DNA sequences have been identified in only two systems, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. The picture that emerges from centromere studies in the yeasts is rather confusing; the budding yeast centromeres are quite small (120 bp) and do not resemble the large (50–100 kb) arrays of repetitive DNAs that make up *S. pombe* centromere regions (Clarke, *Trends Genet. 6:* 150 [1990]). Although it is thought that the *S. pombe* centromeres may provide a simple model for the larger, more structurally complex centromeres of higher eukaryotes, we have decided to begin characterizing a higher eukaryotic centromere directly.

Our approach to isolating *A. thaliana* centromeric DNA is based on the strategy initially used to clone yeast centromeres. The first stage involves determining a genetic map position for the target centromere and identifying molecular markers that flank the centromere. Analysis of the genomic regions encompassing the centromere can then be initiated using these flanking markers. In addition, cloning of the centromere can be achieved by isolating the genomic DNA between the flanking markers.

We have focused on mapping and characterizing *A. thaliana* centromere 1. The initial mapping stage of the project has been completed this year, as summarized in Figure 1. To map *A. thaliana* centromere 1, we relied on a set of aneuploid mutants that contain a single telocentric chromosome 1 derivative in addition to the diploid set of ten metacentric *A. thaliana* chromosomes. Two such telotrisomic lines exist (Tr1A and Tr1B), each carrying one of the two arms of chromosome 1 as an additional telocentric. Genomic DNA was prepared from Tr1A plants carrying two copies of the A chromosome arm from one ecotype parent and one dose from another ecotype parent. Similar Tr1B material was also generated, and genomic DNA was prepared. RFLP markers located in the center of chromosome 1 were positioned on the A or B arm by determining the dosage of the RFLP alleles after Southern hybridization. The markers were then ordered by reference to recombination breakpoints. The centromere was positioned between the most proximal markers as illustrated in Figure 1.

Our focus now shifts to assembling a long-range

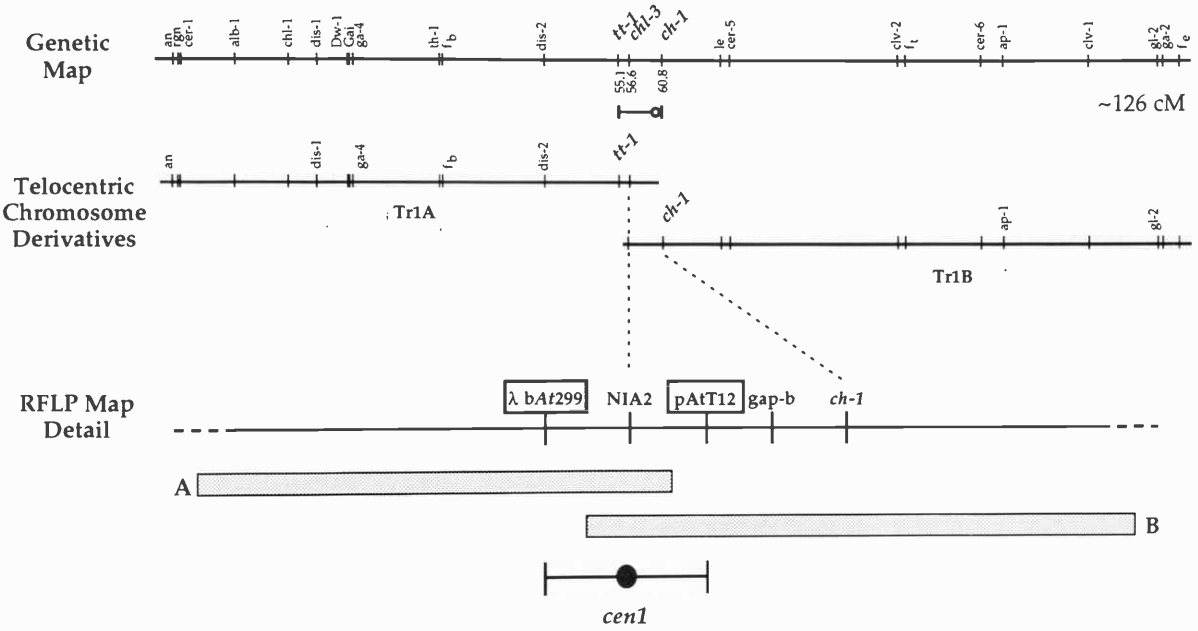

FIGURE 1 The genetic map of *A. thaliana* chromosome 1 is shown at the top of the figure (Koornneef, *Genetic maps*, Cold Spring Harbor Laboratory [1987]). The approximate position of *cen1* relative to classical genetic markers was determined by Koornneef (*Genetica 62:* 33 [1983]) by analyzing crosses between marked strains and telotrisomic plants carrying the telocentric chromosome derivatives. Correlations between the classical genetic and RFLP maps (Nam et al., *The Plant Cell 1:* 699 [1989]) are indicated by the dashed lines. The stippled boxes shown below the RFLP map indicates the coverage of the overlying RFLP markers by the telocentric chromosome derivatives Tr1A and Tr1B. The position of *cen1* is shown at the bottom of the figure, bracketed by the RFLP markers bAt299 and pAtT12 (shown in boxes; window size = roughly 4 cM).

restriction map of the region to determine the size and DNA organization of the centromere. Such information will supply a foundation for the next phase: isolation of centromeric sequences in YAC vectors and functional analysis of these sequences.

DNA HYPOMETHYLATION MUTANTS

Although DNA methylation has been implicated in a number of cellular processes in eukaryotes, very little is known about the nature and regulation of the enzymatic machinery that carries out cytosine methylation or the function of this DNA modification. In collaboration with Rob Martienssen's laboratory (see this Section), we are taking a genetic approach to address these questions, using *A. thaliana* as a experimental organism. Plants, such as *A. thaliana*, provide an opportunity to dissect a fundamental process that cannot be easily addressed in well-developed genetic models such as nematodes, *Drosophila*, and the yeasts, which lack detectable levels of cytosine methylation.

At present, three independent, allelic hypomethylation mutations have been recovered from M2 populations of EMS-mutagenized seeds by screening for plants that have centromeric satellite repeats susceptible to digestion by methylation-sensitive endonucleases. Both satellite arrays and rDNA are hypomethylated in the mutants, although methylation at some single-copy sequences is unaffected. Measurement of global methylation levels indicate that the cytosine methylation is reduced up to 80% in the mutants. Strikingly, the mutants do not display a morphological phenotype that can be associated with hypomethylation.

Study of the hypomethylation mutants is now focused on isolation of more severe alleles or second-site mutations that reduce or abolish the residual levels of cytosine methylation. In addition, the extant hypomethylation mutants are being investigated to assess the role that cytosine methylation plays in a variety of processes, including epigenetic regulation of transgenes, recombination, rDNA expression, DNA replication timing, and chromosome behavior.

It is anticipated that study of these mutants, as well as isolation of mutations that cause hypermethylation or alterations in the sequence distribution of methylation, will provide an understanding of the biology of DNA methylation in eukaryotes.

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STRUCTURE AND COMPUTATION

This section includes five laboratories interested in the detailed structural properties of proteins and computational biology. Dr. John Anderson is a macromolecular crystallographer currently attempting to solve the structure of the *Pvu*II restriction endonuclease. His laboratory is also pursuing studies of transcription factors. Dr. Jim Pflugrath is also a crystallographer whose laboratory has recently solved the structure of T7 lysozyme. Dr. Rich Roberts' laboratory has had a long-standing interest in restriction enzymes and methylases. Recently, in collaboration with Drs. Jim Pflugrath and Xiaodong Cheng, crystals of the first cytosine methylase have been obtained. Dr. Jeff Kuret studies protein kinases and is working on the structure of the cAMP-dependent protein kinase from yeast in collaboration with Dr. Pflugrath. Dr. Kuret is also studying casein kinase I. Finally, Dr. Tom Marr is a computational biologist who joined this section in 1989 and whose laboratory has expanded considerably during the last year. Dr. Marr is interested in database problems particularly those arising from the Human Genome Project.

MACROMOLECULAR CRYSTALLOGRAPHY

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Structural Studies of *Pvu*II Endonuclease and Its Interaction with DNA

G.K. Balendran, J. Keller, M. Slawecki, J.C. Wu, J.E. Anderson [in collaboration with I. Schildkraut, New England BioLabs]

The restriction endonuclease from *Proteus vulgaris*, R·*Pvu*II, recognizes the DNA sequence CAGCTG. In the presence of magnesium, R·*Pvu*II cleaves between the central guanine and cytosine bases. We have cocrystallized R·*Pvu*II with an oligodeoxynucleotide carrying its recognition sequence. To prevent cleavage of the oligo, EDTA is included in the crystallization buffer. The crystals are space group $P2_12_12_1$ with unit cell dimensions a = 95.1 Å, b = 85.6 Å, c = 47.7 Å, and they diffract to at least 2.6 Å resolution. We have collected native data to 3 Å resolution. To obtain phases for these data, we are preparing isomorphous heavy atom derivative crystals in two ways. The first is the conventional method of soaking native cocrystals in solutions of various heavy atom reagents. Two reagents, platinum *ter*-pyridinium chloride and *para*chloromercuribenzoic sulfate, yielded isomorphous crystals, and data sets are being collected for each of these. The second approach takes advantage of the presence of DNA in the crystal. The heavy atom is introduced by covalent modification of the oligo, substituting an iodine atom for the methyl group of thymine at two symmetrically related positions in the sequence. This simplifies the task of locating the iodine atoms and hence of calculating the phases, because we expect the conformation of the DNA in the crystal to be similar to the well-known structure of B-DNA. Isomorphous cocrystals were grown with two such iodinated oligos. Analysis of data collected from them indicated that the percentage of oligos actually containing iodine was too low to provide phases. Iodinated oligos have been resynthesized, taking care in processing and handling to ensure a useful iodine occupancy, and data collection is under way. Once

phases are determined using the various derivatives, we will calculate a multiple isomorphous replacement (MIR) electron density map and begin building a model of the complex.

To truly understand the enzymology of R·*Pvu*II, we need the structure of the apoprotein. We have two crystal forms of R·*Pvu*II grown in the absence of DNA that diffract to better than 2.5 Å. One crystal form is space group P422 with $a = b = 176.2$ Å, $c = 50.6$ Å, and the other is space group $P2_12_12$ with $a = 106.6$ Å, $b = 84.6$ Å, $c = 46.9$ Å. We have collected a partial native data set from the $P2_12_12$ crystal form, which is more stable but less abundant than the P422, and have initiated a search for heavy atom derivatives using the conventional soaking technique.

Structural Studies of AP-1 Proteins

C.K. Cheung, J. Keller, J.E. Anderson [in collaboration with T. Curran, Roche Institute]

The protein products of the proto-oncogenes c-*fos*, c-*jun*, and a number of related genes form a major component of eukaryotic transcription factor AP-1 activity. Heterodimers of Fos-related and Jun-related proteins, and homodimers of Jun-related proteins, bind to DNA at AP-1 sites (TGACTCA) in the promoters of many genes. Once bound, they can activate or repress transcription, depending on the composition of the dimer and the context within which it binds DNA. During the last year, we have concentrated our efforts on purifying and crystallizing complexes of bacterially expressed rat Fos- and Jun-related proteins with cognate DNA. We have purified and prepared complexes of intact Jun with oligodeoxynucleotides containing AP-1 sites. We have also prepared protein-DNA complexes using truncated Fos and Jun proteins that contain the DNA-binding basic region/leucine zipper (bZIP) domains of each protein, along with some additional flanking residues. Although the concentration and purity of these preparations are suitable for crystallization, none have yet produced crystals. The reason for this may lie in the stability of the folded structure of the proteins. Intact Jun, a 334-residue transiently expressed protein that is normally part of a multiprotein transcription complex, may not fold properly in the absence of the other transcription factors, even in the presence of cognate DNA. The truncated proteins contain sequences outside of the bZIP domain that may not fold properly in the absence of the rest of the protein. We therefore decided to focus on proteins containing only Fos or Jun residues corresponding to the respective bZIP domains. We obtained *Escherichia coli* strains expressing Fos and Jun bZIP proteins from Dr. Curran. We have purified the bZIP proteins, and crystallization trials are now under way.

Structural Studies of S100β

T. Malone, F. Papanikolaou, J.W. Pflugrath [in collaboration with D. Marshak, Cold Spring Harbor Laboratory]

Predominant among water-soluble brain proteins is S100β, an acidic 10.5-kD protein that contains the sequence requirements necessary to form two EF hand calcium-binding loops. Besides the postulated calcium signal mediation function, S100β binds zinc very tightly and has been implicated in its dimer form as a neurite extension factor. In both Alzheimer's disease and Down's syndrome, elevated levels of S100β have been detected. To understand better the function of S100β and the role that calcium plays in its dimerization, we will determine its structure to atomic resolution.

Although we had previously crystallized bovine S100β, the analysis of these crystals was not pursued because we could not purify the protein to homogeneity from brain extracts, making reproducible crystals difficult to obtain. We were much more successful with the recombinant rat S100β produced in the bacterium *Escherichia coli*. We now obtain 15–20 mg of pure S100β from a liter of bacterial cell culture in 3 days. The recombinant protein was as active as our earlier bovine brain preparations in an assay for neurite extension factor activity. This indicates that the lack of amino-terminal acetylation and loss of some of the initiator methionine in the recombinant protein do not alter its function and activity.

Early crystallization trials of the recombinant S100β yielded crystals that did not diffract well in an X-ray beam. By adjusting the pH of the trials, we were able to obtain crystals with a different morphology that diffract to beyond 2.2-Å resolution. These crystals are thick diamond-shaped plates and have space group $C222_1$ with cell dimensions 36 x 88 by 61 Å. A diffraction data set of 14,890 measurements collected on our FAST area detector was merged into 2910 unique structure factors with a R_{merge} of 4.9%

that was 94% complete to 2.7 Å resolution. We plan to use the molecular replacement method with search model phases calculated from the atomic coordinates of the homologous intestinal calcium-binding protein to determine the structure of S100β.

Yeast cAMP-dependent Protein Kinase

J.W. Pflugrath, T. Malone, X. Cheng, J. Kuret

An initial electron density map that we obtained for the yeast cAMP-dependent protein kinase proved to be only partially interpretable. In an effort to overcome this, we will recollect all of our diffraction data at the nearby National Synchrotron Light Source. In addition, we have changed the crystal harvesting buffer to one that stabilizes the crystals for months instead of a few weeks. This increased stability allowed us to soak crystals for a longer time than was previously possible in heavy atom compounds. Unfortunately, such soaks did not reveal any new substitutions.

Area Detector Software

J.W. Pflugrath [in collaboration E.M. Westbrook, Argonne National Laboratory]

In the past year, the device-independent area detector software system MADNES was adapted for use on the larger CCD-based detector of M. Strauss and E. M. Westbrook of Argonne National Laboratory for use at synchrotrons. This detector, which has 1024 by 1024 pixels, is being tested at beamline X8C of the National Synchrotron Light Source.

Three-dimensional Structure of Bacteriophage T7 Lysozyme

X. Cheng, J.W. Pflugrath [in collaboration with X. Zhang and F.W. Studier, Brookhaven National Laboratory]

T7 lysozyme is a unique enzyme in several ways. (1) Most of the well-studied lysozymes, such as hen egg white and phage T4 lysozymes, are endoacetylmuramidases that cut the glycosidic bond between sugar residues of NAM and NAG on the peptidoglycan layer of bacterial cell walls. T7 lysozyme, however, is an *N*-acetylmuramyl-L-alanine amidase that cuts the amide bond between peptide and sugar. It has recently been identified to be a Zn^{++} metalloenzyme. (2) T7 lysozyme can specifically bind to T7 RNA polymerase and inhibit its transcription. Therefore, this protein has been used in an inducible T7 expression system to reduce the basal level of target gene expression and allow the cloning of relatively toxic genes. (3) Unlike other phage lysozymes that are synthesized late in phage infection and simply perform cell lysis, T7 lysozyme is expressed early and is involved in the transcription shutoff and DNA replication, as well as in lysis and progeny phage release. These unique properties of T7 lysozyme make it an interesting protein for structural analysis.

CRYSTALLIZATION

T7 lysozyme is a 17-kD protein encoded by T7 gene 3.5. It has been cloned, overexpressed, and purified to homogeneity. However, extensive trials to obtain the crystal of wild-type T7 lysozyme have been unsuccessful. X. Zhang and F.W. Studier constructed a lysozyme mutant (named AK6) that deletes amino acids 2 through 5 (Arg-Val-Gln-Phe) from the amino terminus. The AK6 mutant has normal cell wall cutting activity but is completely unable to bind or inhibit T7 RNA polymerase.

Optimum conditions to crystallize AK6 mutant were found: 5 µl of 10 mg/ml protein solutions were mixed with the same volume of 20% (w/v) polyethylene glycol 4000, 50 mM ammonium sulfate, and 50 mM sodium citrate at pH 6.6 and equilibrated versus 1 ml of the latter solution. At 16°C, small crystals began to appear within 10 minutes and reached a size of 0.5 x 0.6 x 1.5 mm overnight (Fig. 1). After crystals of AK6 mutant were obtained, we crystallized wild-type lysozyme by microseeding with crushed AK6 crystals. The size of the wild-type crystal reached about 0.3 x 0.4 x 0.6 mm after 3–4 weeks, whereas the same size crystal was obtained after 1 week when the wild-type crystals were subsequently used for microseeding. No crystals of the wild-type lysozyme were ever observed without microseeding.

The crystals displayed space group $C222_1$ with cell dimensions a = 46.5 Å, b = 62.5 Å, and c = 110.0 Å. There is one molecule in each of the eight asymmetric units. The crystals diffracted beyond 2.2 Å and were stable in the X-ray beam for 6 days.

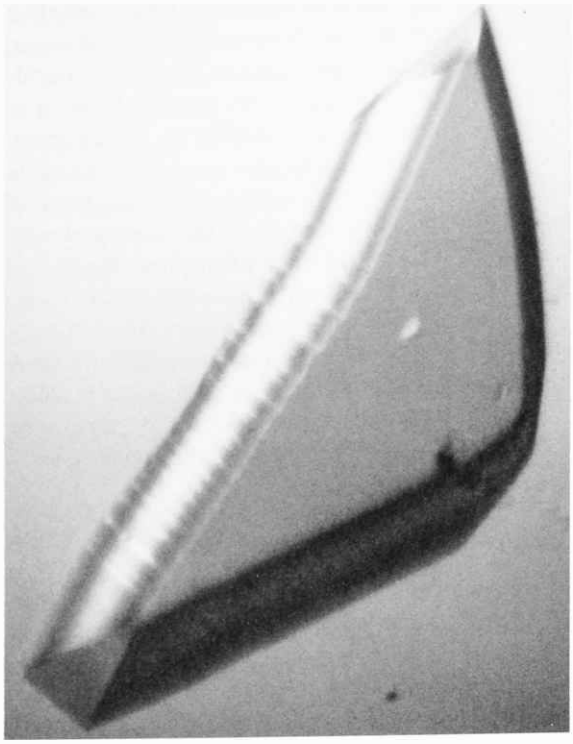

FIGURE 1 Crystal shown is from AK6 mutant lysozyme from bacteriophage T7.

HEAVY ATOM DERIVATIVE, ELECTRON DENSITY MAP, AND REFINEMENT

We obtained two mercury derivatives by cocrystallizing protein with mercury chloride and soaking native crystals in *p*-chloromercuriphenyl sulfonate. Both showed the same single mercury site in isomorphous difference Patterson and anomalous Patterson maps (Fig. 2). Initial 2.5 Å phases were obtained by multiple isomorphous replacement from these two derivatives, along with mercury anomalous scattering signals. The overall figure of merit was 0.80 at 2.5 Å resolution. The initial 2.5 Å phases produced electron density maps that enabled us to trace the polypeptide backbone and fit the complete sequence of 146 amino acids. The crystal structure was subsequently refined to the current crystallographic R factor of 21.5% without water molecules for reflections between 10 and 2.2 Å resolution with the use of restrained refinement and molecular dynamics by the program X-PLOR.

PROTEIN STRUCTURE

The most striking feature of the T7 lysozyme is a Zn^{++}-containing cleft built by three α-helices, five β-strands, and four loops (Fig. 3). The five β strands (β1: residues 14–18; β2: residues 48–51; β3: residues

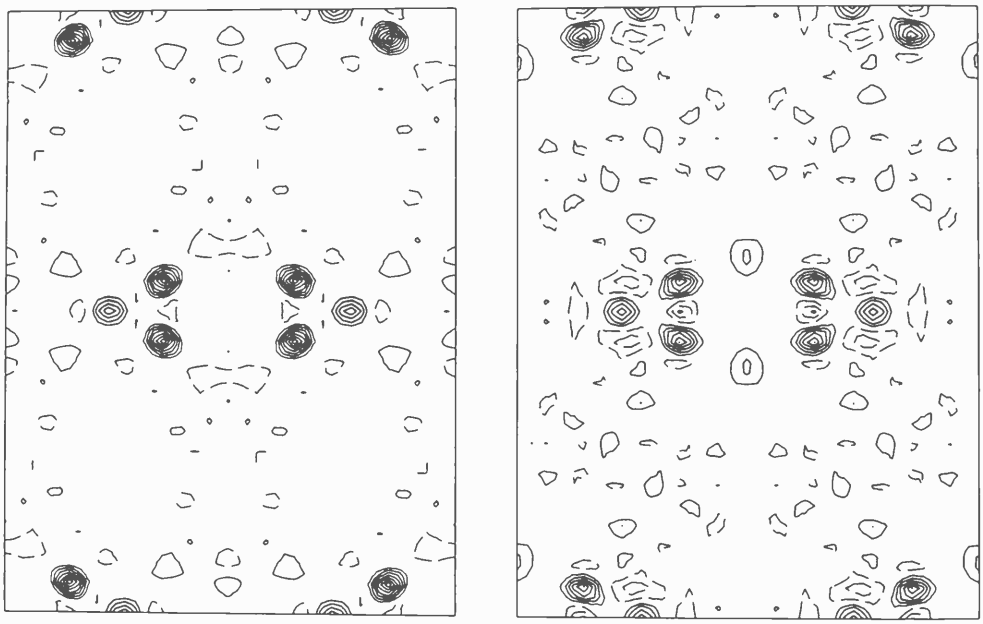

FIGURE 2 Harker section at $w = 1/2$. Peaks show mercury sites in isomorphous difference Patterson map (*left*) and anomalous Patterson map (*right*).

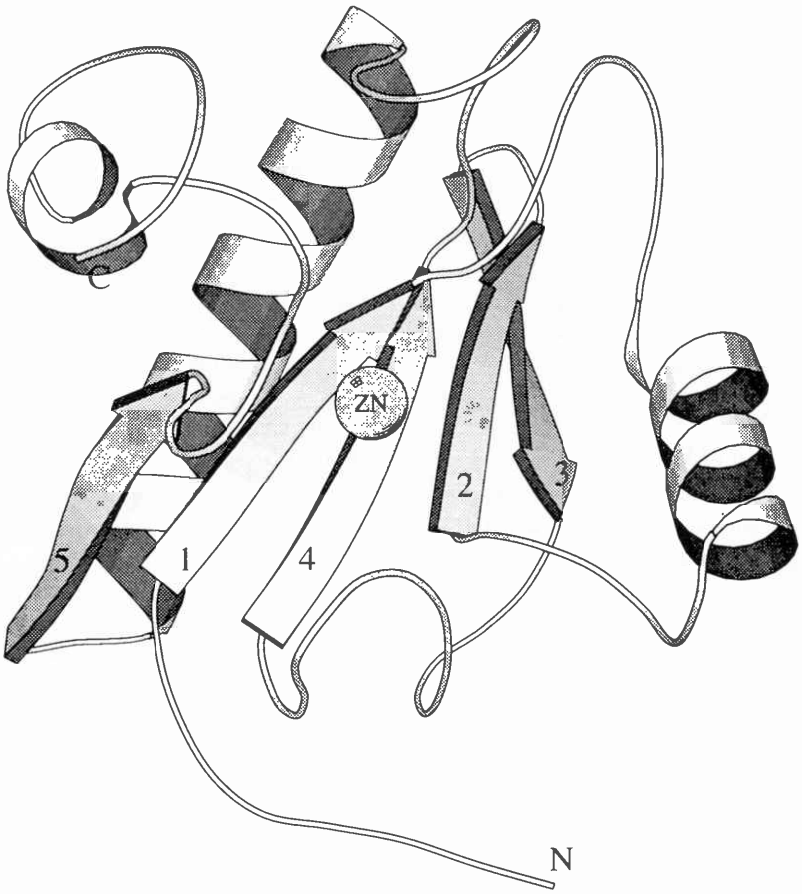

FIGURE 3 Topological diagram for the structure of T7 lysozyme. The central region contains five adjacent β strands, numbers 5, 1, 4, 2, and 3. The active-site zinc atom is bound to side chains of His-122 and Cys-130, which are part of the loop region outside strand β5. In addition, His-17, which is the last second residue of strand β1, is also bound to zinc. (Figure made by Mol Script [Kraulis, *J. Appl. Cryst. 24:* 946 (1991)].)

55–59; β4: residues 77–81; β5: residues 117–121) form a sheet: β1, β2, β4, and β5 are parallel, and β2 and β3 are arranged in an antiparallel hairpin structure. This sheet constitutes the lining of the bottom of the catalytic cleft. The long, 18-residue helix (α2: residues 98–115) is underneath this sheet. The four loops (*l*1: residues 29–47; *l*2: residues 60–76; *l*3: residues 82–96; *l*4: residues 122–134) form the two edges of the catalytic cleft. Loops *l*1 and *l*2 are on one side facing loops *l*3 and *l*4 on the other side. The overall dimension of the cleft is 22–26 Å long, 10–11 Å deep, with two open ends of different widths. Loops *l*1 and *l*3 form the wider end (20 Å), and loops *l*2 and *l*4 form the narrower end (10 Å). The helix α1 (residues 29–39) is part of loop *l*1, and α3 (residues 135–139) is near the carboxyl terminus.

A zinc cofactor was identified in the cleft that is likely to be the substrate-binding site. Recent biochemical study showed that the T7 lysozyme requires Zn^{++} for its cell-wall cutting activity but not the transcription inhibition activity, suggesting a catalytic role, rather than structural role, for the Zn^{++}. The zinc atom is coordinated by three protein ligands His-17, His-122, and Cys-130, and Tyr-46 is connected to Zn^{++} through a fourth ligand (possibly an ordered solvent molecule). Potential possible catalytic residues within the cleft are His-36, Gln-39 on one side and Lys-128 on the other. The side chains of

His-36, Gln-39 and Lys-128, have the right orientation to interact with the bound substrate molecule. As expected, no homology is found by comparing this metalloenzyme with hen egg white and T4 lysozymes in amino acid sequences, the respective backbones, or the presumed modes of catalysis. We think that the catalytic mechanism of T7 lysozyme is similar to that of zinc proteases such as carboxypeptidase A and thermolysin.

Mutants of T7 lysozyme that have decreased activity to inhibit T7 RNA polymerase but retain catalytic activity have been located in the amino terminus and residues Arg-33, Gly-40, and Leu-42. Arg-33 is within the helix α1, and together with Gly-40 and Leu-42, it is part of the loop *l*1 that resides on the surface of the molecule. All of these three residues face away from the catalytic crevice, and the possible catalytic residues in loop *l*1 all face inward. The distance between the amino terminus and this region in loop *l*1 is about 10 Å in the AK6 mutant that lacks the interaction with T7 RNA polymerase. It is conceivable that the additional four amino acids in the wild-type protein will bring the two regions closer and form a surface that specifically interacts with T7 RNA polymerase.

Crystallization and Preliminary X-ray Diffraction Studies of the M·*Hha*I Methylase

X. Cheng, J.W. Pflugrath [in collaboration with S. Kumar and R.J. Roberts, Cold Spring Harbor Laboratory]

The M·*Hha*I methylase from the bacterium *Haemophilus haemolyticus* catalyzes the transfer of a methyl group from *S*-adenosylmethionine to C-5 of the internal cytosine in the tetranucleotide DNA sequence GCGC. We searched for suitable M·*Hha*I crystallization conditions using the hanging-drop vapor diffusion technique combined with a sparse matrix screening method. We discovered crystals growing after 1 day from polyethylene glycol at 16°C. After careful refinement of precipitant, salt concentrations, and pH, we were able to grow crystals of the same morphology at room temperature as well. The crystals grown at higher temperature are larger in size and reached a maximum size of approximately 0.9 x 0.3 x 0.1 mm after 1 week. These conditions have consistently yielded crystals from separate preparations of the protein. Since the M·*Hha*I protein was purified and crystallized in the presence of *S*-adenosylmethionine, we believe that the crystal obtained is possibly a more informative cocrystal between M·*Hha*I and *S*-adenosylmethionine, although this has not yet been confirmed.

X-ray diffraction data were collected from single crystals on our FAST area detector attached to a GX-21 X-ray generator producing monochromated CuK_α radiation (1.54 Å). Crystals remain stable in the X-ray beam for at least 3 days. At a crystal-to-detector distance of 90 mm and swing angle of 20°, we were able to complete our native data set to 3.0 Å (>90% completeness) and partially complete to 2.5 Å (>60%) from two crystals.

Crystals were of monoclinic space group $P2_1$ and have unit-cell dimensions of a = 55.2 Å, b = 75.0 Å, c = 90.2 Å, and β = 103°. Assuming that the crystals have an average packing density of approximately 2.4 $Å^3$ per dalton, the molecular weight of the asymmetric unit is approximately 76,000 (= 2 x 38,000). This is close to twice the predicted molecular weight (37,000) of one polypeptide of M·*Hha*I methylase and suggests that there are two molecules in the asymmetric unit. Experiments are under way to search for useful heavy atom derivatives and to cocrystallize the protein with oligonucleotides containing the recognition sequence GCGC.

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NUCLEIC ACID CHEMISTRY

R.J. Roberts A. Dubey, S. Klimasauskas, C.-L. Lin, D. Roberts, M. Sha, J. Earle-Hughes, S. Kulakauskas, D. Macelis, G. Otto, M. Wallace, S. Klein, S. Kumar, C. Marcincuk, J. Posfai

One major research effort in this laboratory is directed at m5C-methylases, enzymes that are common to organisms ranging from bacteria to mammals. The function of the methylases is to transfer a methyl group from *S*-adenosylmethionine (SAM) to the C5 position of a cytosine residue in a specific double-stranded DNA sequence. Methylated DNA sequences have many different in vivo biological functions. Prokaryotic m5C-methylases methylate the target sequences to prevent digestion by their cognate restriction endonuclease. In eukaryotes, m5C is mainly localized in CpG islands and may be involved in gene regulation and cell differentiation. From a biochemical and structural standpoint, relatively little is known about how m5C-methylases work. Comparison of the primary structure of prokaryotic m5C-methylases reveals an overall common architecture and also interesting differences: There are five highly conserved sequence motifs about 10–20 amino acids long and five moderately conserved motifs. We have recently shown that DNA sequence recognition specificity resides in the so-called variable region that lies between conserved motifs VIII and IX.

Sequence Specificity Domain of m5C-Methylases

S. Klimasauskas

Prokaryotic DNA methylases recognize the same short sequences on DNA as do the better-known restriction enzymes, which they usually accompany. To identify the region responsible for DNA sequence recognition in the mono-specific DNA methylases, we have constructed a series of hybrids between the mono-specific methylases, M·*Hpa*II (recognition sequence: Cm^{5}CGG) and M·*Hha*I (recognition sequence: Gm^{5}CGC), and tested the specificity of these hybrid enzymes. These constructions take advantage of the conserved sequence motifs that are present within the m5C-methylases so that the boundaries in the hybrids are defined by the sequence motifs. The first and the second junctions mark the boundaries of the so-called variable region located between conserved motifs VIII and IX, whereas the third junction separates motifs IX and X. Two approaches were used to make hybrid methylases. In the first approach, a plasmid was constructed with the two methylase genes in tandem and arranged in the same orientation. The polymerase chain reaction (PCR) was used to construct exact deletions from the plasmid containing both methylase genes such that the amino terminus of one methylase becomes joined to the corresponding carboxyl terminus of the second methylase. An alternative method (Splicing by Overlap Extension) used two PCR-generated intermediate fragments, one of which contained a 20-nucleotide extension overlap, so that fusion during PCR led to the production of the required hybrid.

Each methylase gene was transferred into the vector, pUHE25-2, that contains a *lacI*-repressible expression system driven by an early bacteriophage T7 promoter. Upon induction, it leads to hybrid methylase synthesis in amounts above 10% of total soluble proteins in the cell. The corresponding clones carrying the genes for the wild-type M·*Hpa*II and M·*Hha*I have been used in separate experiments for large-scale production, purification, and protein crystallization experiments (X. Cheng and S. Kumar, unpubl.).

All plasmid DNAs overexpressing the hybrid methylases were tested for their susceptibility to cleavage, in vitro, with the *Hpa*II and *Hha*I restriction enzymes. This allowed a determination of the extent of methylation in vivo. In the case of hybrids with detectable activity, the specificity as well as a qualitative estimation of the efficiency of methylation could be determined. For hybrids providing detectable protection against R·*Hpa*II, an additional direct measure of activity was performed. This analysis

took advantage of R·*Msp*I, an isoschizomer of R·*Hpa*II, which cleaves both methylated and unmethylated *Hpa*II sites. The R·*Msp*I fragments were ^{32}P-labeled at their 5′ ends and digested to mononucleotides, and the labeled mononucleotides were analyzed by thin-layer chromatography. The ratio of methylated and unmethylated cytosines at *Hpa*II sites, as estimated by this method, correlates well with the extent of protection of each hybrid against the action of R·*Hpa*II.

We have constructed 11 plasmids coding for hybrids between the two methylases containing various combinations of domain swaps. All of the hybrids proved to be considerably weaker methylases than their wild-type parents. Six were inactive by our assay, and five of them retained partial methylation activity. Despite the poorer enzymatic properties, the hybrids retained sequence specificity. Two of the constructs contained exact swaps of the variable regions, but in neither case were we able to detect methylation activity in vivo. Thus, we were not able to determine the specificity potential of this region directly. However, other hybrids allow us to define the boundaries of the specificity domain by excluding regions nonessential for this function. In all five cases, the specificity matched that of the parent methylase which contributed the variable region, located between conserved motifs VIII and IX. This was the only sequence held in common between the active hybrids and for the first time provides unequivocal evidence that the specificity determinants of the mono-specific m5C-methylases are located within the variable region. Correlation of the hybrid methylase structure with the efficiency of methylation suggests that conserved motif IX may interact with the variable region, whereas motif X most probably interacts with the amino-terminal half of the molecule.

Hybrids between the *Msp*I and *Hpa*II Methylases

M. Sha

The *Msp*I and *Hpa*II methylases both recognize the same DNA sequence, CCGG, but have different variable regions and methylate a different base in the recognition sequence. M·*Msp*I methylates the first cytosine and M·*Hpa*II methylates the second cytosine. To discover how the methylase determines which base to methylate within its recognition sequence, we have constructed a series of hybrids between M·*Msp*I and M·*Hpa*II and have tested their recognition specificity.

Many hybrids have been constructed, but two have proved highly informative. HM1 and HM2 are two hybrid methylases designed to share the same sequence except for the variable regions. HM1 contains the amino-terminal region of the *Hpa*II methylase up to the variable region and continues with the variable region and carboxyl terminus of M·*Msp*I. HM2 also contains the amino-terminal region of the *Hpa*II methylase, but the M·*Msp*I sequences are fused beyond the variable region so that only motif IX and the extreme carboxyl terminus are from M·*Msp*I. Thus, the two hybrids differ only in the source of their variable regions. An extensive series of experiments shows that HM1 has *Msp*I methylation specificity and HM2 has *Hpa*II methylation specificity.

Nearest-neighbor experiments indicate that HM1 methylates the first C in the CCGG sequence and HM2 methylates the second C in the CCGG sequence. We also compared the relative transformation efficiency of the hybrid methylases into *Escherichia coli* strains having either *mcr*A or *mcr*BC backgrounds. HM1 was restricted in the *mcr*BC strain about 1000-fold (*Msp*I is restricted about 10,000-fold) but not in the *mcr*A strain. HM2 was restricted in the *mcr*A strain about 5-fold (*Hpa*II is restricted about 3000-fold) but was not restricted in the *mcr*BC strain. Hybrid methylases HM1 and HM2 are weakly active in comparison with their wild-type progenitors as demonstrated by an in vitro methyl group transfer assay. It is likely that this low activity is caused by imperfections in the final folding and raises the possibility that mutations could be found that will lead to increased activity by restoring the proper folding. Such mutants may reveal intermotif interactions. Random mutagenesis and selection to find such mutants is in progress.

DNA Binding by the *Msp*I Methylase

A. Dubey

The *Msp*I methylase (M·*Msp*I) recognizes the sequence CCGG and catalyzes the formation of 5-methylcytosine at the first cytosine residue. We have

investigated the sequence-specific DNA-binding properties of M·*Msp*I using gel-mobility shift assays and DNase I footprinting. M·*Msp*I binds to DNA in a sequence-specific manner either alone or in the presence of the normal methyl donor *S*-adenosyl-L-methionine as well as the analogs, sinefungin and *S*-adenosyl-L-homocysteine. M·*Msp*I shows the highest binding affinity to DNA containing a hemimethylated recognition sequence (K_d = 3.6 x 10^{-7} M) but binds less well to unmethylated DNA (K_d = 8.3 x 10^{-7} M). Surprisingly, it shows specific, although poor, binding to fully methylated DNA (K_d = 4.2 x 10^{-6} M). M·*Msp*I binds approximately 100-fold more tightly to DNA containing its recognition sequence, CCGG, than to nonspecific sequences in the absence of cofactors. In the presence of *S*-adenosyl-L-methionine, *S*-adenosyl-L-homocysteine, or sinefungin, the discrimination between specific and nonspecific sequences increases greatly. During binding, equilibrium is reached so rapidly that the normal kinetic properties cannot be studied using a gel-shift assay. DNase I footprinting studies indicate that 16 base pairs of DNA are covered by M·*Msp*I, with the recognition sequence CCGG located asymmetrically within the footprint.

Purification and Crystallization of M·*Hha*I

S. Kumar

No previously characterized DNA-binding motifs have been identified within the group of m5C-methylases, suggesting that a novel method is used for DNA binding and sequence recognition. For several years, we have been attempting, unsuccessfully, to obtain crystals of an m5C-methylase that might be suitable for structure determination by X-ray crystallography. Our most recent effort with one of the smallest methylases known, M·*Hha*I (recognition sequence: GCGC), has been spectacularly successful.

M·*Hha*I (M_r = 37,000) was purified from an IPTG-inducible overexpression strain constructed by S. Klimasauskas. An unusual property of the enzyme was exploited to greatly simplify the purification and to enhance the yield of M·*Hha*I. After induction and cell lysis, the majority of the enzyme (>70%) remained associated with the disrupted cellular debris. Back-extraction of this pelleted material with high-salt (0.4 M NaCl) buffers selectively solubilized M·*Hha*I. The resulting soluble fraction (termed S2) was greatly enriched for M·*Hha*I, which made up approximately 80% of the fraction. Nucleic acids were removed by protamine sulfate precipitation, and the cleared S2 supernatant was fractionated by ion-exchange chromatography on a Mono-S (cation exchange) FPLC column. This single column procedure yielded 0.75–1.0 mg of M·*Hha*I per gram of bacterial cell paste, with 95% purity or better. Remaining contaminants could be removed by an additional ion-exchange chromatography step using a Mono-Q (anion exchange) FPLC column.

A significant finding made during the development of the purification scheme was that M·*Hha*I exists as a heterogeneous mixture of two forms. On Mono-S columns, M·*Hha*I was observed to elute as two peaks: one containing the enzyme bound with its cofactor, *S*-adenosylmethionine (AdoMet), the other without AdoMet. This was based on the observation that M·*Hha*I from the "AdoMet-bound" peak was capable of methylating DNA in vitro in the absence of exogenous AdoMet. Addition of exogenous AdoMet to the cleared S2 fraction at micromolar concentration prior to FPLC chromatography yielded a single peak, presumably consisting of a homogeneous preparation of AdoMet-bound M·*Hha*I. The binding of AdoMet to the methylase was sufficiently stable to survive chromatography on the optional Mono-Q FPLC column.

Crystallization of purified M·*Hha*I bound to AdoMet and preliminary X-ray diffraction studies were carried out in collaboration with Xiaodong Cheng in Jim Pflugrath's laboratory (see this Section). Crystals were obtained in a remarkably short time using the hanging drop vapor-diffusion method and reached a size of approximately 0.9 x 0.3 mm. AdoMet-bound M·*Hha*I crystallizes in space group $P2_1$ with unit cell dimensions: a = 55.2 Å, b = 75.0 Å, c = 90.2 Å. The asymmetric unit appears to consist of two molecules of M·*Hha*I. Crystals diffracting to 2.5 Å have been obtained.

Currently, work is continuing toward the determination of the crystal structure of the AdoMet-M·*Hha*I complex. We are also attempting to cocrystallize the enzyme with DNA substrates and competitive inhibitors (*S*-adenosyl homocysteine and sinefungin). In parallel, biochemical analysis of cofactor binding by site-specific mutagenesis is also planned. The latter studies will use comparative sequence analysis to predict AdoMet-binding sites within the protein to targeted for mutagenesis.

Selection of Mutant Restriction Endonuclease and Methylase Genes

S. Kulakauskas

Prokaryotic m5C-methylases show ten conserved sequence motifs. To understand the function of these motifs and to study interactions between them, it would be useful to have a system that would allow the in vivo selection of mutations in methylase genes and that would also allow the subsequent isolation of suppressor mutations. To this end, a plasmid carrying the *Hpa*II restriction-modification system has been used to obtain temperature-sensitive mutants in the *Hpa*II methylase gene. We expected that at elevated temperature, the function of the methylase would be partially or fully inactivated, causing the cellular DNA to become sensitive to the action of the restriction endonuclease in vivo. Such mutants would die by self-restriction at 42°C but would survive at 30°C where the methylase would be fully active. An alternative, and equally interesting, thermosensitive phenotype could result from a change in the specificity of the methylase or the restriction endonuclease.

After mutagenesis with hydroxylamine, the plasmid carrying the *Hpa*II restriction endonuclease and methylase genes was transformed into *E. coli* cells, and transformants were tested for the thermosensitive phenotype by replica-plating. Candidates were analyzed by introducing a deletion mutation into the restriction endonuclease gene in vitro and testing the thermosensitivity of these new constructs. All of the initial mutants lost their thermosensitive phenotype, confirming that the restriction endonuclease was responsible for cell death. Plasmid DNA was prepared from the deletion mutants grown at 30°C and 42°C and then challenged with the *Hpa*II restriction enzyme in vitro to test for methylation. Only 1 of 11 mutants analyzed contained DNA that was differentially methylated at the two growth temperatures. Sequence analysis has shown that this mutant contained a C→T transition in conserved motif 2 causing an amino acid change from cysteine to tyrosine. No function has previously been assigned to this motif. We are now selecting second-site suppressor mutations that may give insight into the internal interactions among motifs. The remaining 10 thermosensitive mutants are also being analyzed. Future experiments will concentrate on the isolation of similar mutations in the *Hha*I restriction-modification system, now that crystals of the *Hha*I methylase are available.

An interesting finding has emerged from our studies of hybrids between the *Hpa*II and *Hha*I methylase genes (Klimasauskas et al. 1991). Two hybrids, H3 and H4, can effectively repress β-galactosidase activity in a strain carrying a *metF*::*lacZ* fusion after the hybrid methylase genes are overexpressed in vivo. However, other hybrids and the wild-type methylase show no such effects. This phenotype was not abolished by the introduction of a complementing *metF*$^+$ plasmid, indicating that it was not merely the result of methionine starvation. The sequence of the *metF* gene contains nine sites with the sequence CCGG, the recognition sequence for both the H3 and H4 hybrids. The effect is *metF*-specific since replacement of the *metF*::*lacZ* fusion and mutation of the *lacZ* gene to wild type did not lead to repression of β-galactosidase activity. Our current hypothesis is that the hybrids, H3 and H4, bind tightly to the CCGG sites but are defective in the catalytic step of methylation. Their continued presence bound to the DNA of the *metF* gene could interfere with transcription, causing a decrease in β-galactosidase expression. We are presently testing this idea and using it as a means of selecting mutant methylases.

We are constructing a system for the selection of mutant *Hpa*II methylase genes (or other hybrids) using the toxic effect of the *Bacillus subtilis sacB* (levansucrase) gene. A 79-bp oligonucleotide, containing four CCGG sites, the *Hpa*II recognition sequence, was inserted between the promoter and the coding sequence of the *sacB* gene. Expression of the levansucrase gene makes *E. coli* sensitive to sucrose; *sacB*-containing cells cannot grow in the presence of 5% sucrose. Cells containing both the *Hpa*II methylase gene and this *sacB* gene construct will allow us to select methylase mutants in vivo. Mutant methylases that can bind tightly should block expression of *sacB* and allow cell growth on sucrose-containing media.

Representation and Detection of Sequence Motifs by Artificial Neural Nets

J. Posfai

A question of great interest to molecular biologists concerns the meaning of similar sequence segments

or motifs in different proteins. These are often described as consensus patterns, sequence signatures, or weight matrices, and heuristic rules are used to find them. No matter which of the current methods is chosen, many arbitrary assumptions are usually made when defining them. One new technique, that of artificial neural nets, requires fewer assumptions and imposes few restrictions on possible answers. We are exploring the use of this technique for the identification of common sequence features.

Artificial neural nets were originally intended to simulate the structure and activity of a simplified brain. Units called "neurons" receive stimuli on their inputs, change their internal states and give state- and input-dependent responses as their outputs. Such neurons are organized into a "network," where outputs from neurons in one layer are connected to the inputs of neurons in the next layer. The system first undergoes a "teaching process" as known examples of some objects are presented to the input-layer neurons. The neurons in this layer respond to the stimulus and propagate a transform of their excitation pattern to the layer that lies below, the so-called "hidden" layer. The signals then propagate to the third (output) layer, and the outputs of this layer constitute the network's response to the stimulus. This output pattern is compared to the known answer, and to make them match, the connections between the neurons are modified by a learning algorithm. After repeated rounds of teaching, the connections in the system usually stabilize, and the system is expected to then give mostly correct answers not only to the examples of the teaching set, but also to previously unseen examples.

Using this technique, we have studied protein sequence segments that contain disulfide-bond-forming cysteines. Because disulfide bonds occur in hundreds of sequences, neither a simple consensus pattern nor a simple rule can describe why some cysteines form disulfide bridges and others do not. For the training set, we chose SWISSPROT entries that had full and definite annotations on disulfide bridges. Segments of 11 amino acids, centered on the disulfide-bond-forming cysteines, were selected for the teaching set of true examples. Eleven banks of 20 neurons formed the input layer of the network, one bank being assigned to each amino acid position and every bank allowing a unipolar representation of the 20 possible amino acids. Forty neurons, each connected to every input neuron and to both output neurons, constituted the middle layer. Two output neurons represented the YES and NO answers to the question: Can the cysteine in the center of the input segment form a disulfide bond? The outputs on these neurons had continuous outputs in the range of [0,1], high output values meaning good chances either for YES or NO. A standard back-propagation technique was selected as the learning algorithm.

At the start of the teaching session, the internal states of the neurons and the strengths of the connections between the neurons are initialized by random values. One by one, the 640 examples of the training set are presented to the network. After each example, the connections in the network are adjusted, according to back-propagation rules, to match the actual response to the expected one. After 26 rounds over the 640 examples, the network learned the patterns of the teaching set and was able to recognize all the patterns, at a tolerance level of 0.1. Previously unseen patterns of the test set were characterized correctly in 75% of the cases, at a tolerance level of 0.4. Despite its simple structure, the network has acquired the ability to recognize a fuzzy sequence feature and is able to make predictions about this feature.

An expansion of the input layer, using longer sequence segments, combined with an expansion of the hidden layer should improve the performance of the network. A more substantial modification would take into account the sequence neighborhood of the recipient cysteine as well. Another extension might replace the 20-neuron banks by banks of three times four neurons, to match the genetic coding scheme exactly. The major problem with this approach comes during the teaching phase. One cannot be completely confident about the false examples, since the absence of a suitable recipient cysteine may leave an otherwise satisfactory disulfide-bridge-forming cysteine unpaired and would lead to the incorrect recording of a false example. The replacement of the "supervised" teaching method by an "unsupervised" one may solve the problem.

Error Detection in DNA Sequences

R.J. Roberts, J. Posfai, C. Lin

We have developed a program that can detect frameshift errors within coding regions of DNA se-

quences. The program is based on the idea that an insertion or deletion error present within a coding sequence leads to the interruption of a reading frame, meaning that the correct translation of that DNA sequence would require one or more frameshifts. If the coding sequence is similar to that of a known protein sequence, then such frameshift errors can be detected by comparing the conceptual translations of the new DNA sequence in all six reading frames with every sequence in the protein sequence database. The program that implements these ideas is called DETECT and can serve as an aid to an experimentalist who is determining a new DNA sequence. This program allows obvious errors to be located and suggests regions of a sequence where new data should be collected before the sequence is considered "finished."

The output from the program is both a graphic display showing the relative locations of matching segments that serve as the basis for detecting a potential error and a more detailed listing of the precise location of these segments within the DNA sequence. The graphic output is extremely informative when a sequence contains more than one frameshift error, since the errors and their location are immediately obvious. In many cases, the combination of matching sequence segments in different reading frames and the position of termination codons within those reading frames can lead to very precise pinpointing of the location of the error.

We have tested the program using both raw experimental data and sequences from the EMBL database that are known to contain frameshifts. We have also tested it extensively using unidentified open reading frames that flank known annotated genes in the GENBANK database. In the latter series of tests, many potential errors have been found, and in some cases, functions can be suggested for the "corrected" versions of the reading frames on the basis of their similarity to known gene products. Clearly, as more DNA sequences are determined and new genes are identified, the power of this approach will increase substantially. It should be noted that the use of this program can also identify features of sequences that are of more than usual biological interest. For instance, ribosomal frameshifting is known to account for bona fide products in many instances. Such frameshifts will also register as potential errors during our analysis as will some sites of posttranscriptional editing. Similarly, some pseudogenes or intron-containing genes will also register as hits by our program.

Comparative Methods of Sequence Analysis

G. Otto

We are continuing our work on probabilistic methods of comparative sequence analysis. The major focus is on the comparison of protein sequences sharing a common function. The goal of these comparisons is to define significant sequence patterns that correlate with and are predictive of function. In this context, functions are taken to be specific ligand-binding activities. The elements of the derived patterns are often structurally relevant, tending to be those amino acids in close contact with the bound ligand. There is a large element of phylogenetic analysis in these methods. It has become clear that the best representation of sequence similarities predictive of function is actually a phylogenetic tree of related sequence patterns. A single, maximally inclusive pattern is just the root of this larger tree. Beyond the pragmatic goal of predicting protein function it will be interesting to examine the set of these pattern trees for similarities that might reflect the evolution of protein diversity.

Our work on comparative methods involves a number of mathematical issues. We departed from standard comparative techniques by defining the measure of similarity as the probability that an optimal alignment of sequences is due just to the random matching between unrelated sequences. We proposed a basic, empirical test that any probabilistic measure must satisfy. An estimator of these probabilities for pairwise comparisons was developed that satisfied the test quite well. This measure has been incorporated into a program for the comparison of one sequence with a database and has been generalized to the case of multiple sequence comparisons, providing an estimate of the probability of a given multiple alignment occurring among a random set of proteins. We have also developed a probabilistic measure for the matching of consensus patterns with protein sequences and have written a program that searches a database for pattern matches of varying qualities. However, many new developments are required for this approach to achieve its full power.

The sheer volume of sequence data and the additional structure imposed on these data by biological theory and experiments have generated considerable interest among mathematicians and computer scientists. It is also recognized that the measure of sequence similarity arises in many fields and is of gen-

eral scientific interest. We are beginning collaborations with a number of these mathematical scientists with the aim of developing a coherent interdisciplinary effort in the New York area.

Identification of Functional Domains of Integration Host Factor

D. Roberts

Integration host factor (IHF) is a site-specific DNA-binding protein of *E. coli*. Binding to DNA generates a sharp bend of 140° (Kosturko et al., *Nucleic Acids Res. 17:* 317 [1989]; Thompson and Landy, *Nucleic Acids Res. 16:* 9687 [1988]). IHF is a heterodimer composed of two homologous subunits, IHFα and IHFβ, that are encoded by two separate genes, *himA* and *hip/himD*. IHF is required for a variety of cellular processes (for review, see Friedman, *Cell 55:* 545 [1988]), including integration, excision, and packaging of bacteriophage λ, replication of plasmid pSC101, partitioning of phage P1, and transposition of Tn*10*. We have been interested in understanding the structural elements of IHF that are responsible for the specific binding and bending of DNA.

We have mapped functional domains of IHF by analysis of point mutations and hybrid proteins. Point mutations were isolated either by random mutagenesis and selection for loss of IHF function or by site-directed mutagenesis. Hybrid proteins were constructed by exchanging segments between IHFα and IHFβ. The construction of hybrids was guided by the crystal structure of the homologous protein HbS (Tanaka et al., *Nature 310:* 376 [1984]; White et al., *Proteins 5:* 281 [1989]). In each subunit of this structure, there are two amino-terminal α helices and one carboxy-terminal α helix that form a globular base that supports a flexible arm. For IHF, it has been proposed that the arms fit into the minor groove of DNA, and additional protein/DNA contacts support the bending of the DNA around the protein (Yang and Nash, *Cell 57:* 869 [1989]).

Our results are consistent with (and add to) the structural model. (1) Amino acid substitutions in the IHF arms reduce IHF activity, consistent with a role for the arm in DNA binding. However, the arms of IHFα and IHFβ are not identical. IHF requires at least one α-like arm for activity, and amino acid substitutions in IHFβ are better tolerated than changes in IHFα. We are currently testing the idea that the sequence specificity resides in the IHFα arm and the β arm provides nonspecific binding. (2) The carboxy-terminal α helix is also important for IHF function. Mutations of basic amino acids in this region reduce IHF activity. In contrast to the flexible arm, this helix is interchangeable between IHFα and IHFβ. We are testing the idea that the basic amino acids in this region provide nonspecific contacts for DNA bending.

Restriction Endonucleases

J. Earl-Hughes, S. Klein, D. Macelis, R.J. Roberts

The collection of type II restriction endonucleases continues to grow and more than 2000 have now been characterized; 179 different specificities are known. During the last year, we have characterized a number of new restriction endonucleases, although none of these have provided new specificities. In light of the increasing difficulty of finding new enzymes with interesting recognition sequences, we have recently discontinued this program of research, which first began in 1973. Future efforts will focus on the use of recombinant DNA methods to alter the specificity of existing enzymes.

The restriction enzyme database, REBASE, continues to grow and provide primary information about restriction enzymes to researchers worldwide. During the last year, we have essentially completed the transfer of the data management system from ORACLE to SYBASE. In the new implementation, many additional fields, describing useful properties of enzymes, are present and much future work will be required to consolidate the known properties of restriction enzymes into the new database and to provide cross-references to other databases relevant to REBASE. We are just beginning to develop an interface into the SYBASE version that will allow online entry of information and extensive capabilities for browsing. We anticipate that during 1992, the ORACLE version of the database will disappear and be replaced completely by the SYBASE version.

In addition to electronic mailings of useful reports from the database, we have also implemented an anonymous FTP site at Cold Spring Harbor Laboratory that enables users to log on anonymously and ac-

cess information from REBASE. This has proved very popular, and many of our previous users have chosen the FTP route as the most appropriate to obtain data files. We also have plans to include REBASE on various CD ROMS that will be distributed widely through the National Library of Medicine and through EMBL.

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PROTEIN KINASE STRUCTURE AND FUNCTION

J. Kuret G. Carmel A. Desai F. Giorgini P. Kearney T. Mitcheson A. Vancura P.C. Wang

Protein kinases are important components of many regulatory pathways because they can integrate several input signals and coordinate a response through the phosphorylation of substrate proteins. In an effort to determine the common three-dimensional structure kinases adopt, and the implications of that structure for selective recognition of protein substrates and catalysis of the phosphotransferase reaction, we study TPK1, the *Saccharomyces cerevisiae* homolog of the cAMP-dependent protein kinase catalytic subunit. This kinase, along with its isozymes, regulates the ability of that organism to grow. In a separate project, we seek to elucidate the biological function of casein kinase-1, an enzyme found in all eukaryotic cells in which it has been sought. It is unrelated to casein kinase-2, which is described elsewhere in this volume. Our progress in applying the techniques of X-ray crystallography and molecular genetics to the study of protein kinases is described below.

Yeast Casein Kinase-1

A. Desai, J. Kuret, T. Mitcheson, A. Vancura, P.C. Wang

Casein kinases are enzymes that phosphorylate casein (an acidic, heavily phosphorylated family of proteins found normally in milk) efficiently. Although casein is an unlikely target for casein kinases in living cells, it is a useful and surprisingly specific tool in the isolation, assay, and characterization of these enzymes because few of the many protein kinases cloned or purified are capable of phosphorylating it. Two classes of casein kinases are recognized in eukaryotic cells, termed casein kinase-1 (CK1) and casein kinase-2. Our goal is to discover the biological role of CK1 using lower eukaryotes as a model system.

This year, we deduced the primary structures of two *S. cerevisiae* and two *Schizosaccharomyces pombe* CK1 cDNAs. Comparison of their structures

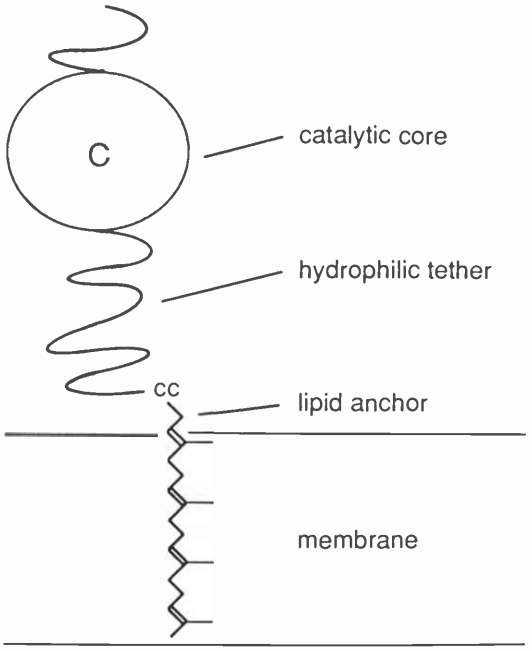

FIGURE 1 Proposed model of yeast CK1 structure.

reveals a conserved structural organization (Fig. 1). All four sequences begin Met-Ser- or Met-Asp and are probable substrates for N^{α}-acetylation. This is followed by a typical, 280-residue eukaryotic protein kinase domain that is highly conserved. Sequence similarity ends abruptly with a long stretch of hydrophilic residues that are predicted to be highly flexible in the folded protein. The final segment of these proteins contains a 12-residue sequence that includes a pair of carboxy-terminal cysteine residues. This region is similar to the carboxy-terminal sequences shared by members of the GTP-binding protein family, including *YPT1*, *SEC4*, *YPT3*, and *SAS1/SAS2*. In those proteins, carboxy-terminal cysteine residues are thought to be linked to prenyl groups through thioether bonds and contribute to membrane localization. In addition to cysteine residues, the carboxy-terminal regions of most of these proteins contain lysine residues that vary in number and distance from the cysteines. As in the case of cellular Ki-*ras*, these positively charged amino acids may assist in targeting proteins to membranes.

The catalytic core of yeast CK1 shares more than 50% sequence similarity with three mammalian CK1 isozymes and with HRR25, a protein kinase associated with repair of damaged DNA in *S. cerevisiae*. All of these enzymes share an unusual sequence motif in subdomain VIII, where the common peptide triplet Ala-Pro-Glu is replaced by Ser-Ile-Asn (Fig. 2). In the cAMP-dependent protein kinase, the third residue (Glu) of this triplet forms a salt bridge with a conserved arginine residue located in subdomain XI. Although the Asn substitution can no longer bond in this manner, it may still make contact with domain XI through hydrogen bonds. Not surprisingly, the invariant arginine residue normally found 11 residues carboxy-terminal to a conserved hydrophobic amino acid in domain XI is not apparent in this protein kinase family.

Subcellular fractionation in conjunction with Western blotting was used to determine whether CK1 is membrane-associated or soluble. To facilitate detection of the enzyme, CKI1 was tagged with the influenza hemagglutinin epitope recognized by monoclonal antibody 12CA5. The tag was placed at the amino terminus of CKI1 to avoid interference with the putative site of prenylation found at its carboxyl terminus. When cells expressing tagged CKI1 were lysed, boiled immediately in 2% SDS, and subjected to Western analysis, epitope-tagged CKI1 was detected as a 66-kD protein (Fig. 3, lane 3). As expected, this protein was not detected in strains that did not express the epitope-tagged CKI1 (Fig. 3, lane 4). When cells were lysed in the absence of SDS, epitope-tagged CKI1 underwent rapid proteolysis to smaller immunoreactive forms. The inclusion of protease inhibitors during cell lysis reduced but could not prevent proteolysis. Nevertheless, membranes prepared in the presence of protease inhibitors clearly contained intact epitope-tagged CKI1 along with a major proteolysis product of 52 kD (Fig. 3, lane 2). In cytosolic fractions, however, only the 52-kD fragment was detected and no intact epitope-tagged CKI1 (Fig. 3, lane 1). We conclude that epitope-tagged CKI1 associates exclusively with yeast cell membranes and that proteolysis occurring at the carboxyl terminus releases truncated forms of it into the cytosol.

As mentioned above, *S. cerevisiae* contains two CK1 genes, and we have named them *CKI1* and *CKI2*. To determine if CK1 supplied an essential function in yeast cells, we disrupted the *CKI* genes alone and in tandem. Although haploid spores that are deficient in *CKI1* or *CKI2* germinate and grow normally, spores that are *cki*$^{-}$ either fail to germinate or germinate and arrest as small cells prior to bud formation. Thus, although neither of the two *CKI* genes is essential by itself, at least one *CKI* gene is

FIGURE 2 Sequence alignment of CKI1, CKI2, and the cAMP-dependent protein kinase (bovine C_α). Sequences were first aligned using the FASTDB program, then adjusted by eye, and divided into 11 subdomains.

required for yeast cell viability. In the coming year, we will continue our genetic approach to CK1 function in both *S. cerevisiae* and *S. pombe*.

Yeast cAMP-dependent Protein Kinase

G. Carmel, F. Giorgini, P. Kearney, J. Kuret, J. Pflugrath
[in collaboration with R. Sweet, Brookhaven National Laboratory]

Despite clear amino acid sequence homology among members of the eukaryotic family of protein kinases, and the probable existence of a common enzymatic mechanism of phosphoryl transfer, each protein kinase possesses a unique (although potentially overlapping) recognition selectivity for protein substrates. For the handful of kinases studied in detail, the ability of a protein to serve as a substrate is determined by the amino acid sequence surrounding its phosphorylatable hydroxyamino acid (i.e., serine, threonine, or tyrosine), with the location and spacing of charged amino acids being especially important. Because we wish to understand the overall folding pattern of eukaryotic protein kinases and to identify the structural features of kinases that are involved in catalysis, regulation, and substrate selectivity, we initiated a crystallographic study of the well-characterized cAMP-dependent protein kinase catalytic subunit from yeast, TPK1.

In previous years, we described the overexpression, purification, crystallization, and derivatization of a truncated version of TPK1, termed TPK1Δ. The crystals grow to over a millimeter in length, are hexagonal dipyramids in shape, and diffract beyond 2.8 Å resolution. Because these crystals possess a very long unit cell, collection of X-ray diffraction data at Cold Spring Harbor Laboratory (CSHL) is

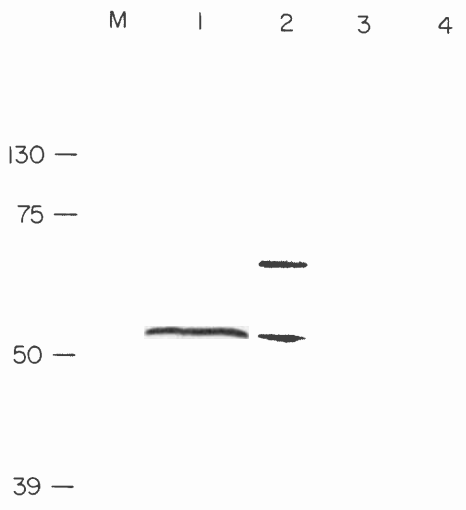

FIGURE 3 CKI1 is a particulate enzyme. Cytosol, membranes, and whole-cell lysates were subjected to Western analysis using monoclonal antibody 12CA5. (Lane *1*) Cytosol; (lane *2*) membranes; (lane *3*) whole-cell lysate prepared in 2% SDS with immediate boiling; (lane *4*) control lysate prepared in 2% SDS with immediate boiling from cells not expressing epitope tagged CKI1. Prestained markers (lane M) included phosphorylase b (130 kD), bovine serum albumin (75 kD), ovalbumin (50 kD), and carbonic anhydrase (39 kD).

difficult. To overcome this problem, we collaborate with Dr. Robert Sweet, who operates a beamline at the National Synchrotron Light Source at Brookhaven National Laboratory. The synchrotron delivers X-rays that are two orders of magnitude greater and of shorter wavelength than those available at CSHL, and it overcomes many of the physical problems associated with the analysis of crystals such as ours.

We will return to the synchrotron in the coming year and will collect complete and accurate native and derivative data sets. The resulting electron density map, combined with the coordinates of the recently solved mammalian homolog of TPK1, should allow us to bring this project to a successful conclusion.

A Solid-phase Screen for TPK1 Substrate Selectivity

G. Carmel, J. Kuret

Synthetic peptides of known amino acid sequence are useful in defining the substrate specificity of protein kinases. This is because their structures can be varied systematically to identify critical residues involved in recognition and because the contribution of these residues can be quantified through steady-state kinetics. Nevertheless, the synthetic peptide approach has limitations. First, the design of useful synthetic substrates requires a priori knowledge of a kinase's phosphorylation selectivity, which, of course, is frequently unknown. Second, peptide synthesis can be expensive and time consuming. A new approach that combines inexpensive synthesis of recognition sequences with an ability to screen them in complex mixtures is clearly desirable.

We have developed a strategy for producing and screening substrate recognition sequences in bacteria and have applied the method to TPK1Δ. The strategy uses cassette mutagenesis to create a phosphorylatable serine residue that is flanked on both sides by degenerate amino acids known to be important in substrate recognition, including arginine (e.g., cyclic nucleotide and calcium-dependent protein kinases), glutamic acid (e.g., casein kinase-2), and proline (e.g., $p34^{cdc2}$); also included are alanine, glutamine, and glysine residues, which contribute small hydrophobic, hydrophilic, and nonexistent side chains, respectively.

The carrier protein used to house the cassette is BCY1, the regulatory subunit of the cAMP-dependent protein kinase from *S. cerevisiae*. BCY1 is useful because it (1) contains a single phosphorylation site for TPK1 that is located in a well-characterized "hinge" region, (2) is expressed in *E. coli* at high levels in a soluble and dephosphorylated form, and (3) is easily purified (by cAMP-agarose affinity chromatography) to facilitate kinetic studies in solution.

The resultant library of recognition sites, housed within BCY1, is processed like other bacterial expression libraries and is "probed" with a purified protein kinase in a radiometric, solid-phase phosphorylation assay. Individual bacterial colonies that express a phosphorylatable sequence are thereby identified. The plasmid DNA from these colonies is isolated and sequenced through the cassette region to reveal the preferred location of amino acid residues involved in substrate recognition.

The results of a screen with TPK1Δ are summarized in Table 1. The pattern of deduced amino acid sequences is dominated by the requirement for arginine residues in positions p^{-3} (10/11) and p^{-2} (11/11). In addition, the p^{-1} position most frequently

TABLE 1 Deduced Substrate Selectivity of TPK1Δ

Clone	Deduced amino acid sequence							
Position:	−4	−3	−2	−1	p	+1	+2	+3
1	...Q	E	R	Q	**S**	E	Q	Q...
2	...E	R	R	G	**S**	A	Q	G...
3	...Q	R	R	A	**S**	R	A	G...
4	...G	R	R	R	**S**	G	R	G...
5	...Q	R	R	G	**S**	G	Q	A...
6	...A	R	R	G	**S**	Q	Q	G...
7	...Q	R	R	G	**S**	A	A	E...
8	...Q	R	R	P	**S**	Q	A	G...
9	...P	R	R	A	**S**	Q	P	E...
10	...P	R	R	G	**S**	E	A	E...
Amino acid	Frequency[a]							
A	1	0	0	**2**	–	2	4	1
R	0	**9**	**10**	1	–	1	1	0
Q	5	0	0	1	–	3	4	1
E	1	1	0	0	–	2	0	3
G	1	0	0	**5**	–	2	0	5
P	2	0	0	1	–	0	1	0

[a]Frequency refers to the number of times an amino acid is found in a given position. The data are derived from clones 1 through 10.

(8/11) contains the small residues alanine or glysine, whereas the p^{-4} position frequently (6/11) contains glutamine. Clear trends in other positions are not apparent. Nevertheless, some unfavorable substitutions are discernible. Proline residues are underrepresented at all positions except p^{-4}. Glutamic acid residues are acceptable carboxy-terminal to the phosphorylatable serine but rarely are they found on the amino-terminal side of this residue.

The consensus recognition sequence deduced for TPK1Δ by our method is similar to that established with synthetic peptides for the mammalian cAMP-dependent protein kinase. Consistent with this behavior in vitro, the mammalian kinase can substitute for TPK1 and its isozymes in vivo. That the selectivities of the two enzymes are so similar is surprising, considering they share only 50% sequence identity in their catalytic domains. The substrate selectivity screen described herein will complement our crystallographic efforts to characterize the structural features of TPK1Δ responsible for substrate recognition.

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COMPUTATIONAL MOLECULAR BIOLOGY

T. Marr W. Chang S. Cozza A. Reiner J. Salit M. Zhang

This marks the second year of the new Computational Molecular Biology group at Cold Spring Harbor Laboratory. Five people were recruited in 1991: Steve Cozza, a software engineer, joined the group in February; Bill Chang, a postdoctoral fellow, came to my laboratory in August—Dr. Chang received his Ph.D. in theoretical computer science from University of California, Berkeley; Andy Reiner, a visiting computer scientist, joined us in May; Jacqueline Salit, a software engineer, joined the group in September; and Michael Zhang, a postdoctoral fellow and theoretical physicist came to us in October from the Courant Institute of Mathematical Sciences at New York University. Our group is working on computerized methods for studying genome organization and function.

We are working on new database technologies, new analytic methods, and new software approaches that allow us to study the genomes of organisms at scales of resolution spanning several orders of mag-

nitude. The system will allow us to examine classes of sequences, such as repetitive elements, multi-copy genes, and motifs, from a global perspective so that we can get a handle on the gross patterns of distributions of distinct types of genetic elements. The system will also allow us to view high-resolution local genome organization, such as detailed contiguous maps (contigs) of regions containing genes or even sequence motifs embedded within sequenced genes or sequenced genomic segments.

When viewed as templates for information processing, genomes serve two primary functions: (1) templates for self-duplication and (2) templates from which collections of polynucleotides become processed in combinatorial ways. Nucleotide sequences accrue biological function and meaning at many levels of organization. Genes usually exist in one copy per genome (most genes) or they can exist in multiple copies per genome (e.g., histone genes). Genes sometimes form large co-evolved clusters within a genome, e.g., the mouse T complex. Many of these complexes are conserved between genomes; there are numerous examples of orthologous gene collections between humans and mice. In addition, there are many genes and gene families that are essentially identically conserved from yeasts to humans.

In addition to the more traditional view of genes, and how they are distributed within and between genomes, there exists a wide variety of evolutionarily conserved short sequences. These are sequences that, when studied in detail, perform one particular function and are reused in different combinations with other such sequences. We call these sequences short functional elements (SFEs). SFEs can be thought of as being the shortest sequences about which it makes sense to study, or *atomic elements* in database terminology. In general terms, SFEs include, but are not limited to, the following types of sequences: (1) sequences that are sites for the binding of proteins to DNA or RNA, such as sites that bind transcription regulatory proteins, sites that bind replication initiation proteins, and sites that bind translation inhibiting proteins; (2) sequences that affect the secondary and tertiary structures of DNA and RNA such as coiling in DNA or looping in RNA; (3) repetitive elements; (4) sequences that have intrinsic kinetic or thermodynamic lability, such as long runs of homo-purines or homo-pyrimidines; (5) sequences that in general are involved in the regulation of replication, transcription, and translation that are not mentioned above; (6) signal peptides such as those polypeptides that allow migration of a protein into the nucleus; (7) ligand-binding sites in proteins; (8) amino acid "motifs"; and (9) restriction sites.

Because nucleotide sequences accrue biological function and meaning at many levels of organization, it is useful to view genomes as a *sequence of sequences* (Fig. 1). Gene and/or genome mapping typically begins by knowing only partial information about the sequence of sequences associated with a particular gene or other target sequence(s). Finding the location(s) of a target sequence(s) in a genome involves stepwise refinement of characterization of the landmark sequences within or around the sequence of sequences.

We make a distinction between two basic types of maps: reference maps and working maps. Reference maps are maps that are, by scientific consensus, considered to reflect the best understanding of the relative order of, and/or distance between, various map elements. Reference maps fall into two basic classes, *landmark maps* and *marker maps*. Landmark maps are maps constructed from physically mapped elements, where the distance between elements is measured in units of base pairs. Examples of landmark maps are the standard cytogenetic banding maps used in mammalian genetics and the polytene salivary gland chromosome maps of the fruit fly. Marker maps are maps constructed from polymorphic markers ordered by genetic linkage analysis, where the distance between elements is measured in units of centiMorgans. We use reference maps primarily for navigational purposes, but also to provide us with reference intervals into which we place a variety of working maps and map elements.

Working maps and map elements are by far the largest number of objects that we need to deal with in the database. Fortunately, the variety of working maps and map elements is limited in number. We have also found that the organism-specific representations of all working maps and map elements vanish once one gets beyond the familiar reference landmark maps. Thus, working maps and map elements can be handled in a general way. The following is a list of objects, or named-fragments, that will be supported by our database and software: (1) genes, DNA segments, and polymorphics markers (loci); (2) DNA and protein sequences, including SFEs; (3) genomic restriction fragments and maps; (4) rearrangements, deletion segments, and breakpoints; (5) restriction maps of clones; (6) probed clones and contigs; (7)

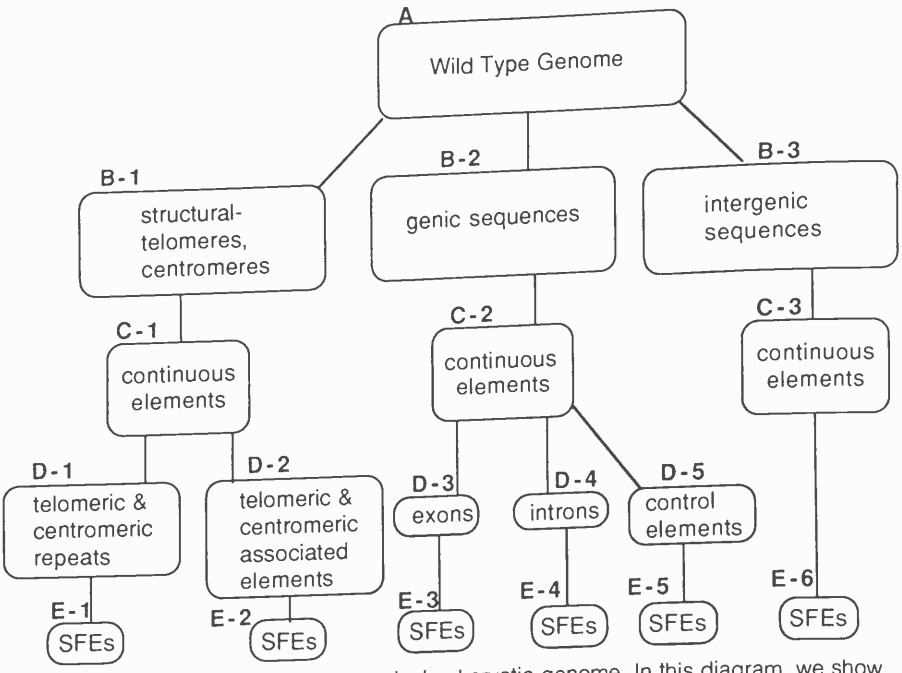

FIGURE 1 A hierarchical view of a typical eukaryotic genome. In this diagram, we show broad classes of types of DNA sequences. Note that we have not attempted to indicate the order of these sequences, but rather to show simple distinctions between them. Gene and genome mapping involves finding particular sequences embedded within or around landmark sequences. We are working on computer methods and software that allows users to examine locations and orderings of sequences across several orders of magnitude of resolution. SFE stands for short function element (see text for description of SFE).

probed somatic cell lines; and (8) composite collections and maps of these objects.

In addition to having the capability of storing the above information, provision is made so that users of the database can enhance the basic information in the database by being able to assign connections between (1) working maps, map elements, and reference landmark intervals and/or marker intervals; (2) objects placed in and between reference intervals (so that reference intervals can be bridged) and store these connections by name in the database; and (3) map objects and interval maps between different species.

Connections to reference maps are made by recording when a probe known to be within a reference interval is connected to a map element. So, by making successive connections between probed map elements and by observing which interval the anchoring probe resides in, the system can form contigs using a variety of map elements. This is simply another type of "fingerprinting" scheme, but we are using a variety of signals to detect underlying genome continuity by "walking" in random directions until reference probes are encountered and bridged. Some of these connections may be able to be done automatically, but our intention is to make the system highly interactive, so that experts in different biological domains can incorporate their knowledge into the system.

Progress on this system has been substantial. We have implemented prototype software for a significant portion of framework maps of the human genome using our new technology. In collaboration with David Beach (see Genetics Section), we have used our software to assemble a molecular map of most of the fission yeast genome. About 98% of a 2000 cosmid-clone library of the fission yeast has been assembled into contiguous regions, or contigs, since June of 1991. The collection of anchoring probes includes more than 70 genes and over 500 probes made from ends of inserts in the library. Figure 2 shows an example of the results obtained by using our software.

An example of how we utilize reference maps using the fission yeast data follows. Note in Figure 2

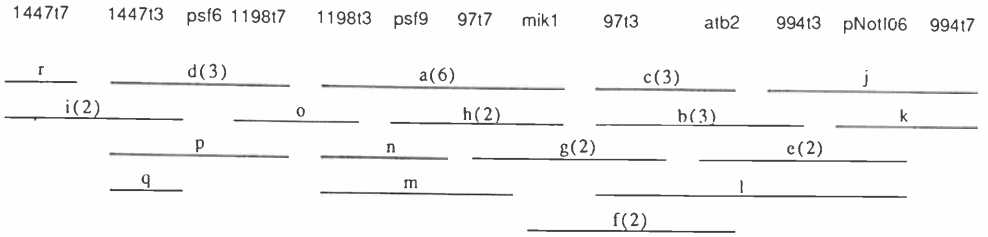

FIGURE 2 The top row shows the order of probes used to construct complex contigs for the fission yeast genome. The lines represent simple contigs that were constructed by hybridization. The letters on the lines are codes used to denote simple contigs, and the number in the parentheses shows the number of cosmid clones anchored by probes.

that we have two anchoring probes: the genes named "*mik1*" and "*atb2*." These genes also have been mapped and ordered on the classic (genetic) map, and they have been probed onto both *Not*I and *Sfi*I genomic restriction fragments. We now have complete long-range genomic restriction maps of the fission yeast genome using both *Not*I and *Sfi*I (by Dr. Igor Garkavtsev, see Genetics Section). Therefore, we have independent evidence, using the consensus genetic map and macro-restriction maps, that allows us to localize and orient the contig shown in Figure 2 to the distal region of the "short arm" of chromosome 2 of the fission yeast. Many other contigs have been mapped in this way.

Initially, we are focusing our attention on studying the genomes of human, mouse, and fission yeast through collaborations with the Genome Database group at Johns Hopkins University (human), Dr. Lee Silver at Princeton University (mouse), Drs. Eric Lander and Nathan Goodman at the Massachusetts Institute of Technology (mouse), and Dr. David Beach at Cold Spring Harbor Laboratory (fission yeast). Later on, we will most likely include the genomes of the budding yeast, nematode, and fruit fly. These all are organisms whose genomes are being studied in detail by researchers all over the world, and individual speciality databases are emerging to support those particular organisms. Our approach is to derive information from these other databases, abstract the details we need, and provide mechanisms that allow our collaborators to enhance our database.

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NEUROSCIENCE

There are few problems in science as significant as understanding the brain. Neuroscience at Cold Spring Harbor Laboratory took a major step forward this year with the dedication and opening of the Beckman Neuroscience Center and the recruitment of three neuroscientists. The Laboratory's effort in neuroscience utilizes molecular, cellular, and genetic techniques to understand learning and memory processes, development of neurons, and neurodegenerative diseases. One major focus is the study of learning and memory in *Drosophila melanogaster*. This animal is being used because of the ease of identifying mutants that disrupt learning and of subsequently cloning the important genes. A second focus is the study of cellular factors which affect the growth and differentiation of neurons. We look forward to continued expansion in the neurosciences and to establishing Cold Spring Harbor Laboratory as a preeminent center for brain research.

NEURONAL GROWTH AND DIFFERENTIATION

D.R. Marshak E. Azmitia M. Meneilly
Y.-S. Bae L. Peña
N. Chester I.J. Yu

This laboratory conducts research on the biochemical basis of neuronal growth and differentiation. Overall, the questions that we approach in our research involve the decisions of neuroblasts to cease proliferation and to subsequently elaborate neuritic processes prior to terminal differentiation. These questions thus involve understanding how signal transduction systems that control cell proliferation in the neuroblast are altered upon becoming postmitotic and learning which growth factor molecules control these switches within cells. Specifically, we are interested in the action of a growth-stimulating protein, S100β, produced by astrocytes in the brain. The mechanism of action of S100β is compared with that of other growth factors, such as those in the family of neurotrophins or the family of heparin-binding (fibroblast) growth factors. The involvement of such neurotrophic factors in degenerative diseases, such as Alzheimer's disease, has prompted our interest in the role of such factors in neuropathological processes. There appears to be interesting interplay among factors important to development and the degeneration of the brain in the aged.

Mechanism of Action of S100β

L. Peña, E. Azmitia, D.R. Marshak

S100β is a 91-residue protein that causes neurite outgrowth from cortical neurons of the brain and is mitogenic for glial cells in culture. During development, S100β is synthesized and released by astroglia during their proliferation concomitant with the initiation of neurite outgrowth from postmitotic neurons. Critical to understanding the role of S100β in neuronal differentiation is unraveling the mechanism of action of this factor. We have taken two approaches to this problem: (1) in vitro experiments to identify a receptor molecule for S100β and (2) in vivo experiments in rats to analyze the release of S100β in the hippocampus.

We have initiated studies of radioactively labeled S100β binding to neuronal membranes. Iodination of S100β results in high specific activity without loss of biological activity in the neurite outgrowth assay. High-affinity saturable binding has not been satisfactorily demonstrated. This raises the possibility that

S100β binds with low affinity to neuronal membranes (and possibly glial membranes) in a relatively nonspecific manner. Activity might then arise from internalization and binding to a cytoplasmic target or from interaction with integral membrane proteins through lateral diffusion. To address these possibilities, cross-linking studies have been initiated using water-soluble, homobifunctional reagents. Additionally, immunohistochemical studies by electron microscopy will aid the identification of the target cellular compartment of S100β action in neurons.

For in vivo studies, radioactively labeled S100β was injected into the dorsal aspect of the ventral midbrain of adult rats. This area contains the Raphé nuclei, centers of serotonergic neuronal cell bodies that have been shown to respond to S100β by E. Azmitia and his colleagues at New York University. Autoradiographs of these tissues at various time points suggest uptake by glia and perhaps selected neurons of the Raphé. However, no retrograde transport of the injected S100β was seen. It appears that S100β is unlike the neurotrophins, which are target-tissue-derived factors that are taken up presynaptically and transported in a retrograde fashion to the neuronal cell body. As a glial-derived factor, S100β appears to act locally on adjacent neurons and glia. Lesion studies of the serotonergic projections to the hippocampus were performed by E. Azmitia, using a serotonin analog, 5,7-dihydroxytryptamine. This compound causes selective degeneration of serotonergic fibers and a corresponding decrease in S100β production in the hippocampus. Treatment of lesioned animals with a serotonin receptor type-1a agonist, ipsapirone, causes a large increase in the hippocampal S100β. These studies suggest that serotonin regulates S100β production in vivo through a type-1a receptor-mediated mechanism.

Protein Kinase Modulation during Neuronal Differentiation

L. Peña, Y.-S. Bae, I.J. Yu, D.R. Marshak

Studies of protein kinase modulation are designed to compare the mechanisms of neurotrophin action with those of S100β and heparin-binding growth factors. Studies of S100β action have centered on the role of the factor as a mitogen as well as a differentiative factor. In an autocrine fashion, S100β causes proliferation of astroglial cells, the source of the protein in vivo. In recent studies, L. Pena has begun to study the effects of S100β on neuroblasts, prior to the commitment to stop cell division. Chicken embryo forebrain neurons from embryonic day 6 appear to respond to increased thymidine incorporation in response to S100β in primary culture. Neuroblastoma cell lines have varied responses: Some differentiate and others proliferate. Distinguishing the differences in signal transduction pathways, including protein kinases, will help us to understand how neurons decide to differentiate.

A more complete study of $p34^{cdc2}$ kinase in cells that respond to nerve growth factor (NGF) has been conducted. The rat pheochromocytoma cell line, PC12, undergoes morphological and biochemical differentiation into sympathetic neurons in culture under the influence of NGF. The enzyme $p34^{cdc2}$ kinase, which is critical to the induction of mitosis, appears to be down-regulated during NGF-stimulated differentiation. This process may involve a related protein, p46, that cross-reacts with $p34^{cdc2}$ antibodies. We are currently trying to obtain a cDNA clone and sequence of this related molecule.

Characterization of the Heparin-binding Growth-associated Molecule

D.R. Marshak [in collaboration with W.H. Burgess, American Red Cross]

Heparin-binding growth-associated molecule (HB-GAM), isolated from bovine brain, was characterized biologically, physically, and chemically (Hampton et al. 1991). The complete amino acid sequence of HB-GAM was established by analysis of overlapping peptides derived from the protein. The protein is highly basic and is cysteine-rich. The mass of bovine HB-GAM was confirmed by plasma desorption, time-of-flight mass spectrometry. The measured mass was 15,291, compared with the predicted mass of 15,289, and this accuracy (0.01%) is well within the experimental limits of the method. There do not appear to be any posttranslational modifications other than proteolytic cleavage of the precursor to the released form. HB-GAM was not mitogenic for a variety of fibroblast, endothelial, and keratinocyte cell lines. However, the protein is highly active in promoting neurite outgrowth from embryonic chicken embryo forebrain neurons. Interestingly, the factor acts maximally when used to coat the surface of the

culture dish, and it has little activity when presented as a soluble factor. This finding, together with evidence that extraction of HB-GAM from brain requires high salt and that HB-GAM binds heparin, suggests that the protein may exist within large complexes of the basal lamina or other substructure. This material may be important to neuronal development in association with cell-surface interactions.

Investigation of Serotonin Systems in Neuronal Differentiation

E. Azmitia, I.J. Yu, M. Meneilly, D.R. Marshak

As described above, in vivo data from lesion studies in rats indicated a role for the neurotransmitter, serotonin, in the release of S100β. In addition, studies of cultured neurons of the Raphe nuclei show that S100β is a potent neurotrophic factor for these serotonergic neurons. Unfortunately, reagents for studying the serotonergic systems are not as available as they are for catecholaminergic and cholinergic systems. Therefore, we set out to produce antibodies to two proteins that are critical to these studies: (1) tryptophan hydroxylase, the mixed function oxidase that is the rate-limiting step in serotonin biosynthesis, and (2) serotonin receptor type 1a, which has been linked pharmacologically to S100β induction and cAMP-mediated responses in glia. We chose to produce these antibodies in rabbits using antigens based on synthetic peptides that correspond to particular regions of the molecules. Regions chosen for antigenic sites are unique to these proteins and are thought to be located on the proteins' surfaces. High-titer antisera were thus prepared and are excellent tools for immunohistochemical studies as well as biochemical analysis of proteins.

Involvement of S100β in Alzheimer's Disease and Down's Syndrome

D.R. Marshak [in collaboration with W.S.T. Griffin, University of Arkansas, P. Whitaker, SUNY Stony Brook, and J. Korenberg, Cedars-Sinai Medical Center, Los Angeles]

The gene for S100β is localized to human chromosome 21, near the telomere of the long arm (21q). This localization suggests a potential involvement in Down's syndrome (DS), since this region of the chromosome is duplicated in the disease. Many of the genes related to Alzheimer's disease (AD) have been localized to chromosome 21. In addition, besides the developmental, neurological symptoms of DS, middle-aged DS patients acquire a degeneration of the brain very similar to that of elderly AD patients. Because of the neurotrophic activity discovered for S100β, it is possible that abnormal levels or regulation of S100β might participate in the degeneration related to these diseases. We studied the levels of S100β in AD temporal cortex and hippocampus and found that the protein was 20-fold elevated in AD over age-matched controls. In addition, the mRNA and biological activities of S100β were found to be approximately 10-fold elevated. The excess S100β was localized to reactive astrocytes adjacent to dense-core amyloid plaques. These data indicate that elevated levels of S100β might play a role in the neuropathology of AD and DS. In DS, elevated levels of S100β were seen in samples of young brain tissue (3–6 months) returning to normal by 9–12 months. Although preliminary, these data suggest that there may be developmental patterns of S100β expression that are altered in DS. In one set of DS patients carrying a ring chromosome 21, an extra copy of the gene for S100β was not found (Falik-Borenstein et al. 1992). Thus, abnormal gene expression, rather than a gene-dosage effect, could explain the high levels of S100β thought to occur in DS and shown to occur in AD.

Analysis of CREB Phosphorylation in Astroglia

N. Chester, D.R. Marshak

Astroglial cells are thought to serve an important role in producing growth factors and other molecules in support of neuronal function. One of these factors, S100β, is the subject of much of the interest in our laboratory. Astrocytes also possess receptor molecules for a variety of neurotransmitter and hormonal signals thought to modulate the expression of genes for the protein growth factors through complicated signal transduction systems. Hippocampal astrocytes contain serotonin type-1a receptors, which are linked to the production of cAMP as a second messenger.

One of the targets of the cAMP-dependent protein kinase is a molecule called CREB (cAMP response-element-binding protein). This protein binds to special DNA sequences that mediate the cAMP-responsive genes, including c-*fos*. Another gene that is cAMP-responsive is that for S100β. Thus, the regulation of phosphorylation of CREB in astrocytes may be a key element in S100β expression. Our studies have focused on another phosphorylation site that modifies the CREB protein at points distal to that for cAMP-dependent protein kinase or calcium-dependent kinases. This site is phosphorylated by casein kinase II, an enzyme that is cell-cycle-regulated and depressed in Alzheimer's disease. We expect that our studies will lead to a more complete understanding of S100β expression in astrocytes under normal and abnormal physiological conditions.

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MOLECULAR AND CELLULAR BIOLOGY OF LEARNING

R. Davis

J. Alexander, J. Cherry, B. Dauwalder, C. Funk, P.-L. Han, A. Nighorn, Y. Qiu, C. Skoulakis, E. Skoulakis, K. Wu

Our laboratory continues with its long-term goals of probing the molecular and cellular biology of learning. We approach these goals using the techniques of genetics, molecular biology, biochemistry, behavior, and anatomy. In general, techniques of genetics and behavior are used to identify genes required for normal learning/memory, the techniques of molecular biology and biochemistry are used to clone the required genes and to characterize the gene products, and anatomical methods are used to trace the flow of information in the brain and to understand where the genes are required to be expressed for normal learning/memory. One focus continues to be on the *Drosophila dunce* locus, the best-characterized "learning gene" of the fly. In the last year, we have completed our studies of the gene's structure, probed the spatial

expression pattern of the gene, and continued with transformation studies designed to alter the spatial and/or temporal expression pattern of the gene. We have expanded our studies of other genes important for learning/memory in the fly, including the *rutabaga* locus and nine other partially characterized loci.

The laboratory moved from the Baylor College of Medicine to Cold Spring Harbor Laboratory in September of 1991. We are excited about the prospects of future growth in the neurosciences at Cold Spring Harbor. The move forced a split in the laboratory. Cindee Funk and Pyung-Lim Han remained in Houston and continue to pursue their goals, keeping in constant communication with the majority of the group at Cold Spring Harbor Laboratory. We said goodbye this year to our friend and colleague, Yuhong Qiu. Yuhong finished a very nice thesis on the *dunce* locus and has begun postdoctoral work at M.D. Anderson Cancer Center in Houston. And we have welcomed Joan Alexander, Carrie Skoulakis, and Jim Cherry into the laboratory this year.

Spatial Expression Pattern of *dunce*

A. Nighorn

The *dunce* locus is the prototype of *Drosophila* learning/memory genes. The gene was defined in a search for mutants that disrupt fly learning and from our subsequent cloning and biochemical experiments, shown to code for the enzyme cAMP phosphodiesterase (PDE).

Using antisera prepared against the *dunce* PDE, we performed a detailed survey of the spatial expression pattern by immunohistochemistry. We observed a striking concentration of the PDE within the neuropil of mushroom bodies, structures that have previously been implicated on other grounds to be involved in insect learning and memory. The enhanced immunoreactivity is also found in larval mushroom bodies, and in situ hybridization to adult brain sections shows that the enhanced level of protein within the mushroom bodies is due in part to elevated levels of *dunce* RNA within mushroom body perikarya.

These were important observations for several reasons. Since the mushroom body neuropil contains dendritic elements, axonal processes, and synaptic contacts, the enzyme must be synthesized in cell bodies and transported to become concentrated in processes, perhaps at synapses. The PDE potentially plays a direct role in regulating synaptic transmission. In addition, mushroom bodies have been implicated in other studies as neuroanatomical centers responsible for integrating sensory information and regulating complex behaviors in other insects. The enrichment of the *dunce* PDE in mushroom bodies suggests that these structures mediate learning/memory through the action of the products of identified learning/memory genes. Finally, these observations along with other information have allowed for the formulation of a circuit model describing the pathways and integration of information within the *Drosophila* brain.

Structure and Function of *dunce*

Y. Qiu [in collaboration with S. Beckendorf and T. Malone, University of California, Berkeley]

The *dunce* locus presented a special challenge in elucidating the sequences of its RNAs. This is due to the large number of RNAs detected by RNA blotting experiments (at least ten), their large sizes (4.2–9.6 kb), and their scarcity. From the sequence analysis of more than 40 different cDNA clones collected from oligo(dT)-primed and primer-extension cDNA libraries, the sequence analysis of the corresponding genomic coding regions, RNA blotting experiments with exon-specific probes, and primer-extension and S1-nuclease protection experiments, we have deduced the structure of this complex locus described below and depicted in Figure 1.

Exons that comprise *dunce* are distributed over more than 148 kb. This makes *dunce* the largest *Drosophila* locus characterized to date. Complementary DNA clones have identified at least six different RNA structures. The alternatively spliced forms predict the presence of multiple isoforms of the PDE product. Transcription of *dunce* begins at a minimum of five sites (one is not shown in Fig. 1): One transcription start site (tss) is at an undefined exon upstream of exon 0.9, a second is within exon 0.9 (class IA), a third at exon 2.1 (class II), a fourth at exon 2.7 (classes III and IV), and a fifth somewhere to the right of coordinate 0. The fifth transcription start site has been deduced from genetic evidence. Flies carrying chromosomal deficiencies removing the sequences upstream of coordinate 0 retain approximately 50% of the normal PDE activity. The

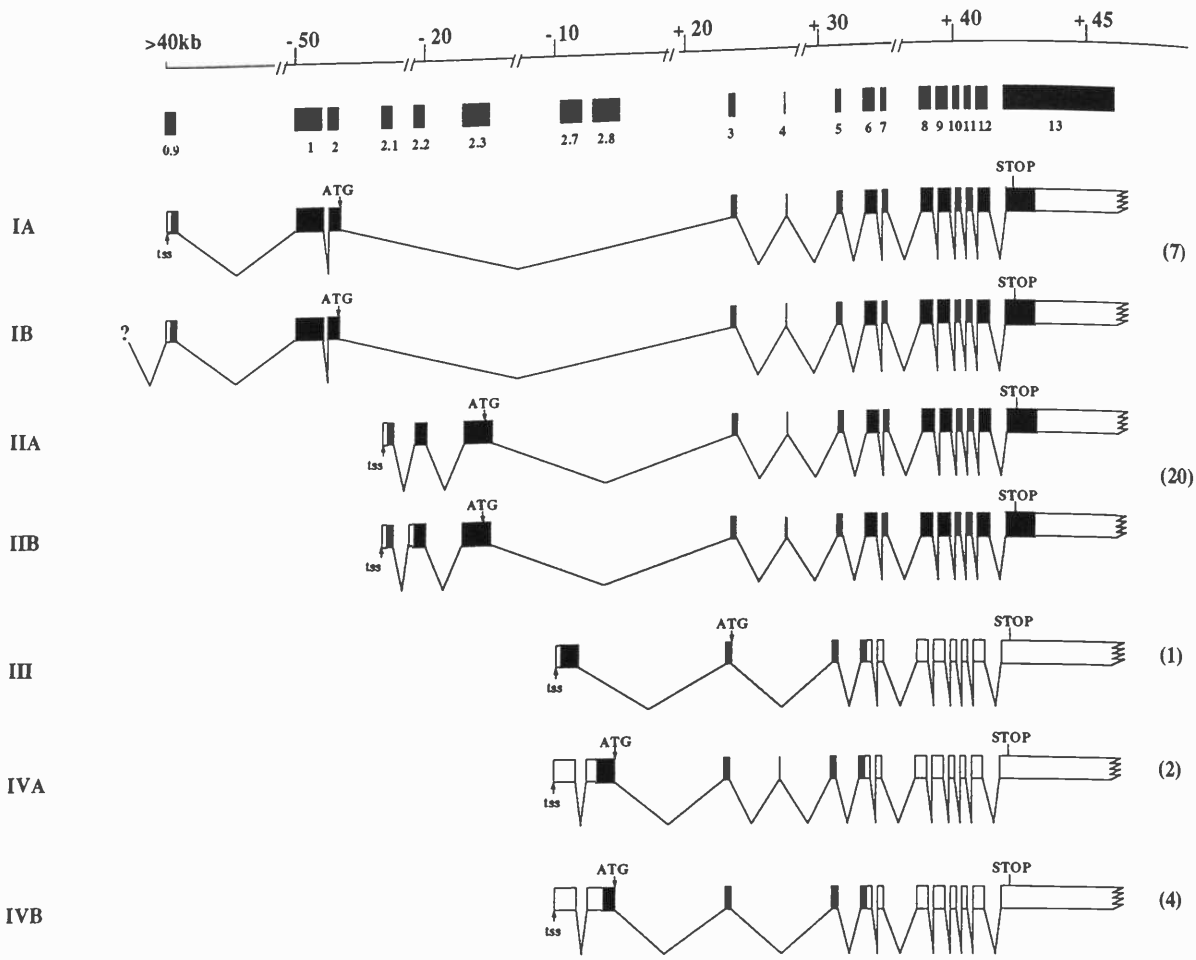

FIGURE 1 Schematic representation of the *dunce* locus. The coordinate system extends from −50 to +45 and is measured in kilobase pairs. *dunce* exons are represented as numbered boxes. Various RNAs as deduced from cDNA clones are shown with several structural classes denoted as IA through IVB. (*Closed boxes*) Protein coding regions; (*open boxes*) untranslated regions. Transcription start sites are designated tss. The number of clones isolated from each class is indicated in parentheses on the right.

transcription start site located to the right of coordinate 0 has not been identified molecularly. The most unusual feature of the locus is the existence of numerous unrelated genes (not shown) nested within the intron defined by exons 0.9 and 1.0 and within another intron defined by exons 2 and 3. The overall picture of the *dunce* locus is one of remarkable complexity.

Transformation and Rescue of *dunce*

B. Dauwalder

An important question is whether the phenotypes associated with mutation at the *dunce* locus are caused by a developmental requirement for the cAMP PDE or a physiological requirement during adulthood. To answer the question of "developmental versus physiological" role, we generated transgenic flies carrying *dunce*-coding sequences driven by the hsp70 promoter in the genetic background of several *dunce* mutations so that the gene could be turned on by the external cue of heat shock at various times during development or during adulthood.

A *dunce* cDNA clone and a rat cDNA homologous to *dunce* were used to construct transformation vectors, and these were inserted into flies carrying two different *dunce* alleles (dnc^1 and dnc^{ML}). Induction of the transgene after heat shock was monitored by enzymatic assay of whole-fly homogenates. For behavioral rescue experiments, flies were heat-

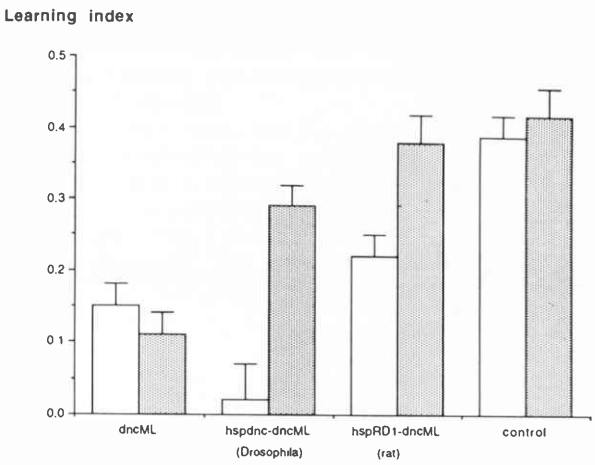

FIGURE 2 Rescue of the *dunce* learning deficiency after heat-shock-induced expression of the *Drosophila dunce* cDNA as well as a homologous rat cDNA (RD1). dnc^{ML} mutant flies homozygous for the *Drosophila* transposon (hspdnc-dnc^{ML}) or a transposon constructed with the rat gene (hspRD1-dnc^{ML}) were heat-shocked, rested for 2 hr, and trained. Performance of the flies is expressed as a learning index in which 1.0 represents perfect learning and 0.0 represents no learning. Flies were tested without heat shock (*open bars*) or after heat shock (*stippled bars*).

shocked and subsequently trained using a negatively reinforced classical conditioning paradigm. The learning scores of transformed flies were significantly higher after heat shock than those of their nonheat-shocked siblings or those of nontransformed *dunce* mutants (Fig. 2). This effect was observed for both the fly and rat cDNA clones, showing that the rat cDNA meets the biochemical needs for learning in the fly. These data suggest that a functional cAMP PDE is an active component of learning/memory physiology. In addition, induction of the transgene during adulthood partially rescued the female sterility of the *dunce* mutants. Thus, the transgenes rescue both phenotypes when induced during adulthood.

Mushroom Body Genes

J. Alexander, B. Dauwalder, P.-L. Han, A. Nighorn, C. Skoulakis, M. Skoulakis, K. Wu

Our laboratory has screened for new learning/memory mutants using *P*-element-mediated enhancer trap mutagenesis. Using histochemical methods, about 100 stocks (MB stocks) have been isolated with *P* elements near genes preferentially expressed in mushroom bodies. Each of these genes potentially represents one required for normal olfactory learning.

From preliminary behavioral tests of approximately 30 of these lines, 9 of these with the *P* element near or in unknown genes have been shown to have defective olfactory learning/memory in preliminary tests. One stock, named MB21, showed normal learning but a faster memory decay than normal flies. Line MB1116 showed a very labile memory several minutes after training, but the memory decay rate slowed such that by 3 hours after training, the scores were similar to those of control flies. MB2225 displayed a memory curve which parallels that of control flies, being reduced at all time points tested. The 90-minute retention of the other six lines was reduced relative to control flies. These stocks and the genes identified by the enhancer detector are currently the subject of additional behavioral and molecular studies.

rutabaga Structure and Expression

P.-L. Han [in collaboration with L. Levin and R. Reed, Johns Hopkins School of Medicine]

Several of the lines identified by enhancer detection with preferred expression of β-galactosidase in mushroom bodies were shown to have a *P* element insertion at the cytogenetic position corresponding to the *rutabaga* locus, the second best-characterized learning gene of *Drosophila*. To determine whether these insertion elements had indeed landed in the *rutabaga* locus, the genomic DNA flanking the insertions was isolated and compared to that isolated from an adenylyl cyclase (AC) gene thought to be the *rutabaga* function. Seven insertions were identified within 2 kb upstream of the transcription start of the AC gene. In addition, we were able to show that several of these insertions were new alleles at the *rutabaga* locus. By RNA in situ hybridization with AC probes and immunohistochemistry with anti-AC antibodies, we demonstrated that the AC RNA is preferentially expressed in mushroom bodies, that the AC immunoreactivity was concentrated in mushroom body neuropil, and that the insertion elements of the new *rutabaga* alleles compromised the expression of the AC gene. These observations add great strength to the idea that mushroom bodies are neural centers for insect learning and that the products of known learning mutants have their effects by altering mushroom body cell physiology.

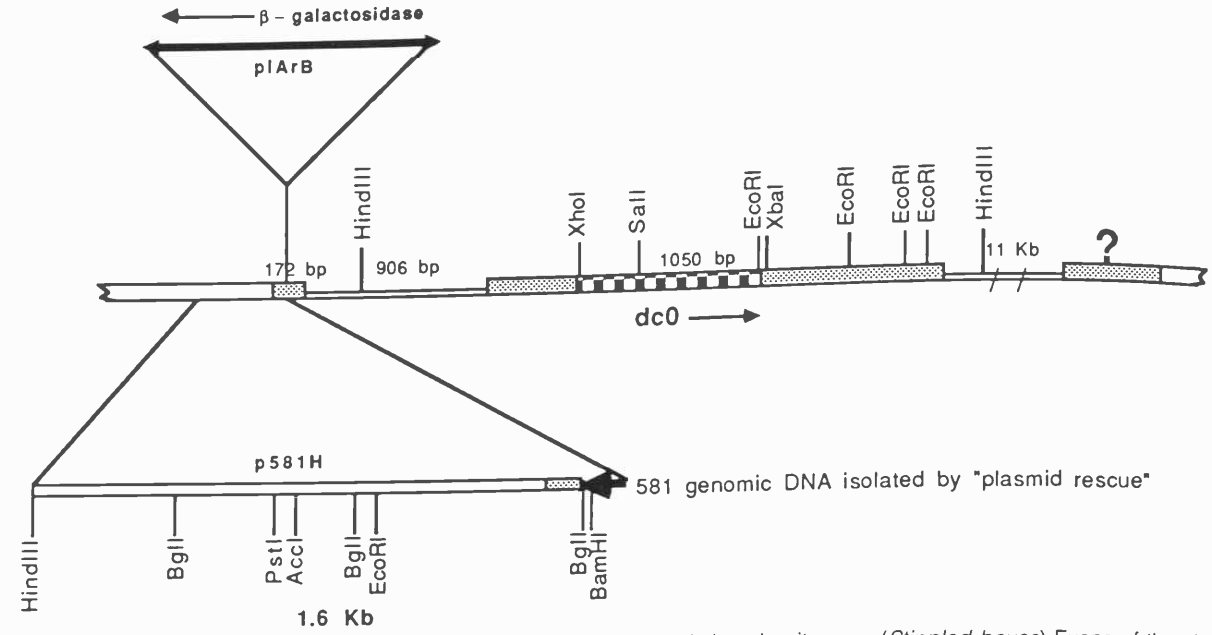

FIGURE 3 Insertion element p1ArB in the protein kinase A (*dco*) catalytic subunit gene. (*Stippled boxes*) Exons of the *dco* gene; (*open boxes*) introns and flanking DNA; (*checkered box*) protein-coding region. The precise location of the insertion was determined by sequence analysis.

Protein Kinase A

E. Skoulakis

Stock MB581 was shown to have an insertion element at the cytogenetic position of the gene for the catalytic subunit of protein kinase A (*dco*). The MB581 insertion is associated with recessive lethality. Since MB581 could be an insertion in the PKA gene, the genomic DNA flanking the insertion was cloned and characterized.

Sequence analysis of the DNA adjacent to the MB581 insertion demonstrated that the transposon was inserted in exon 1, 71 bp downstream from the transcriptional start site of the *dco* gene (Fig. 3). This disruption reduces PKA RNA as determined by in situ hybridization to homozygous MB581 embryos. The resulting reduction in PKA is reflected in a 40% decrease in PKA activity in MB581 heterozygous adults using in vitro enzymatic assays. In situ hybridization with PKA probes to adult brain sections demonstrated that the β-galactosidase expression in MB581 flies accurately reflects the PKA message distribution. Thus, the preferential expression of PKA in mushroom bodies along with the *rutabaga*-encoded AC and the *dunce*-encoded cAMP PDE lends strong support for a role of the cAMP cascade in these neurons to serve learning/memory processes.

Mushroom Body Enhancers

P.-L. Han, A. Nighorn, Y. Qiu

An important goal is to be able to direct the expression of foreign genes specifically in the mushroom bodies of transgenic flies. To achieve this, we have begun to search for a mushroom body enhancer in the vicinity of (1) the *rutabaga* promoter, (2) one of the *dunce* transcription start sites, (3) the PKA gene, and (4) an unknown gene identified in the enhancer detector screen as showing very specific mushroom body expression. We have provisionally identified one fragment in the vicinity of a *dunce* transcription start site that can specifically direct the expression of a β-galactosidase reporter gene to mushroom bodies. Follow-up studies are currently in progress.

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GENETICS OF LEARNING AND MEMORY IN *DROSOPHILA*

T. Tully M. DelVecchio, C. Jones, R. Mihalek, M. Regulski, D. Wood, S. Boynton, C. Brandes, V. Cambiazo, S. Koss, T. Preat, J. Xaio

We are pursuing a genetic dissection of learning and memory in fruit flies. To date, this problem has been tackled at both the genetic and behavioral levels. We have identified two new genes—*latheo* (*lat*) and *linotte* (*lio*)—involved with learning and memory, and molecular cloning of these genes has begun. We also have isolated a temperature-sensitive allele of the *pale* gene, which is known to encode a tyrosine hydroxylase and which we suspect may be involved with associative learning. Similar suspicions have prompted us to study the effects on learning of two other genes: *period* (*per*) and *Amyloid precursor protein-like* (*Appl*). In wild-type flies, we have produced long-lasting memory after classical conditioning of an olfactory avoidance response: Adults show some memory 7 days after extended training, and larvae can retain odor-shock associations through metamorphosis.

We will continue to study the behavioral properties of learning and memory and to isolate new genes. With this approach, we will be able to organize functional sets of learning/memory genes. Once these genes are cloned, such information will enable us to piece together the molecular relationships among their gene products.

Identification of New Genes Involved with Learning and Memory

T. Tully, S. Boynton, R. Mihalek, T. Preat, M. Regulski, C. Jones, M. DelVecchio, D. Wood

In the past year, we have completed a behavior-genetic characterization of two new genes involved with learning and memory in fruit flies: *latheo* and *linotte*. These genes were identified from a P-element mutagenesis, in which 3-hour memory retention after classical conditioning of an olfactory avoidance response was assayed in 1600 mutant strains each homozygous for a single, autosomal P-element insertion. Figure 1 shows the memory retention curves of $latheo^{P1}$ and $linotte^{P1}$ mutant flies, relative to wild-type controls. The primary effect of each of these mutations is on initial learning levels. Thereafter, mutant memory decay curves run parallel to those of wild-type flies.

Associative learning (and memory) is a hypothetical construct that cannot be measured directly. In our experimental context, it is defined as a *change* in olfactory avoidance behavior that *cannot* be attributed to effects on olfactory acuity (naive fly's ability to

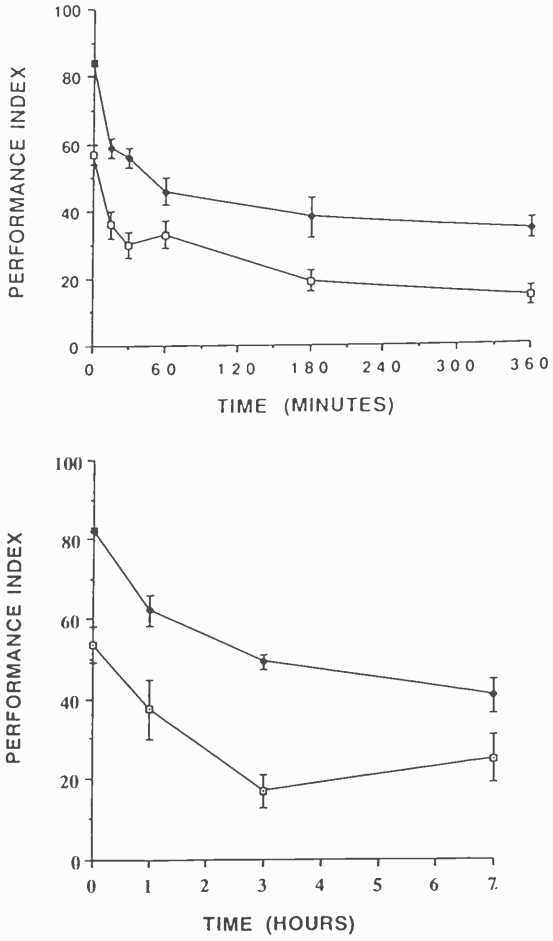

FIGURE 1 Learning and memory in two new P-element insertional mutants. Flies were classically conditioned by exposing them for 60 sec to one odor (either octanol or methylcyclohexanol) paired with 12 1.25-sec 60-volt pulses of electric shock delivered at 5-sec intervals. After a 45-sec rest, the flies then were exposed for 60 sec to a second odor (either methylcyclohexanol or octanol) without shock. After training, flies were transported to the choice point of a T maze between converging air currents of octanol or methylcyclohexanol. They were allowed to choose between these two odors for 120 sec, at which time, the flies were trapped in their respective T-maze arm, anesthetized, and counted. A normalized percent correct was calculated as the {[(fraction of flies that correctly avoided the shock-paired odor) x 2] -1} x 100. A second group of flies was then trained and tested to the reciprocal odor combination, and the normalized percents correct from the two groups were averaged to produce one Performance Index (PI). PIs could range from 0 (flies were distributed 50:50 in the T maze) to 100 (all flies avoided the shock-paired odor). Learning is defined as $t = 0$ time point of the retention curve. (*Top*) Mutant *latheo*^{P1} flies (*open squares*) and their *white* (C-S4) controls (*closed diamonds*). (*Bottom*) Mutant *linotte*^{P1} flies (*open squares*) and their Canton-S wild-type controls (*closed diamonds*).

smell the odors) or shock reactivity (naive fly's ability to sense electric shock and to escape from it). Before concluding that associative learning per se was aberrant, we had to show that these two important "peripheral" behaviors were normal in mutant *latheo* and *linotte* flies. We developed task-relevant assays of olfactory acuity and shock reactivity by assaying avoidance of each odor (octanol or methylcyclohexanol) versus air, or of electric shock versus no shock, in the T maze of the teaching machine. The behavior of *latheo*^{P1} and *linotte*^{P1} homozygous mutants appeared to be normal in both of these assays.

Our initial genetic concern was to show that the learning deficits observed in the *latheo*^{P1} and *linotte*^{P1} mutant strains actually resulted from the P-element insertions, rather than spurious changes in the genetic backgrounds of the strains. We addressed this issue with two corroborative approaches: (1) generation of P-element excisions and (2) complementation mapping with chromosomal deficiencies. Behavioral analysis of excision lines from *linotte* flies showed that seven out of ten were wild-type revertants, indicating clearly that the original P-element insertion was responsible for the behavioral deficits in mutant flies. In contrast, no wild-type revertants were obtained among 14 *latheo* excision lines. Instead, a continuous distribution of learning scores—ranging from *latheo*^{P1}-like to near wild-type—was observed, and about 5% of excision events produced lethal mutations. These results indicated that *latheo*^{P1} and the excision alleles were hypomorphic alleles of an essential gene.

Since P-element DNA had been cloned, we easily identified the cytological locations of the *latheo* and *linotte* P insertions via in situ hybridization of a P-element DNA probe on polytene chromosomes. The *latheo* insert was localized to 49F7-8, and the *linotte* insert was localized to 37D, on the second chromosome. This cytological mapping placed the *latheo*

gene near *vestigal* in a region that has been saturated for ethylmethanesulfonate (EMS)- and γ-ray-induced lethal mutations. Lethal complementation analysis between these alleles and the lethal excisions of *latheo* mapped the *latheo* gene unambiguously to one lethal complementation group. Subsequent behavioral analysis of flies heterozygous for $latheo^{P1}$ and an EMS-induced lethal allele showed clearly that the behavioral deficit mapped to the same lethal complementation group.

On the basis of the cytological positions of $latheo^{P1}$ and $linotte^{P1}$, we also obtained stocks carrying chromosomes with deletions of the same regions of the second chromosome—$Df(2R)vg^{b}$ and $Df(2R)vg^{56}$ for *latheo* and $Df(2L)VA^{12}$ and $Df(2L)TW^{50}$ for *linotte*. In each case, *mut/Df* flies yielded learning scores lower than those of +/*Df* controls. These results corroborated the results from behavioral experiments with excision alleles, indicating again that the P-element insertions were responsible for the behavioral deficits.

Since all existing learning/memory genes are X-linked, the autosomally linked *latheo* and *linotte* genes are new. Moreover, we still are chasing several additional putative mutants. Importantly, all of the genes we identify have been disrupted by P-element insertions, thereby "sequence tagging" the new genes, which, in turn, will expedite their cloning. Such molecular experiments are under way for *latheo* and *linotte*.

We also have isolated an EMS-induced temperature-sensitive allele of the *pale* gene, which recently has been shown to be the structural gene for a tyrosine hydroxylase in fruit flies. Since this enzyme is involved in the synthesis of dopamine and since dopamine metabolites are required for normal cuticle schlerotization, the two extant mutations of *pale* were embryonic lethals. In contrast, flies homozygous for our new $pale^{ts}$ allele survive at permissive temperature, thereby enabling us to study the effects of this mutation on learning and memory in adults. These experiments are relevant because past work on flies homozygous for temperature-sensitive alleles of the *Dopa decarboxylase* (*Ddc*) gene reported a significant learning deficit at restrictive temperature. In *Drosophila*, this enzyme is involved in the synthesis of both dopamine and serotonin. Consequently, we do not know from the *Ddc* experiments whether dopamine or serotonin is the relevant (putative) transmitter involved with associative learning. Mutant $pale^{ts}$ flies, in contrast, have normal levels of serotonin but no detectable levels of catecholamines in brains 24 hours after shifting them to the restrictive temperature. Analysis of $pale^{ts}$ may yield a dissection of the transmitter systems implicated in associative learning. The next phase of this project is to assay learning in these mutants, and to verify the learning deficits in mutant *Ddc* flies.

Biological Rhythm Mutants Show Normal Associative Learning

T. Tully [in collaboration with D.A. Gailey, Brandeis University]

We investigated a long-standing claim in the literature that mutant flies with long-period rhythms also show learning deficits. We assayed learning in three different contexts—courtship suppression toward immature males, conditioned inhibition of courtship toward females, and classical conditioning of olfactory avoidance responses—using mutant flies hemizygous for per^{s}, per^{L1}, per^{L2}, and per^{0} and transgenic flies carrying a copy of per^{+} on either a per^{0} or per^{L1} background. We replicated the original claim that per^{L1} males performed poorly in conditioned inhibition of courtship toward females, but we went on to show that per^{L1} males learned normally when they were exposed longer to females during training. We also showed that per^{L1} males suppressed their courtship of immature males normally and that the original claim for aberrant behavior was an artifact of the way a "learning index" was calculated. Furthermore, per^{L1} flies showed normal classical conditioning. We sequenced the per^{L2} mutation and discovered that it had the same nucleotide substitution as the per^{L1} mutation. Mutant per^{L2} flies performed normally in each learning assay, however, as did flies from each of the transgenic strains. Taken together, these data indicate that long-period biological rhythm mutants do not have learning deficits.

Human Amyloid Precursor Protein Functions in Fruit Flies

T. Tully, M. DelVecchio [in collaboration with K. White, Brandeis University]

A major component of extracellular amyloid plaques in brains of Alzheimer's disease patients is amyloid β-protein, which is a secreted cleavage product of β-

amyloid precursor protein (APP). A single gene generates—by alternative splicing—several APP isoforms, most of which contain a protease inhibitor domain and are found ubiquitously. The APP_{695} isoform, however, does not contain a protease inhibitor domain and is more abundant in the nervous system. Although abnormal cleavage has been implicated in the formation of amyloid plaques, the normal function of APP_{695} is not known.

K. White and co-workers have reported previously that the fruit fly *Appl* gene encodes a protein (APPL) that shows significant homology with APP, especially APP_{695}. We were interested creating *Appl*$^-$ mutant flies in an effort to disrupt, and thereby identify, the normal function of APPL. We identified an X-chromosome deficiency and a Y-linked X-chromosome duplication, which in combination produced a fly containing no APPL protein. Surprisingly, these *Appl*$^-$ flies were viable and fertile, with no apparent morphological abnormalities externally or in paraffin sections of adult head (at the light microscope level).

To determine whether the absence of APPL leads to any behavioral defects, we first compared conditioned odor avoidance responses between *Appl*$^-$ and *Appl*$^+$ flies in our standard classical conditioning experiments. We discovered that the mean learning score for *Appl*$^-$ flies was only 52% of that for *Appl*$^+$ controls. Further control experiments revealed that untrained *Appl*$^-$ and *Appl*$^+$ flies avoided to the same extent the odors used during the classical conditioning experiments, but *Appl*$^-$ flies were not able to escape from electric shock as well as *Appl*$^+$ flies. This failure of *Appl*$^-$ mutants to show normal shock reactivity—a necessary "peripheral behavior" for conditioning experiments—prevents us from concluding that associative learning per se is defective in these mutants.

The shock reactivity procedure induces an escape response, one behavioral component of which is locomotor reactivity. Fast phototaxis is another behavioral assay in which an escape response is induced, and thus we quantified this behavior in several genetic variants of *Appl*. We found a strong correlation between APPL and phototaxis: When APPL was present, phototactic scores were high, and when APPL was absent, phototactic scores were low. The mean phototactic score for *Appl*$^-$ flies was only 62% of that for *Appl*$^+$ flies. Consequently, we decided to use the fast phototaxis assay to study transgenic flies expressing various mutant or normal forms of APP(L) on an *Appl*$^-$ genetic background.

Since the *Appl* gene spans at least 40 kb of genomic DNA and the *Appl* promoter is not well delineated, we made a transgene from *Appl*$^+$ cDNA and the heat-shock promoter (hsp). Transgenic *Appl*$^-$ flies carrying one copy of this hsp-*Appl*$^+$ construct yielded mean phototactic scores significantly higher than that of *Appl*$^-$ mutants, indicating some "rescue" of the aberrant behavior associated with *Appl*$^-$. Compared to the mean phototactic score of *Appl*$^+$ controls, this rescue is only about 50%. Such partial rescue is not too surprising considering the facts that hsp-driven gene expression is ectopic and only one copy of our transgene was present in *Appl*$^-$ mutants. Thus, brain-specific levels of expression of *Appl*$^+$ may not have been completely normal in our transgenic flies.

That 50% rescue is relevant and specific to the *Appl*$^+$ transgene was demonstrated by the effects on phototactic behavior of an hsp-*Appl*$^-$ mutant construct carried by *Appl*$^-$ transgenic flies. The *Appl* gene sequence in this mutant construct contained a small deletion, which prevented the normal cleavage and secretion of APPL. These transgenic flies showed the same aberrant phototactic scores as *Appl*$^-$ mutants, i.e., no rescue was observed.

Finally, we tested whether the human APP_{695} protein could rescue the defective phototactic behavior of *Appl*$^-$ flies. We assembled an hsp-APP_{695} construct using APP_{695} cDNA. Transgenic *Appl*$^-$ flies carrying this fly/human construct showed the same amount of rescue (50%) as those transgenic *Appl*$^-$ flies carrying the hsp-*Appl*$^+$ construct.

These results strongly suggest a functional homology between human APP and fly APPL. Thus, we may be able to ascertain the normal function of amyloid precursor protein by studying fly behavior. To date, we have shown that flies with no functional APPL show a deficit in locomotor reactivity and *may* show a deficit in associative learning. These latter observations are not conclusive, however, because the locomotor deficit interfered with our ability to measure learning in classical conditioning experiments. Other learning tasks exist for flies, however, that do not require the same kind or degree of locomotor activity. Our next task is to quantify learning in normal and mutant *Appl* flies with such assays.

Flies Have Long-lasting Memory

T. Tully, T. Preat, V. Cambiazo

An important behavioral issue concerning the fruit fly as a model system of memory formation is whether

they are able to form stable, long-lasting memory as do vertebrates. Our usual classical conditioning procedure produces strong learning, but memory decays quickly, yielding retention levels 24 hours after training only 20% of initial learning levels. Moreover, memory decay is complete within 2–4 days.

We recently have lengthened memory retention in adult flies significantly by changing several procedural aspects of classical conditioning, the most important of which appears to be the amount of training. A tenfold increase in the amount of training produces memory lasting at least 7 days, which, when normalized for the life expectancy of a fly, is equivalent to human memory lasting 10 years. This long-lasting level of retention is about 20% of initial learning levels.

More interestingly, we have shown that third instar larvae can be trained to avoid a shock-paired odor and then remember this odor-shock association through metamorphosis. Three-day-old adults, trained as larvae 8 days earlier, show memory retention levels similar to those of 10-day-old adults trained as adults 8 days earlier. Thus, this 20% level seems to represent a stable form of long-lasting memory.

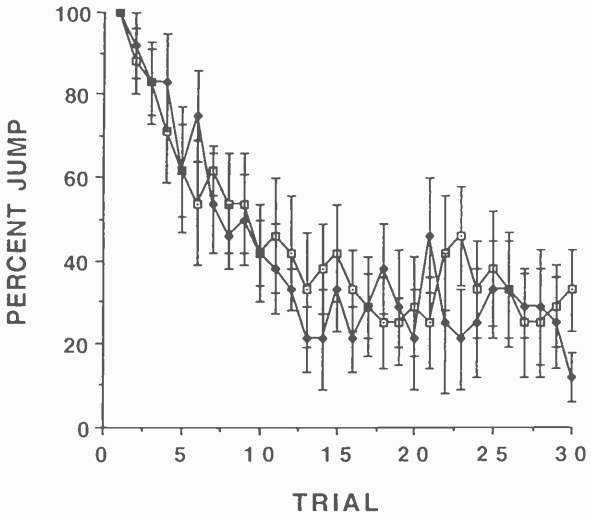

FIGURE 2 Habituation of the jump reflex to olfactory cues in lat^{P1}/lat^{P1} mutants (*open squares*) and $lat^{P1}/+$ control flies (*closed circles*). Individual flies of each genotype were exposed repeatedly to 4-sec pulses of 10% benzaldehyde delivered in a constant current of air. Habituation is shown as a decrement in the percentage of flies jumping during the 4-sec odor stimulus over 30 trials. Mutant *latheo* flies habituated normally, suggesting that their sensory input and motor output systems are normal and that the *latheo* gene may be involved specifically with associative, rather than nonassociative, learning.

The Jump Reflex Can Be Habituated by Olfactory Cues

T. Tully, S. Koss, S. Boynton

We also are interested in knowing whether the molecular mechanisms of memory formation after nonassociative and associative learning are similar. Since learning through different sensory modalities, in fact, may involve different molecular mechanisms and since our associative learning assay is based on odor-shock pairings, we first had to develop an assay for nonassociative learning based on olfactory cues. To do this, we semi-automated the olfactory jump procedure of J. Carlson and co-workers to deliver 4-second presentations of an airborne odor stimulus repeatedly to individual flies. We found, as J. Carlson described, that flies would jump up as if to fly away when presented with a concentrated, noxious odor stimulus. If we presented the same stimulus repeatedly, however, the flies eventually would stop jumping (Fig. 2).

This response decrement in wild-type flies shows many definitive behavioral properties of vertebrate habituation: More stimulus trials are required for a fly to stop jumping if the interval of time between trials (ITI) is longer. Fewer trials are required for a fly to stop jumping if the odor concentration is lower. After habituating, flies will jump in response to the odor stimulus if they have been disturbed or "dishabituated" by a novel, strong stimulus (90 sec of vortexing, in our case).

Preliminary data indicate that the learning/memory mutants *rutabaga* and *amnesiac* show slower than normal habituation and normal dishabituation, whereas mutants with defective locomotion or olfaction show faster than normal habituation and abnormally low levels of dishabituation. Furthermore, one of our new learning mutants, *latheo*, shows normal habituation and dishabituation, suggesting that this gene may be involved specifically with associative learning (see Fig. 2).

In Press, Submitted, and In Preparation

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Koss, S. and T. Tully. 1992. Habituation of the jump reflex in normal and mutant *Drosophila melanogaster*. (In preparation.)

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CSH LABORATORY JUNIOR FELLOWS

In 1986, Cold Spring Harbor Laboratory began a Junior Fellow program to encourage independent research by outstanding young scientists who, during their graduate studies, displayed exceptional promise of becoming leading scientists of the future. The purpose of this program is to provide an opportunity for each Fellow to work independently at the Laboratory for a period of up to 3 years on projects of their choice. The Fellow is provided with a salary, research support, and technical assistance so that they can accomplish their goals free from other distractions. The interaction among research groups at the Laboratory and the program of courses and meetings on diverse topics in molecular biology contribute to a research environment that is ideal for innovative science by these fellows.

Three previous Cold Spring Harbor Fellows, Dr. Adrian Krainer (1987), Dr. Carol Greider (1988), and Dr. Eric Richards (1989), are currently members of the scientific staff at the Laboratory. Dr. David Barford is our most recent Fellow, having joined us in 1991 from Professor Louise Johnson's laboratory at Oxford. Dr. Barford is an X-ray crystallographer who has previously worked on glycogen phosphorylase, but whose present interests lie in the area of protein-mediated signal transduction. Dr. Barford's current focus is on the serine/threonine-specific protein phosphatases. Immediately before coming to Cold Spring Harbor, Dr. Barford spent some time in Dr. P. Cohen's laboratory in Dundee working on protein phosphatase 2A (PP2A) from rabbit muscle. Dr. Barford hopes to crystallize and solve the structure of this phosphatase.

D. Barford

X-ray Crystallographic Studies on Serine/Threonine-specific Protein Phosphatases and Protein Tyrosine Phosphatase

D. Barford

The purpose of my research is to determine the three-dimensional structures of serine/threonine-specific protein phosphatases PP1, PP2A (and the structure of selective enzyme substrate complexes), and protein tyrosine phosphatases by X-ray crystallography. This information will be useful in determining the mechanism of enzyme catalysis and the stereochemical basis for substrate recognition and selectivity. Knowledge of the phosphatase structures will enable a comparative study of the conformation, catalytic mechanism, and basis for substrate specificity to be made between the two classes of phosphatases and with protein kinases and other phosphatase enzymes (e.g., alkaline phosphatase and purple acid phosphatase).

Information about the structures will be used to design protein engineering experiments to alter the substrate specificity of the enzymes in a defined and predicted manner. The structures of the protein phosphatase catalytic subunits complexed to their regulatory subunits and to various tumor viral gene products (e.g., the small T antigens of polyomavirus and SV40) will allow insight into the structural mechanism for phosphatase regulation.

SERINE/THREONINE-SPECIFIC PROTEIN PHOSPHATASE

Complementary DNA (cDNA) clones encoding serine/threonine-specific protein phosphatases have been isolated and sequenced (for review, see Cohen and Cohen, *J. Biol. Chem. 264:* 21435 [1989]). The

cDNAs encoding rabbit skeletal muscle protein phosphatase 1 (PP1), PP2A, and a *Drosophila* phosphatase, PPV, have been incorporated in the baculovirus DNA under the control of the strong polyhedron promoter, allowing expression in the baculovirus/insect cell system. In addition, the cDNA encoding the catalytic subunit of PP2A has been cloned into the T7 bacterial expression vector and expressed under the control of the T7 promoter. Although all phosphatase catalytic subunits are expressed as insoluble protein aggregates, 10–20 mg of protein may be obtained from 1 liter of insect cell or bacterial cell culture. The insoluble material when washed in Triton X-100 and 0.5 M NaCl is 50% homogeneous and may be solubilized in guanidinium chloride and DTT. The solubilized protein can be renatured in renaturation buffer at an enzyme concentration of 2–3 μg/ml. The protein is concentrated by absorption onto phenyl Sepharose followed by elution in low-salt buffers. This procedure yields 3–4 mg of soluble active protein per liter of cell culture relatively easily. Crystallization trials are currently in progress.

BACTERIOPHAGE λgt10 ORF221

The bacteriophage λgt10 genome encodes a sequence (ORF221) that is similar to that of eukaryotic protein phosphatase 1 (35% sequence identity over the amino-terminal 115 residues, with 22% sequence identity overall). This gene has been cloned into an expression vector under the control of the T7 promoter. The gene is expressed at high levels in *Escherichia coli*, corresponding to 30–50% of the soluble cell protein at levels of 20–40 mg per liter of cells. The protein shows phosphatase activity toward glycogen phosphorylase, casein, and para-nitrophosphophenyl substrates, hence confirming its identity as a protein phosphatase. This material will be purified and subject to crystallization trials.

PROTEIN TYROSINE PHOSPHATASE

The cDNA encoding the T-cell protein tyrosine phosphatase is expressed in the baculovirus/insect cell system (Zander et al., *Biochemistry 30:* 6964 [1991]). Purified protein will be subject to crystallization trials.

COLD SPRING HARBOR MEETINGS

56th COLD SPRING HARBOR SYMPOSIUM ON QUANTITATIVE BIOLOGY

The Cell Cycle

May 29–June 5, 1991

ARRANGED BY

David Beach, Cold Spring Harbor Laboratory
Bruce Stillman, Cold Spring Harbor Laboratory
James D. Watson, Cold Spring Harbor Laboratory

461 participants

It is an infrequent occurrence in biology when two seemingly disparate areas of research are fused by a few key experiments. Such was the case in 1988 when it was reported that the function of the maturation-promoting factor (MPF) that regulates meiosis in amphibian oocytes depended on a key cell-cycle-regulated protein kinase that previously had been identified using the genetics of yeast. Indeed, it was soon recognized that the protein kinase was a component of MPF and that another essential component was a cyclin protein, again through the combined efforts of investigators studying early development in amphibian and marine invertebrate embryos and yeast geneticists studying cell growth regulation. Cell cycle research was revitalized, and during the last 3 years, there has been an explosion of information. As a result, the general principles of cell cycle regulation in eukaryotic cells have been established. New and exciting developments have been emerging daily, including the involvement of tumor suppressor proteins in cell cycle regulation and the bridging of cell cycle control to cellular events such as transcription and DNA replication. It was because of this exciting background that it seemed obvious to hold the 56th Cold Spring Harbor Symposium on The Cell Cycle, bringing together almost all of the key investigators in the field.

After opening remarks by Dr. James Watson, the themes of the Symposium were outlined by excellent presentations from Arthur Kornberg, Marc Kirschner, Lee Hartwell, and Richard McIntosh. At the end of a long and truly exciting week, the enthusiastic Tim Hunt concluded with a thoughtful summary.

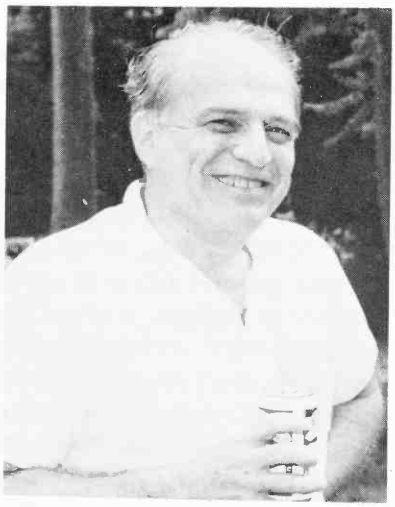
The final program included more than 450 scientists from research centers in Europe, Asia, and the United States. The program included 90 speakers who discussed such fundamental problems as control of mitosis; entry into the cell cycle; the role of anti-oncogenes and tumor suppressor genes in regulating cell growth; DNA replication; cell differentiation and cessation of proliferation; role of oncogenes and growth factors in control of cell proliferation; and chromosome behavior and division. The participating leading researchers in these areas use a variety of approaches from genetics to biochemistry to study control of the cell cycle. A variety of model systems from yeast to humans were discussed, and the meeting provided the opportunity to demonstrate how these different approaches complement each other. The meeting was one of great intensity and scientific interchange.

J.D. Watson

A. Stenlund, B. Stillman, P. Howley

This year's Symposium received support from the the National Cancer Institute/National Institutes of Health, the National Science Foundation, and the U.S. Department of Energy. Essential funds also came from our Corporate Sponsors: Alafi Capital Company; American Cyanamid Company; AMGen Inc.; Applied Biosystems, Inc.; Becton Dickinson and Company; Boehringer Mannheim Corporation: Bristol-Myers Squibb Company; Ciba-Geigy Corporation/Ciba-Geigy Ltd.; Diagnostic Products Corporation; The DuPont Merck Pharmaceutical Co.; Eastman Kodak Company; Genentech, Inc.; Hoffmann-LaRoche, Inc.; Johnson & Johnson; Kyowa Hakko Kogyo Co., Ltd.; Life Technologies, Inc.; Eli Lilly and Company; Millipore Corporation; Monsanto Company; Oncogene Science, Inc.; Pall Corporation; Perkin-Elmer Cetus Instruments; Pfizer Inc.; Pharmacia, Inc.; Sandoz Research Institute; Schering-Plough Corporation; SmithKline Beecham Pharmaceuticals; Sumitomo Pharmaceutical Co., Ltd.; Toyobo Co., Ltd.; The Upjohn Company; Wellcome Research Laboratories, Burroughs Wellcome Co.; and Wyeth-Ayerst Research.

D. Beach



R. Morris

E. Karsenti

T. Hunt, J.M. Bishop, M. Kirschner

PROGRAM

Welcome: J.D. Watson

Introduction
Chairman: M. Mitchison, *University of Edinburg*

START
Chairman: H. Weintraub, *Fred Hutchinson Cancer Research Center*

Cycling
Chairman: D. Botstein, *Stanford University School of Medicine*

Mitotic Regulation
Chairman: E. Karsenti, *European Molecular Biology Laboratory*

Checkpoints
Chairman: R. Morris, *University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School*

Transcriptional Regulation
Chairman: S. McKnight, *Carnegie Institution of Washington*

Centrosomes and Kinetochores
Chairman: M. Yanagida, *Kyoto University*

K. Nasmyth

M. Yanagida, D. Botstein

J. Ruderman

J.M. Mitchison, T. Mitchison

Exit
Chairman: A. Levine, *Princeton University*

Surprising Events
Chairman: K. Nasmyth, *Research Institute of Molecular Pathology, Vienna*

Dorcas Cummings Lecture
Speaker: J. Michael Bishop, *University of California, San Francisco*

DNA Replication
Chairman: B. Alberts, *University of California, San Francisco*

Oncogene Interactions
Chairman: I. Herskowitz, *University of California, San Francisco*

G_2M Regulation
Chairman: T. Hunter, *Salk Institute*

Mitosis
Chairman: J. Ruderman, *Harvard Medical School*

Mitogenesis
Chairman: D. Nathans, *Johns Hopkins University School of Medicine*

Summary: T. Hunt, *Imperial Cancer Research Fund*

G. Vande Woude, H. Weintraub, S. McKnight

The Role of Isoform Diversity in Cytoskeletal Functions

April 24–April 28, 1991

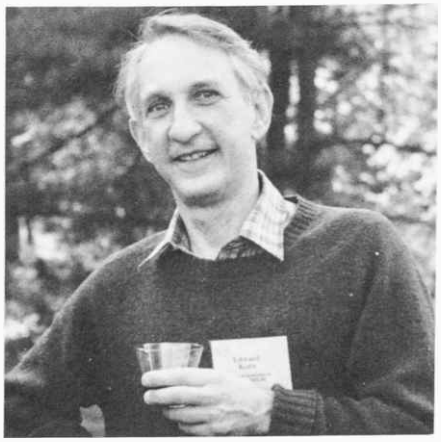
ARRANGED BY

Henry Epstein, Baylor College of Medicine
Donald Fischman, Cornell University Medical College
David Helfman, Cold Spring Harbor Laboratory

119 participants

This meeting focused on the functional significance of the isoform diversity found among cytoskeletal proteins. The cytoskeleton is involved in a variety of cellular processes including cell division, exocytosis, endocytosis, adherence to the substratum, motility, and cell shape. The cytoskeleton of eukaryotic cells is composed of three filamentous systems: actin filaments, intermediate filaments, and microtubules. Each of these systems possesses a major core protein, namely, actin, intermediate filament protein, and tubulin. In a given organism or cell type, considerable isoform diversity of both core proteins and associated proteins is often present. In many cases, the different isoforms exhibit distinct cell- and tissue-specific patterns of expression. A remaining question in biology is to understand the function and significance of the extensive polymorphism found among these proteins. The scientific program encompassed both invertebrate and vertebrate systems. The emphasis of the meeting was also placed on cellular functions as opposed to a particular filament type or protein species in order to focus attention on the role different isoforms play in a cell. Investigators from a wide number of disciplines attended, including cell biologists, biochemists, molecular biologists, and developmental biologists. Topics included cell motility and contractility, molecular motors and intracellular trafficking, cell division, chromosome movement, nuclear structure, isoform switching in development, and neuronal function.

A Ben-Ze'ev, D Helfman, H Epstein



E. Korn

R. Stewart, S. Hawley

This meeting was funded in part by the Muscular Dystrophy Association of America, The Council for Tobacco Research-USA, Inc., Merck Sharp and Dohme, and Carl Zeiss, Inc.

PROGRAM

Cell Motility and Contractility I
Chairman: E. Korn, *National Institutes of Health*

Cell Motility and Contractility II
Chairman: G. Gerisch, *Max Planck Institute, Martinsried*

Isoform Switching during Development and Differentiation
Chairman: A. Ben Ze'ev, *Weizmann Institute of Science, Israel*

Membrane-Cytoskeletal Attachment, Extracellular Matrix
Chairman: M. Beckerle, *University of Utah*

Special Platform Session

Isoform Diversity in Neuronal Function
Chairman: M. Shelanski, *Columbia University*

Intracellular Trafficking and Organelle Movement
Chairman: R. Vallee, *Worcester Foundation for Experimental Biology*

Cell Division, Chromosome Movement, and Nuclear Structure
Chairman: S. Hawley, *Albert Einstein College of Medicine*

Cell Shape and Morphogenesis
Chairman: M. Mooseker, *Yale University*

Closing Remarks
H. Holtzer, *University of Pennsylvania*

M. Beckerle

D. Fischman

Stress Proteins and the Heat Shock Response

April 29–May 2, 1991

ARRANGED BY

Costa Georgopoulos, University of Utah
Richard Morimoto, Northwestern University

296 participants

Cold Spring Harbor was the setting for the largest (to date) and most comprehensive meeting on Stress Proteins and the Heat Shock Response. There were 296 participants in this three and a half day meeting. The program consisted of eight sessions and a total of 61 speakers, the majority of which were selected from the abstracts. A substantial amount of exciting data was also presented in three poster sessions for a total of 152 posters. The scientific highlights of the meeting were the sessions that focused on the biochemical role of stress proteins in chaperoning, transport and protein folding reactions, and the social highlight undoubtedly was the square dance that, like stress proteins, allows for multiple interactions.

This meeting was funded in part by the StressGen Biotechnologies Corp., Biogen, Inc., Berlex Biosciences, Inc., Takara Shuzo Co., Ltd., and Kureha Chemical Industry Co., Ltd.

Back A Tissières, W Neupert, G. Hahn, T Ura, C. Wu
Front C Georgopoulos, S Lindquist, M -L Pardue, R Morimoto

PROGRAM

Introductory Remarks:
R. Morimoto, *Northwestern University*
C. Georgopoulos, University of Utah

Organismal Responses to Physiological Stress:
Activation of Heat Shock Genes in Prokaryotes and Yeast
Chairman: T. Yura, *Institute for Virus Research, Japan*

Activation of Heat Shock Gene Transcription in Larger Eukaryotes
Chairman: C. Wu, *National Institutes of Health*

Other Stress Responses and Developmental and
Tissue-specific Stress Responses
Chairman: M.-L. Pardue, *Massachusetts Institute of Technology*

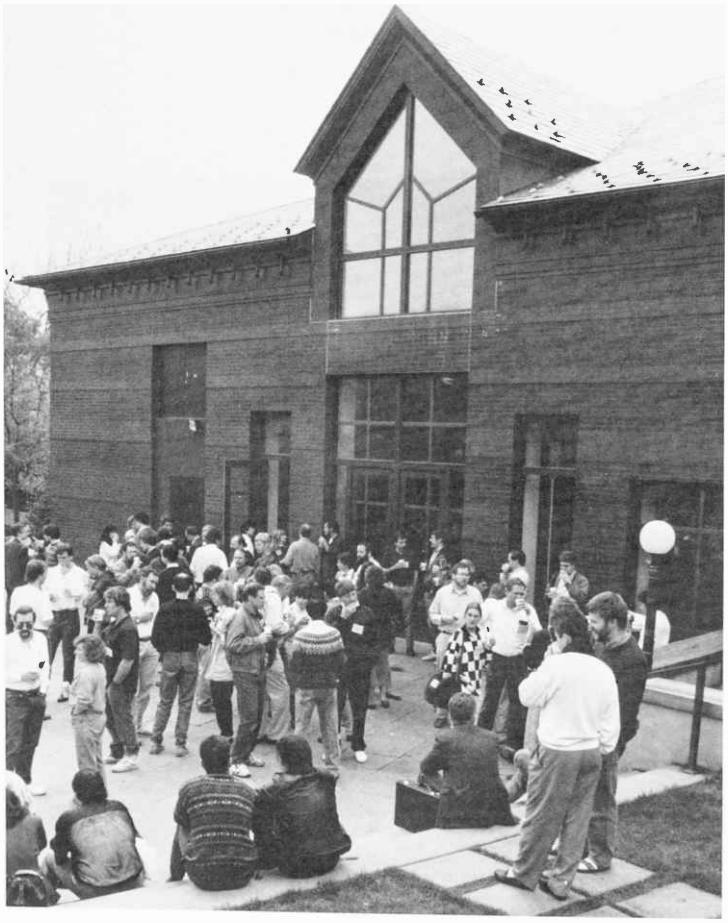
Mechanisms of Protein Folding and the Biochemical Properties of Stress Proteins
Chairman: A. Tissières, *University of Geneva*

Functions of HSP70-related Stress Proteins
Chairman: W. Neupert, *Universitat Munchen*

Biochemical and Cell Biological Properties of Other Heat Shock and Stress-induced Proteins
Chairman: S. Lindquist, *Howard Hughes Medical Institute, Chicago*

Biochemical Properties of Stress Proteins
Chairman: M. Schlesinger, *Washington University School of Medicine*

A Role for the Stress Response in Infectious Disease, Immunorecognition, Neuronal Expression and Injury, Aging, Thermotolerance, and Cancer
Chairman: G.N. Hahn, *Stanford University*



Wine and cheese party at Grace Auditorium

Genome Mapping and Sequencing

May 8–May 12, 1991

ARRANGED BY

Charles Cantor, Human Genome Center, Lawrence Berkeley Laboratory
Maynard Olson, Washington University School of Medicine
Richard Roberts, Cold Spring Harbor Laboratory

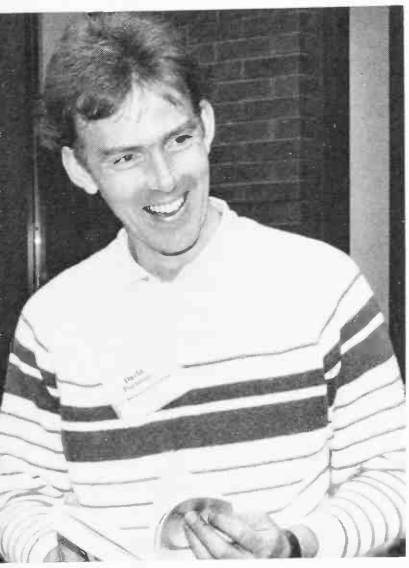
378 participants

The 1991 meeting on Genome Mapping and Sequencing was the fourth in an annual series of meetings designed to provide an overview of systematic efforts to analyze the genomes of the human organism and of a variety of model organisms. All aspects of systematic genome analysis are covered, including biological insights into the molecular organization of chromosomes, applications to research on specific human diseases, experimental techniques, procedures for storing, analyzing, and communicating data, and methods of automating laboratory procedures. Furthermore, as the number of large, long-term genome analysis projects increases, the meeting provides a forum for reporting progress and discussing experience with various models for the scientific management of these projects. The series of meetings would appear to be meeting its goals: in the words of one participant—a veteran contributor to this field—it remains the only regular forum that provides a comprehensive overview of "where things stand." In a rapidly growing field that is still in the process of defining itself, the need for such a forum is obvious.

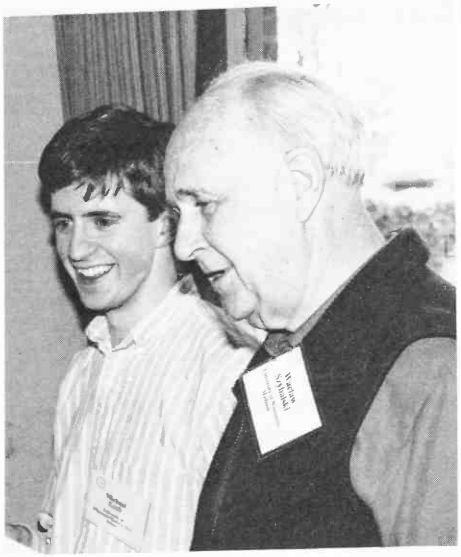
The 1991 meeting clearly demonstrated rapid growth in the amount of effective activity in this field. Although real novelty surfaces infrequently, the power of experimental approaches is increasing rapidly. These gains arise from incremental improvements in the base technologies and the evolution of increasingly effective ways to combine and apply them. Perhaps the best indication of progress is the rapidity and effectiveness with which advances in genome analysis are being applied to important biological problems.

For example, the story behind the most striking biological advance reported at the meeting—the molecular definition of the fragile X site—differs markedly from that behind previous successes in cloning genes associated with genetic diseases. The fragile X syndrome, which is the most common heritable form of mental retardation, encompasses a bizarre combination of genetic, cytogenetic, and phenotypic effects. Three different research groups reported success in defining the site of chromosome fragility, whose genetic behavior is strikingly non-Mendelian. Chromosomal imprinting and hypermutability both appear to be important in the etiology of the disease. The successful laboratories were all able to identify and then clone the DNA at the fragile site by systematic methods; there was no reliance on the end-game serendipity that marked many earlier searches for disease genes. The cooperation of major genome centers in providing rapid access to yeast artificial chromosome (YAC) clones and the increasing power of fluorescent in situ hybridization to metaphase chromosomes both played critical roles. Indeed, there was broad evidence at the meeting that the challenge in identifying the genes that are mutated in human genetic diseases has shifted from an emphasis on recovering DNA from the disease locus to an emphasis on

C. Cantor



D. Porteous



M. Koob, W. Szybalski

S. Brenner, J. Weissenback

developing convincing criteria for proving that a particular candidate gene in the region is actually the one whose mutant alleles cause disease.

This meeting was funded in part by the National Center for Human Genome Research/National Institutes of Health.

K. Davies

PROGRAM

Genome Organization
Chairman: M.L. Pardue, *Massachusetts Institute of Technology*

Large DNA Cloning
Chairman: D.J. Porteous, *Medical Research Council, Edinburgh*

Alu-PCR and Other Methods
Chairman: W. Szybalski, *University of Wisconsin*

Informatics
Chairman: S. Brenner, *Medical Research Council, Cambridge*

Polymorphisms and Linkage Methods
Chairman: H. Donis-Keller, *Washington University, Missouri*

Sequencing/Automation
Chairman: L. Smith, *University of Wisconsin*

Large-scale Mapping Projects
Chairman: G. Evans, *Salk Institute for Biological Studies*

Interesting Loci
Chairman: R. Myers, *University of California, San Francisco*

R. Gemmill, R. Roberts

RNA Processing

May 15–May 19, 1991

ARRANGED BY

Chris Greer, University of California, Irvine
Jim Manley, Columbia University
Alan Weiner, Yale University School of Medicine

437 participants

This year marked the 10th anniversary of the first RNA Processing Meeting. Continuing the trend of the last 2 years, the meeting was oversubscribed and attendance had to be limited. Exciting developments were reported in several areas. The idea that RNA plays an important role in the catalysis of pre-mRNA splicing was strengthened by the demonstration of new interactions involving snRNAs and the pre-mRNA. However, it is also clear that multiple, perhaps dozens, of proteins are required for splicing. In yeast, 1991 was dubbed "the year of the DEAD" in honor of the several putative RNA helicases that are essential for splicing and which contain the characteristic DEAD box motif. In contrast, proteins identified in mammalian systems all appear to be RNA-binding proteins, sharing sequence similarities with genetically defined regulators of alternative splicing in *Drosophila*. Several studies addressed the question of where in the nucleus splicing might take place. Although the details were provocative, and the functional implications uncertain, it is now clear that at least some splicing factors are concentrated in specific subdomains within the nucleus. Progress in identifying factors involved in pre-mRNA polyadenylation continued, with an important conclusion being that this seemingly simple reaction requires a complex array of protein factors.

Novel and refined techniques for tRNA and rRNA structural analysis were used to identify transient, precursor-specific conformations and to probe features that define RNA folding pathways. The conformation of these molecules is surprisingly dynamic, and this conformational fluidity appears to be essential for

N. Proudfoot, J. Manley, J. Alwine

both their function and biosynthesis. Refined structural analyses of the active sites of RNA catalysts were also described. The results establish certain general features common to RNA and protein catalysts and also define many other features, including the functional role of the phosphate backbone and the implications of template interactions, as unique to RNA enzymes. Surprising results were reported in studies of the mechanism of RNA editing. Indirect evidence for covalent guide/target RNA intermediates was presented, a result that led to the discussion of an analogy between guide RNAs and the viroid class of catalytic RNAs. The identification of a novel 3′ processed structure for U6 RNA suggests an intriguing analogy to guide RNAs with interesting implications for U6 interactions in pre-mRNA splicing.

This meeting was funded by the National Science Foundation and the National Institute of General Medical Sciences/National Institutes of Health.

PROGRAM

mRNA splicing I: Ribonucleoprotein Particles and Mechanism
Chairman: I. Mattaj, *EMBL, Heidelberg*

mRNA Splicing II: Protein Factors and Mechanism
Chairman: T. Blumenthal, *Indiana University*

mRNA 3′ Ends
Chairman: N. Proudfoot, *University of Oxford, England*

Stable mRNAs
Chairman: B. Sollner-Webb, *Johns Hopkins University School of Medicine*

RNA Transport/Localization
Chairman: M. Green, *University of Massachusetts*

mRNA Splicing III: Alternative Splicing
Chairman: T. Maniatis, *Harvard University*

Catalytic RNA
Chairman: M. Belfort, *Wadsworth Center, Albany*

Guide RNAs/Modification/Turnover
Chairman: E. Blackburn, *University of California, San Francisco*

T. Blumenthal

T. Maniatis

M. Green

RNA Tumor Viruses

May 21–May 26, 1991

ARRANGED BY

John Coffin, Tufts University School of Medicine
Irvin Chen, University of California, Los Angeles

436 participants

Papers presented at the 1991 RNA Tumor Virus meeting covered all aspects of retrovirus biology, including replication mechanisms and virus-host interactions, and important pathogenic mechanisms, including oncogenesis and immunodeficiency. All important retrovirus groups were represented, and somewhat more than half the presentations involved the human pathogenic retroviruses HIV and HTLV.

Topics of particular note included the nature and normal function of retroviral receptors; a lively discussion of the mechanisms of viral DNA integration; revealing studies on the mechanisms and role of *trans*-activators and "minor" gene products in the expression and replication of complex retroviruses like HIV and HTLV and the roles in infection of model animals; the surprising relationship of some retroviruses and superantigens; and new insights into the mechanism of virion assembly. The traditional retrotrivia contest provided an opportunity for participants to sharpen their sense of retrohistory. Unlike most recent meetings, the unbroken spell of fine weather permitted participants to enjoy the surroundings as well as the science.

Contributions from CSHL Corporate Sponsors provided core support for this meeting.

Back: H. Fan, R. Desrosiers, B. Cullen, A. Rein, L. Ratner, D. Blair, M. Linial
Front: P. Jolicoeur, D. Ito, J. Coffin, K. Chen, K. Beemon, N. Hopkins

PROGRAM

Viral Entry
Chairmen: D. Ho, *Aron Diamond Research Center*
J. Cunningham, *Howard Hughes Medical Institute, Boston*

Reverse Transcription and Integration
Chairmen: H. Temin, *University of Wisconsin, Madison*
S. Goff, *Columbia University*

Genetics, Mutation, and Evolution
Chairmen: M. Linial, *Fred Hutchinson Cancer Research Center*
S. Hughes, *Frederick Cancer Research and Development Center, NCI*

Expression 1: Enhancers and Promoters
Chairmen: N. Hopkins, *Massachusetts Institute of Technology*
F. Clavel, *Institut Pasteur*

Expression 2: *Trans*-activation
Chairmen: M. Yoshida, *University of Tokyo*
C. Rosen, *Roche Institute of Molecular Biology*

Pathogenesis 1: Oncogenesis
Chairmen: N. Rosenberg, *Tufts University School of Medicine*
D. Blair, *National Cancer Institute*

Expression 3: Posttranscriptional Events
Chairmen: K. Beemon, *Johns Hopkins University*
B. Cullen, *Duke University Medical Center*

Pathogenesis 2: Immunological and Neurological Diseases
Chairmen: P. Jolicoeur, *Clinical Research Institute of Montreal*
R. Desrosiers, *Harvard Medical School*

Retrovirus Potpourri
Chairmen: L. Ratner, *Washington University*
A. Rein, *Frederick Cancer Research and Development Center, NCI*

Virion Assembly and Structure
Chairmen: J. Leis, *Case Western Reserve University*
A. Skalka, *Fox Chase Cancer Center*

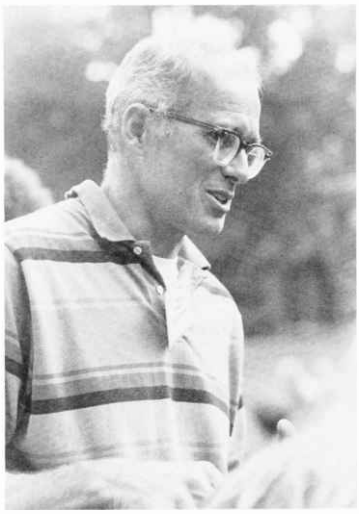
C. Rosen

N. Rosenberg

O. Heidman, O. Danos, P. Charneau, F. Arenzana-Seisdedos, P. Jolicoeur, F. Clavel

Yeast Cell Biology

August 13–August 18, 1991



J. Pringle

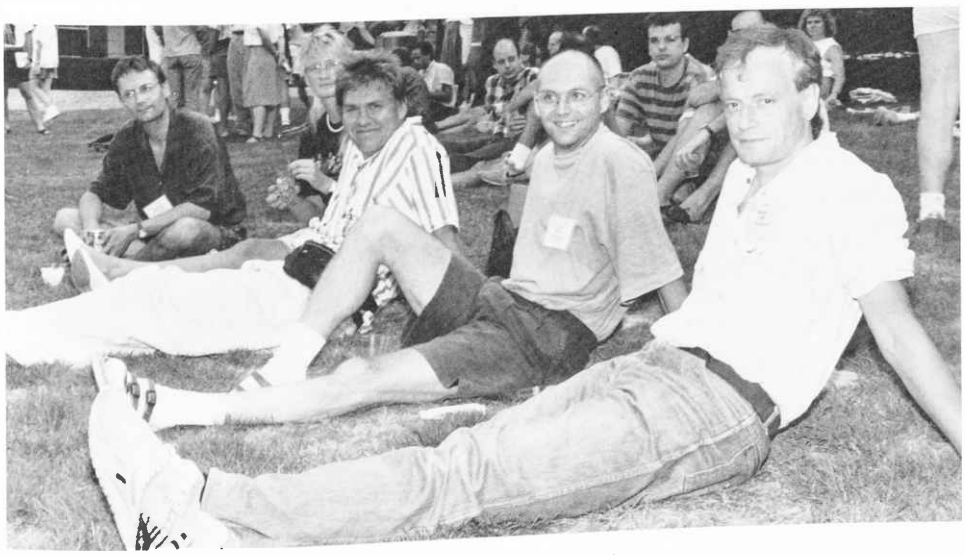
ARRANGED BY

Scott Emr, California Institute of Technology
John Pringle, University of North Carolina
Steven Reed, Scripps Clinic and Research Foundation

363 participants

The 1991 meeting on Yeast Cell Biology was the third in a series of very successful biannual meetings on this subject. At the 1991 meeting, 363 participants presented a total of 110 talks and 140 posters covering a very wide range of topics, including the structure and function of the cytoskeleton and the mechanisms of intracellular movements; plasma membrane structure and function; morphogenesis and cell wall biogenesis; protein processing, secretion, and intracellular targeting; the biogenesis and function of membrane-bounded organelles (nucleus, endoplasmic reticulum, Golgi complex, vacuole, mitochondria, and peroxisomes); cell signaling and the role of calcium; chromosome structure and function; and growth control and the cell division cycle.

The major attraction of yeasts as experimental objects remains the extraordinary ease of classical and molecular genetic manipulations, and nearly all of the presenters had exploited this property. The power of the genetic methods was well illustrated by the striking results obtained by both "forward genetics" (discovery of unanticipated types of proteins involved in familiar processes) and "reverse genetics" (often surprising revelations about the in vivo roles of proteins that had originally been identified biochemically). However, a major theme of this meeting was the increasing sophistication of yeast cell biologists in complementing their genetic methods with powerful biochemical and morphological methods,



M. Kretschmer, G. Straussberger, G. Achatz, K. Köhrer, M. Aebi

such as those used with animal cells; advances in the applications of immunoelectron microscopic techniques provided a particularly striking example. The power of the combined methodologies seems to guarantee that yeast cell biology will continue to move ahead quickly. That this progress will have a major impact on cell biology generally is suggested by two other major themes of this meeting. The first is the continuing stream of revelations about the remarkable degree to which fundamental cellular mechanisms have been conserved throughout the eukaryotic world, from yeasts to humans to flowering plants. The second is the remarkable complexity of intracellular processes in terms both of the sheer numbers of proteins involved and of the degree to which these proteins have fully or partially overlapping functions. This complexity seems unlikely to be sorted out without the kinds of genetic methods that can be applied to yeast. Yeast cell biologists have good reasons to expect another very exiciting meeting in 1993.

This meeting was funded in part by John Labatt Limited, Miller Brewing Company, and Anheuser-Busch Companies, Inc.

PROGRAM

Calcium and Cytoskeleton
Chairman: J. Cooper, *Washington University*

Mitochondria and Peroxisomes
Chairman: M. Douglas, *University of North Carolina*

Secretion and Endocytosis
Chairman: R. Schekman, *University of California, Berkeley*

Cell Cycle
Chairman: H. Iida, *National Institute for Basic Biology, Japan*

Methodology
Chairman: J. Pringle, *University of North Carolina*

Morphogenesis and Cell Wall
Chairman: M. Breitenbach, *University of Vienna*

Protein Sorting
Chairman: E. Jones, *Carnegie Mellon University*

Nuclear Traffic and Vacuole Function
Chairman: J. Woolford, *Carnegie Mellon University*

Signaling and Growth Control
Chairman: J. Thorner, *University of California, Berkeley*

Protein Processing
Chairman: R. Fuller, *Stanford University*

Nucleus and Chromosomes
Chairman: K. Bloom, *University of North Carolina*

S. Reed, B. Futcher

J. McKoy, C. Moore, K. Bloom

Molecular Genetics of Bacteria and Phages

August 20–August 25, 1991

ARRANGED BY

Susan Gottesman, National Cancer Institute
Lucia Rothman-Denes, University of Chicago
Miriam Susskind, University of Southern California

252 participants

S. Gottesman

More than 250 participants were at the 1991 Bacteriophage meeting. About half of the 203 abstracts were presented as talks, and half were presented as posters during the 2-day poster sessions. At least 53 of the abstracts had, as a primary author, a scientist from outside the United States, continuing the strong international flavor of these meetings.

Among the highlights of work presented was the continuing investigation of the basis of positive activation of transcription, with a paper by Richard Ebright (UMDNJ) pinpointing the location of a large number of mutations in the catabolite activator protein (CAP) which do not interfere with DNA binding but block activation. His results suggest that protein-protein interactions are important for CAP activation at promoters such as lac. Sarah French (UVA) provided striking electron microscopy evidence that replication forks can displace active transcription complexes, although when the two collide, replication is slowed.

This meeting continued to provide an important forum for interactions between scientists studying the molecular basis of basic processes and those examining the consequences of the control of these processes for the physiology of the organism and its interaction with the environment.

Contributions from CSHL Corporate Sponsors provided core support for this meeting.

M. Susskind

W. McAlister

PROGRAM

DNA Rearrangements
Chairman: H. Nash, *National Institutes of Health*

DNA Replication and Cell Division
Chairman: N. Kleckner, *Harvard University*

RNA Polymerase
Chairman: G. Gussin, *University of Iowa*

Regulation of Transcription I
Chairman: N. Craig, *University of California, San Francisco*

Regulation of Transcription II
Chairman: W.T. McAllister, *State University of New York, Brooklyn*

Changing Lifestyles
Chairman: C. Miller, *University of Illinois, Urbana*

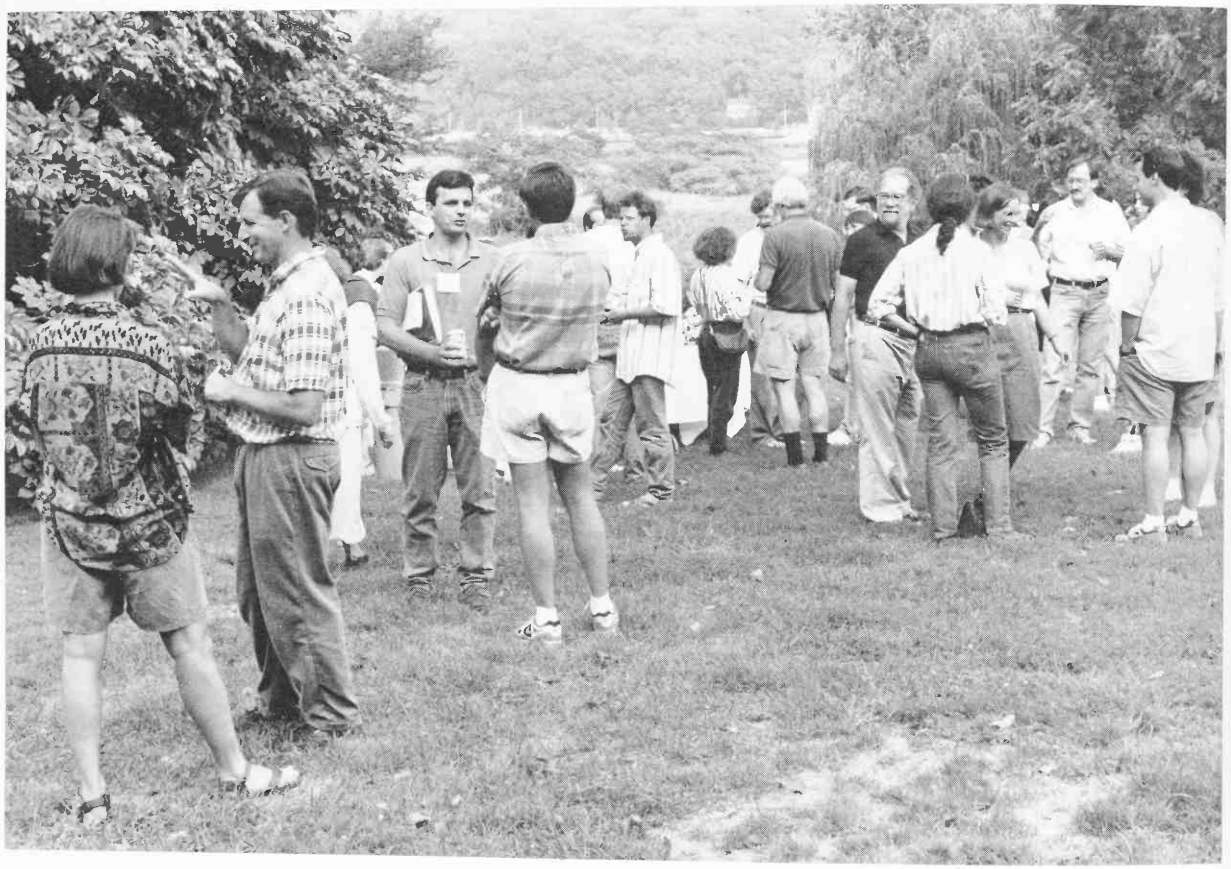
Phage Regulation and Posttranslational Regulation
Chairman: D. Wulff, *State University of New York, Albany*

Protein Structure and Function
Chairman: A. Poteete, *University of Massachusetts*

Surfaces and Structures
Chairman: M. Russel, *Rockefeller University*

Plasmids and Tricks
Chairman: M. Yarmolinsky, *National Institutes of Health*

M. Russell, P. Model



Wine and cheese party at Airslie

Regulation of Eukaryotic mRNA Transcription

August 28–September 1, 1991

ARRANGED BY

Winship Herr, Cold Spring Harbor Laboratory
Robert Tjian, University of California, Berkeley
Keith Yamamoto, University of California, San Francisco

458 participants

The 1991 Cancer Cells meeting, Regulation of Eukaryotic mRNA Transcription, followed upon the success of the 1989 meeting on the same topic. Again, more than 400 scientists sharing overlapping interests in regulation of transcription in eukaryotic cells from yeast to humans attended the meeting. The reports presented by participants spanned a broad spectrum of topics, including the biochemistry general transcription factors, analysis of sequence-specific activators and repressors, structural studies, yeast genetics, and transcriptional regulation in development. Major advances were reported in the cloning of cDNAs encoding general transcription factors, including TFIIA, TFIIB, TFIIE, and TFIIF. It was described how the prototypical general RNA polymerase II factor TFIID, which recognizes the so-called TATA box found in many promoters, can also activate transcription by RNA polymerase III. There was continued debate on the regulatory targets within the transcriptional initiation complex for sequence-specific activators. The candidates range from TFIID or TFIIB as targets of an acidic activator and TFIID-associated proteins as a target for a glutamine-rich activator. Yeast genetics was again shown to be an excellent tool to uncover interactions between elements involved in transcriptional regulation. For example, altered DNA sequence specificity mutants were described for both TFIID and the sequence-specific activator GCN4. Yeast genetics was also used to reveal how the higher-order structure of DNA, as occurs in chromatin, may regulate transcription. Structural studies revealed new DNA-binding motifs. In-

I. Herskowitz, W. Dyan, R. Tjian

W. Herr

deed, the crystal structure of the GAL4 DNA-binding domain revealed a unique zinc-containing structure. The interplay between members of different classes of transcription factors such as steroid receptors and Fos/Jun proteins displayed the complexity of regulatory interactions generating both positive and negative effects on transcription. Posttranslational protein processing was also shown to be key in the regulation of transcription factor activity. Examples of such regulation were revealed by reports on regulation of sporulation in bacteria and the p50 subunit of NF-κB in mammalian cells. The meeting closed with an excellent meeting review by Dr. Ira Herskowitz from the University of California, San Francisco. He emphasized how the mechanisms by which transcription is regulated are so similar in different eukaryotic species.

This meeting was funded in part by the National Science Foundation and the National Institute of General Medical Sciences, division of the National Institutes of Health.

K. Yamamoto, F. Schaufele, J. Miner

PROGRAM

Basal Transcription Factors and RNA Polymerase II
Chairman: R. Roeder, *Rockefeller University*

Initiation and Postinitiation Events
Chairman: N. Hernandez, *Cold Spring Harbor Laboratory*

Activators, Basal Factors, and Multisubunit Complexes
Chairman: J. Greenblatt, *Banting and Best, University of Toronto*

Structure and Function of Transcription Factors
Chairman: P.S. Kim, *Howard Hughes Medical Institute, Whitehead Institute, Massachusetts Institute of Technology*

Factor/Factor Interplay
Chairman: H. Weintraub, *Fred Hutchinson Cancer Research Center*

Regulation of Transcription Factor Activity
Chairman: S. Kustu, *University of California, Berkeley*

Chromatin Function and Regulation
Chairman: R. Kornberg, *Stanford University Medical School*

Transcription in Development
Chairman: R. Losick, *The Biological Laboratories, Harvard University*

Eukaryotic DNA Replication

September 4– September 8, 1991

ARRANGED BY

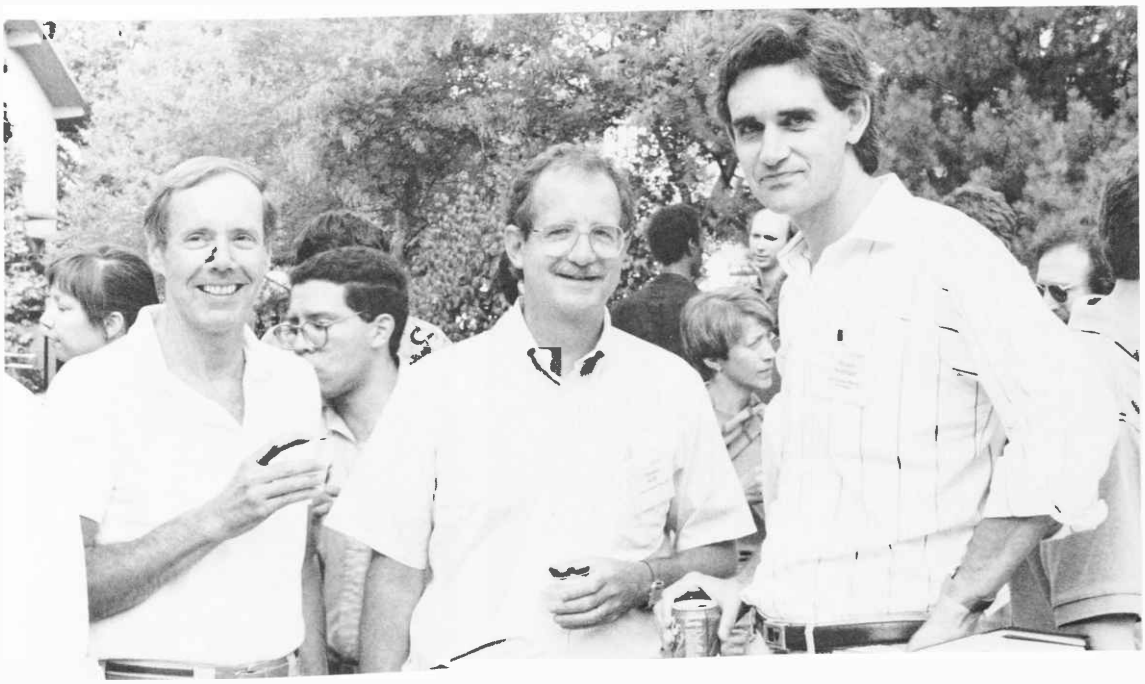
Thomas Kelly, Johns Hopkins University
Bruce Stillman, Cold Spring Harbor Laboratory

315 Participants

The third biannual meeting on Eukaryotic DNA Replication attracted over 300 scientists to an exciting 4 days of high-quality discussion. The topics covered at the meeting included the mechanics of both virus and cellular chromosome replication, the identification and characterization of cellular origins of DNA replication, the function of and interactions between DNA replication, proteins, regulation of DNA replication, and chromosome structure, including telomere function. It was clear that studies on eukaryotic DNA replication have progressed rapidly since the last meeting 2 years ago, but it was equally obvious that many key and important biological problems remain to be solved.

At this year's meeting, we were again fortunate to be entertained by the multi-talented Ron Laskey. A highlight this year was the recording of a dozen of his most famous songs for scientific posterity (tape available from Cold Spring Harbor Laboratory Press).

The organizers wish to acknowledge generous financial support from the National Science Foundation.



S. Linn, T. Kelly, B. Stillman

Wine and cheese party at Grace Auditorium

PROGRAM

Mechanisms of DNA Replication
Chairman: D. Clayton, *Stanford University*

Chromosomal Replication
Chairman: R. Hay, *University of St. Andrews, Scotland*

Regulation
Chairman: C. Newlon, *University of Medicine and Dentistry of New Jersey Medical School*

Viral DNA Replication
Chairman: J. Borowiec, *New York University Medical Center*

Replicons
Chairman: B. Brewer, *University of Washington, Seattle*

Interactions of Replication Proteins
Chairman: S. Linn, *University of California, Berkeley*

Chromosome Structure and Function
Chairman: P. Traktman, *Cornell University Medical College, New York*

H. Klein, C. Newlon

B. Brewer

Synthesis of Ribosomes

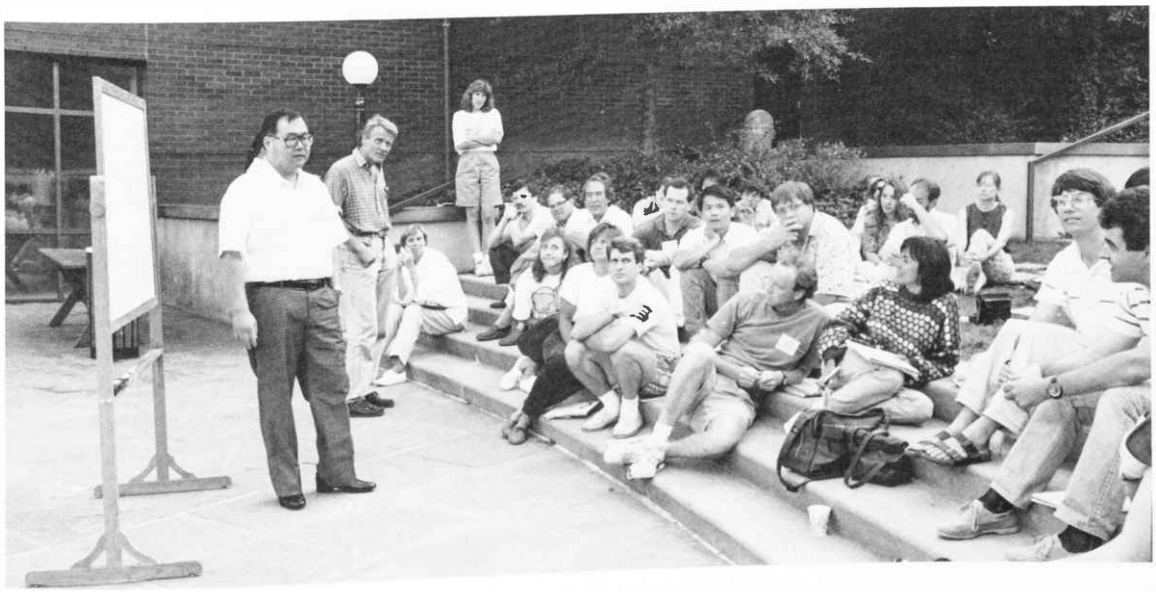
September 11–September 15, 1991

ARRANGED BY

Lasse Lindahl, University of Rochester
Edward Morgan, Roswell Park Memorial Institute
Robert Perry, Fox Chase Cancer Center
Barbara Sollner-Webb, Johns Hopkins University
Jonathan Warner, Albert Einstein College of Medicine

214 participants

The second Cold Spring Harbor meeting on Synthesis of Ribosomes was held September 11–15, 1991. Because of the complexity of the ribosome and because of its importance in the overall economy of the cell, the study of ribosome synthesis leads down many paths, from the regulation of transcription, of splicing, and of translation to the transport of components across the nuclear envelope, to the structure of that enigmatic organelle, the nucleolus. This meeting brought together more than 200 researchers in the field of ribosome biosynthesis, studying prokaryotic as well as eukaryotic systems. Indeed, one of the major motivations behind the meeting is to bring together the "euk's" and the "prok's", who rarely attend the same meetings. Key issues covered included the regulation and the mechanism of transcription of ribosomal RNA in *E. coli* and in higher cells, as well as the initiation of translation of ribosomal protein mRNAs, which appears to be a major mechanism of regulation both in prokaryotes and in eukaryotes, although probably by different mechanisms. Other sessions concerned the proteins and small RNAs involved in the processing of ribosomal RNA precursors, the nature of the nucleolus, and the regulation of ribosome synthesis through development. A successful innovation of this year's meeting was the



Lecture on patio at Grace Auditorium

holding of concurrent workshops on Sunday morning, which livened up a session often characterized by the "morning-after" blahs. Another meeting on Synthesis of Ribosomes is scheduled for 1994.

This meeting was funded in part by Abbott Laboratories, Bio 101, Inc., Bristol-Myers Squibb Company, Connaught Laboratories, Inc., Du Pont Medical Products, Supelco, Inc., United States Biochemical Corporation, and Worthington Biochemical Corp. Grants were also received from the National Science Foundation and the National Institute of General Medical Sciences, division of the National Institutes of Health.

O. Meyuhas, J. Warner, L. Steel

I. Gonzalez, B. Sollner-Webb

PROGRAM

Transcription of rRNA Genes I
Chairman: C. Squires, *Columbia University*

Transcription of rRNA Genes II
Chairman: B. Sollner-Webb, *Johns Hopkins University*

Ribosomal Protein Synthesis I
Chairman: R. Perry, *Fox Chase Cancer Center*

Ribosomal Protein Synthesis II
Chairman: L. Lindahl, *University of Rochester*

Ribosomal RNA Processing
Chairman: J. A. Steitz, *Howard Hughes Medical Institute, Yale University*

Regulation and Development
Chairman: M. Pellegrini, *University of Southern California*

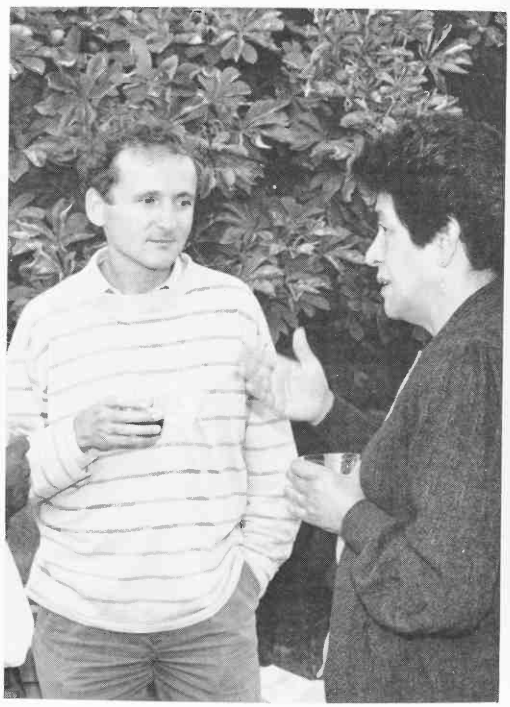
Ribosome Assembly and the Nucleolus
Chairman: J.R. Warner, *Albert Einstein College of Medicine*

Workshops

Transcription by RNA Polymerase I
Chairman: M.R. Paule, *Colorado State University*

The Nucleolus and RNA Processing
Chairman: J.R. Warner, *Albert Einstein College of Medicine*

RNA Structure in Regulation
Chairman: M. Springer, *Institute de Biologie Physico-Chemie, Paris*



P. Labhart, E. Sanchez-De Jimenez

Modern Approaches to New Vaccines Including Prevention of AIDS

September 19–September 23, 1991

ARRANGED BY

Fred Brown, USDA, Plum Island Animal Disease Center
Robert M. Chanock, NIAID, National Institutes of Health
Harold S. Ginsberg, Columbia University College of Physicians & Surgeons
Richard A. Lerner, Research Institute of Scripps Clinic

208 participants

The ninth annual meeting on Modern Approaches to New Vaccines brought together scientists who had a wide variety of research interests ranging from basic molecular biology, immunology, or pathogenesis to clinical infectious diseases and vaccine trials. The meeting provided an opportunity for basic scientists and clinical investigators to exchange their latest observations and broaden their perspective of the diverse interactive components of vaccine development.

Advances relevant to vaccine development were reported for a number of important human viral pathogens such as HIV (the etiologic agent of AIDS) and HBV (the cause of hepatitis B). For example, compelling evidence that serum antibodies provide effective protection against infection by cell-free simian immunodeficiency virus (SIV) or human immunodeficiency virus type 2 (HIV-2) was presented for the first time. Conference participants also learned that an inactivated whole-virus vaccine derived from a proviral DNA clone of SIV induced complete protection against infection by the homologous cell-free virus as well as a disparate strain of SIV that varied significantly in its envelope glycoprotein amino acid sequence from the vaccine virus.

Conference participants described the first successful efforts to generate human monoclonal antibody Fab sequences against hepatitis B virus surface

R. Lerner

F. Brown, R. Chanock, H. Ginsberg

antigen (HBsAg) or the envelope glycoprotein of HIV-1 by antigen selection from a combinatorial library expressed on the surface of filamentous phage. These high-affinity Fabs should prove useful in defining the repertoire of human antibody response to immunization, and full-length antibodies prepared from the Fabs may prove useful in passive immunoprophylaxis as well as immunotherapy.

This meeting was supported in part by the Rockefeller Foundation.

PROGRAM

Immunology
Chairman: R.N. Germain, *NIAID, National Institutes of Health*

Immunology and AIDS
Chairman: J.A. Berzofsky, *NCI, National Institutes of Health*

Bacteriology and Parasitology
Chairman: F. Brown, *USDA, Plum Island Animal Disease Center*

Virology I
Chairman: C.-J., Lai, *NIAID, National Institutes of Health*

AIDS II
Chairman: E. Norrby, *Karolinska Institute*

AIDS III
Chairman: P.R. Johnson, *Georgetown University*

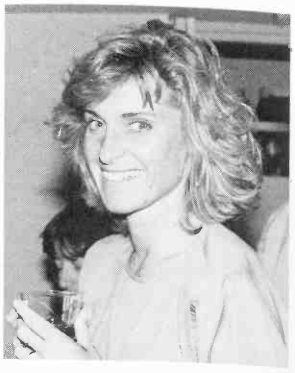
Virology II
Chairman: R.M. Chanock, *NIAID, National Institutes of Health*

Virology II (continued) and AIDS IV
Chairman: H.S. Ginsberg, *Columbia University College of Physicians & Surgeons*

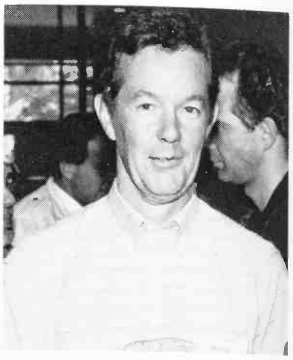
AIDS and Virology
Chairman: R.A. Lerner, *Scripps Research Institute*

Summary
Fred Brown, *USDA, Plum Island Animal Disease Center*

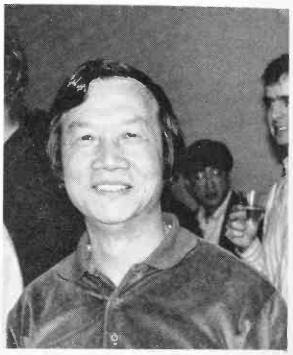
R. Houghten, J. Berzofsky, R. Germain



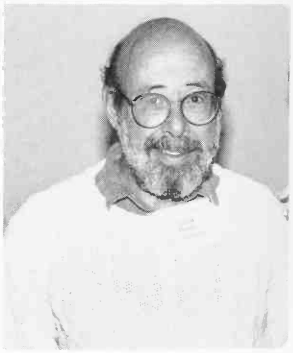
S. Zebedee



M. Bray



C.-J. Lai



A. Prince

Molecular Neurobiology of *Drosophila*

September 25–September 29, 1991

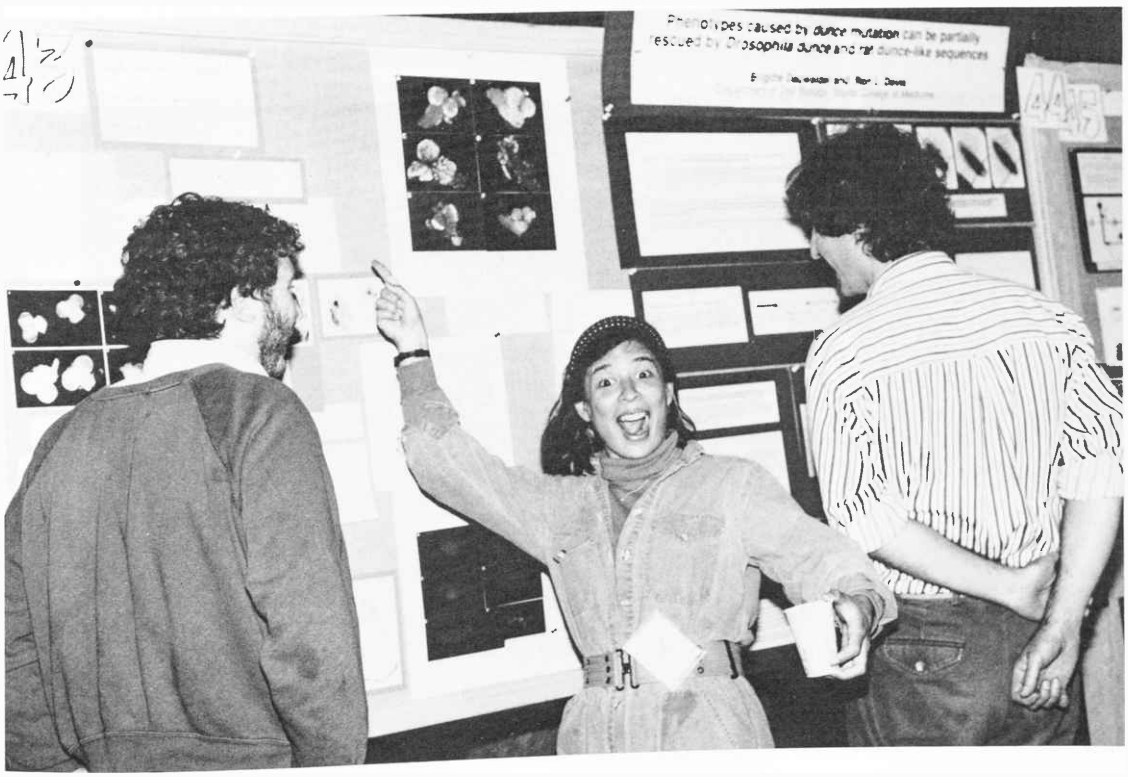
ARRANGED BY

Ralph Greenspan, Roche Institute of Molecular Biology
John Palka, University of Washington

226 participants

The molecular neurobiology of *Drosophila* is a new field of study, rooted in the spectacular advances made in fly molecular biology and in the feasibility of rather detailed studies on the nervous system despite the small size of the organism. The record attendance at the 1991 meeting testifies to the vigor of the field.

Molecular and genetic techniques are being applied to the study of many aspects of nervous system development and function. Genes essential to the initial segregation, proper spatial distribution, and correct number of nerve cell precursors are being identified in increasing numbers, and their interactions are being analyzed. Mechanisms of cell-cell communication are being dissected in ever finer detail. Our understanding of the great diversity of ion currents found in excitable cells is expanding, with several new channel-related genes being identified, cloned, and sequenced. Some of the more subtle aspects of neural function, such as rhythmicity and plasticity, are also being examined at the genetic and molecular levels, as is the evolution of at least one behavior (courtship).



S. Kunes, S. Datta, J. Heilig

The meeting hosted the first Elkins Memorial Lecture; William Zagotta (Stanford University) was selected to present his thesis work on the biophysics of genetically modified potassium channels.

This meeting was funded in part by the National Science Foundation and the National Institute of Neurological Disorders and Stroke, division of the National Institutes of Health.

PROGRAM

Behavior
Chairman: M. Young, *Rockefeller University*

Transduction and Second Messengers
Chairman: R. Davis, *Cold Spring Harbor Laboratory*

Channels and Excitability
Chairman: L. Hall, *State University of New York, Buffalo*

Elkins Memorial Lecture
William Zagotta, *Stanford University*

Guidance and Synaptogenesis
Chairman: H. Keshishian, *Yale University*

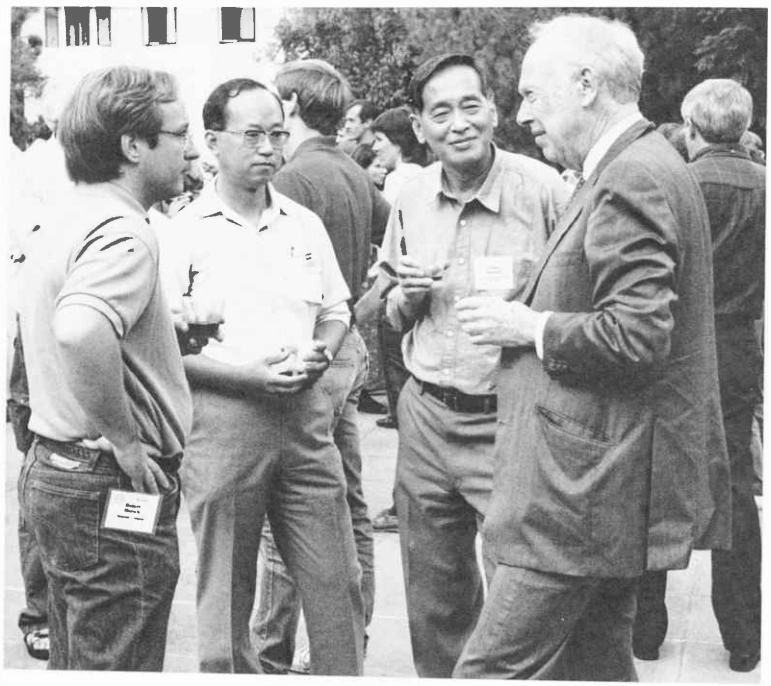
Gene Regulation
Chairman: K. White, *Brandeis University*

Cell Fate-Intercellular Signaling
Chairman: L. Zipursky, *University of California, Los Angeles*

Cell Fate:Intracellular Regulation
Chairman: E. Knust, *Institut fur Entwicklungsphysiologie, Köln*

H. Keshishian

R. Davis



R. Horsch, K. Ikeda, Y. Shimura, J.D. Watson

Molecular Biology of Signal Transduction in Plants

October 2–October 6, 1991

ARRANGED BY

Frederick Ausubel, Harvard Medical School
Nam-Hai Chua, Rockefeller University
Gerald Fink, Whitehead Institute
Venkatesan Sundaresan, Cold Spring Harbor Laboratory

148 participants

The meeting on Molecular Biology of Signal Transduction in Plants represents the first Cold Spring Harbor meeting devoted entirely to plant biology. The development of a plant is regulated by numerous external and internal signals such as light, gravity, and plant hormones. These signals lead to both general and specific biological responses by the plant, and an understanding of the mechanism of signal transduction is essential to understanding plant development. There have been rapid advances in this field over the last 2 years, many of which were presented at this meeting. The subjects covered ranged from light receptors to transcription factors regulating flowering and leaf development. Some other topics that generated much discussion included the role played by calcium in signal transduction; the cloning and characterization of kinases, phosphatases, and G-proteins from plants; and the isolation of several types of mutants in different signaling pathways. A final session was devoted to the presentation of technical advances in the field, including the development of methods for cloning genes using previously generated deletions and the introduction of cloned genes into plants, especially cereals and crop plants.

This meeting was funded in part by the National Science Foundation, the U.S. Department of Energy, and Pioneer Hi-Bred International.

F. Ausubel, V. Sundaresan, N.-H. Chua, Y. Shimura

M. Grant, R. Ranjeva, C. Weiss, K. Hammond-Kosack, T. Ashfield

PROGRAM

Reception of Environmental Signals
Chairman: J. Chory, *The Salk Institute*

Reception of Hormonal Signals
Chairman: J. Ecker, *University of Pennsylvania*

Cell-Cell Recognition
Chairman: J.B. Nasrallah, *Cornell University*

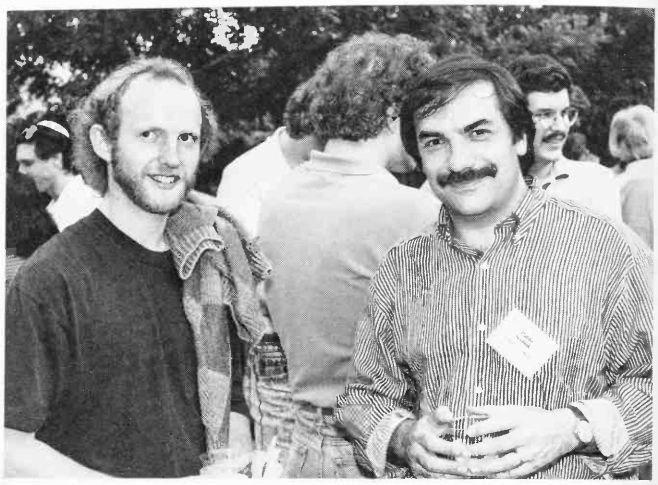
G Proteins and Protein Kinases
Chairman: H. Ma, *Cold Spring Harbor Laboratory*

Targets of Signals I
Chairman: E. Coen, *John Innes Institute*

Targets of Signals II
Chairman: S. Hake, *University of California, Berkeley*

Mutants in Signaling Pathways
Chairman: Y. Shimura, *Kyoto University*

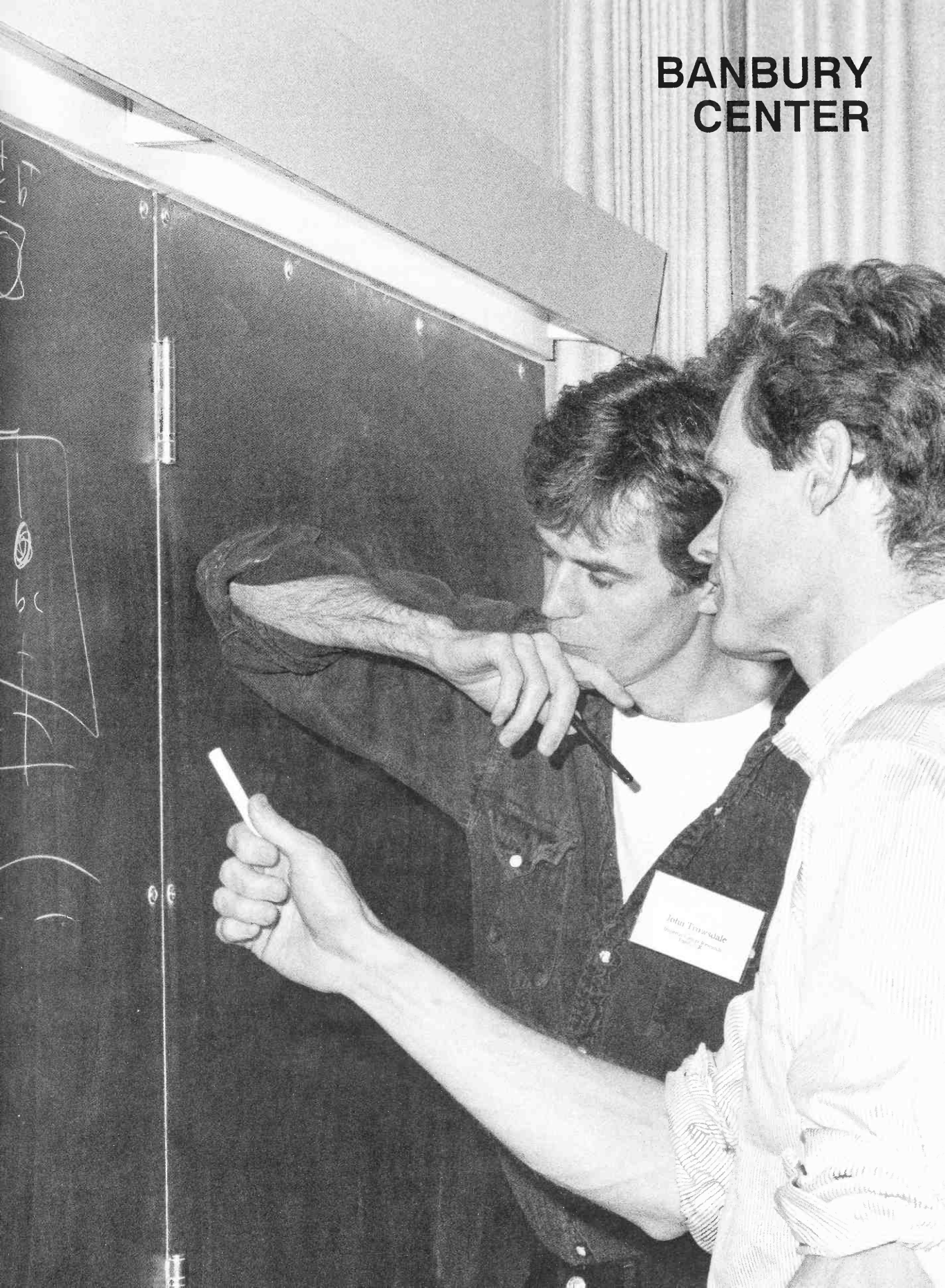
Transgenic Approaches and Technologies
Chairman: R. Horsch, *Monsanto Company*



R. Martienssen, P. Scolnik

Poster session

BANBURY CENTER



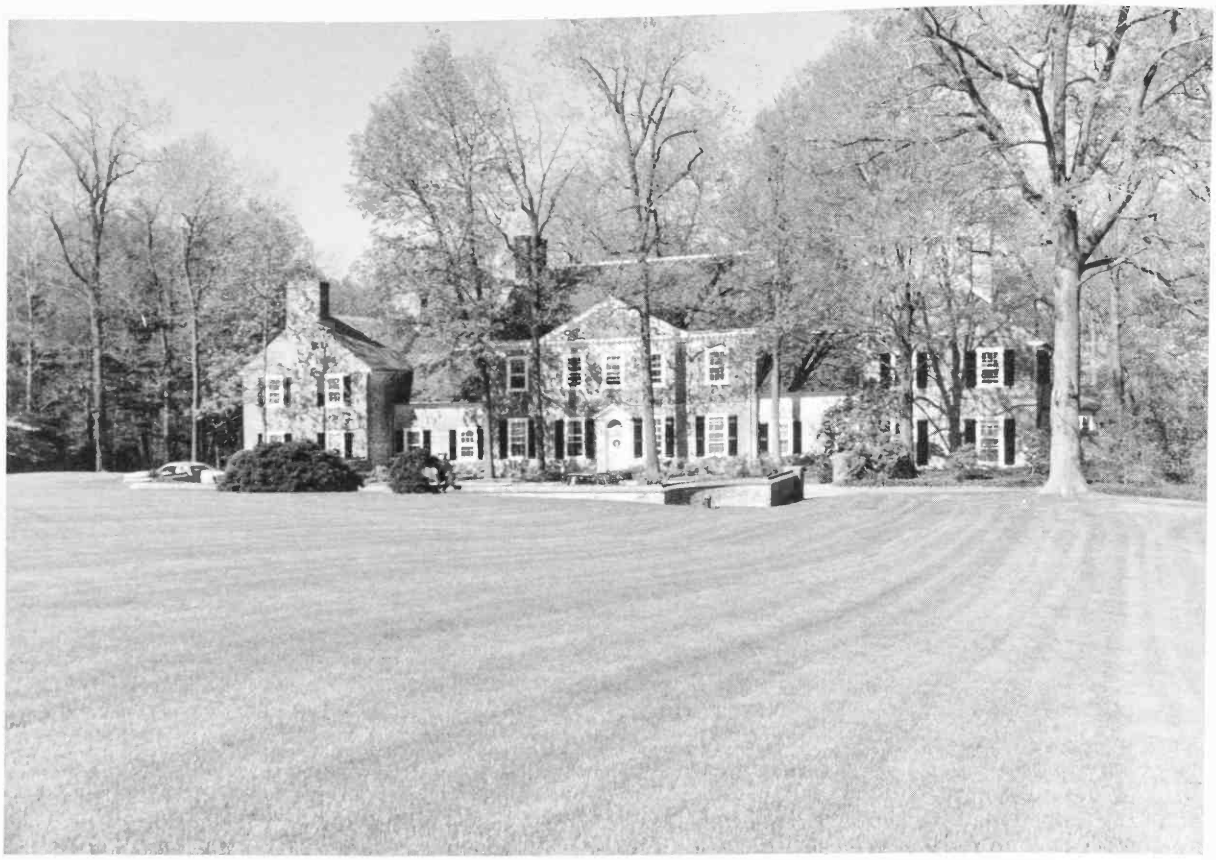
BANBURY CENTER DIRECTOR'S REPORT

The Banbury Center continues to play its role as one of the world's most effective small conference centers. 1991 was one of our busiest years ever, with 15 scientific meetings attended by almost 600 scientists. The gaps between these scientific meetings were filled with five other meetings, so that together with the courses held here during the summer, there seemed to be hardly a moment when the Center was not being used.

Molecular Biology

Two meetings early in the year set very high standards both for their scientific content and for the interest that they aroused. The first of these meetings was on **Adhesion Molecule Receptors and Disease**. Large families of adhesion molecules and their receptors have been identified in recent years, and it has become clear that a variety of responses to pathological conditions are mediated by these molecules. For example, they are involved in the cell movements in inflammation and in the mobilization of the immune system. The field has blossomed with the realization that adhesion molecule receptors are a target for therapies directed at modifying the body's response to inflammation, metastasis, and immune cell function.

Banbury Conference Center



Robertson House provides housing and dining accommodations at Banbury Center

The second meeting was also concerned with the cell surface, but with a very large number of membrane proteins that share a common structural motif—the amino acid chain of the protein loops across the membrane from cytoplasm to the exterior of the cell seven times. The **Seven-transmembrane Segment Proteins** meeting examined the various functions of this family of proteins that include the receptors for pharmacologically active drugs like acetylcholine and dopamine, G proteins that help transmit messages across the membrane, and hormone receptors. The novel element of the meeting was that it compared these proteins, employing a common molecular design, across a wide evolutionary range. Participants thus included scientists working on diverse organisms including bacteria, yeast, the fruit fly, and vertebrates.

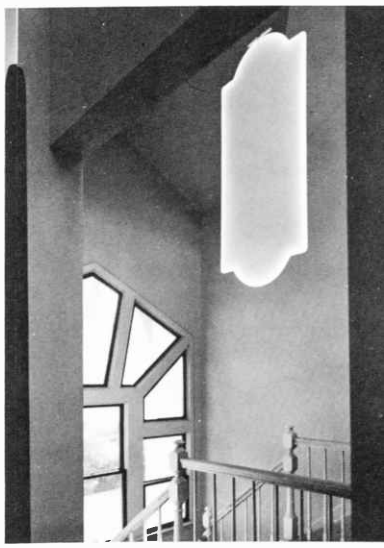
The Molecular Immunobiology of Lyme Disease meeting was particularly appropriate for the Long Island area. Lyme disease was recognized as a distinct clinical entity in 1977, and the causative agent, a spirochete *Borrelia burgdorferi*, was identified in 1982. It is the subject of increasingly intensive research as the disease spreads, and this meeting reviewed the latest findings. Research discussed ranged from the basic (trying to understand how the spirochete infects cells and how our immune system responds to infection), to the practical (trying to devise new diagnostic tests), to the future (examining how vaccines might be developed).

The bacterium *Escherichia coli* is one of the most intensively studied organisms. There is a detailed genetic map of its chromosome that is only 3 mil-

lion base pairs long. It is reasonable to regard *E. coli* as a model organism for genome studies and the Banbury meeting, **The Genome of *E. coli*** was intended to survey the current state of *E. coli* genome analysis. The topics covered included the amount of sequence available, the design and coordination of DNA sequence and genetic databases, interpretation of sequence data, and the ways in which the data can be quickly made available to all *E. coli* researchers. The meeting was outstanding in that it brought together all the leading participants from the United States, Europe, and Japan and that it received financial support from all the agencies that are interested in genome sequencing.

The **Receptor-mediated Virus Entry into Cells** meeting dealt with the earliest steps in virus infection. The mechanisms by which viruses enter cells are poorly understood, but progress has been made recently in identifying the cellular proteins that serve as receptors for viruses. In addition, the proteins in viral coats that are involved in the binding of virus to cell receptors have been analyzed by X-ray crystallography. We brought together scientists studying virus structure, cellular virus receptors, the kinetics of virus-receptor interactions, and what happens when a virus binds to a receptor. One hoped-for outcome of this research is the development of new antiviral drugs that will work by interfering with the process by which cells take up viral particles.

Sammis Hall lounge area



Sammis Hall stairway area

Human Molecular Genetics

In 1991, there were four meetings that continued Banbury Center's interest in human molecular genetics. The most timely of these meetings was **The Molecular Genetics and Cell Biology of Marfan Syndrome** meeting. Marfan Syndrome is a disorder of connective tissue, and the patients are also remarkably tall and thin (Abraham Lincoln is thought by some to have had Marfan Syndrome). Scientists have been mapping the chromosomal location of the gene involved and cloning DNA from that location, in addition to studying proteins known to be important for connective tissue function. Two lines of research came together at this spring meeting when it was found that both the gene mappers and the candidate protein researchers had cloned the same gene: the gene for a connective tissue protein called fibrillin. This was the first occasion on which the two groups had come together to discuss these findings, and their discussions hastened the publication of several papers in *Nature* reporting this work.

Although research on breast cancer is not so advanced, our **Molecular Genetics of Breast Cancer** meeting showed that progress is being made in determining the genetic factors that are involved in breast cancer. The meeting brought together scientists who are following two different lines of research in the pursuit of the genetic causes of breast cancer. Epidemiologists and population geneticists are using linkage studies to map genes in families with a high incidence of breast cancer, and at this meeting, they were able to compare their data from different families. On the other hand, scientists are studying the expression of known oncogenes in breast tumors, trying to dissect out the genetic changes going on in these tumors. These two research strategies are beginning to complement each other; chromosomal location provides clues about which genes might be involved, and genes known to be altered provide resources for gene mapping.

The **Genetics of Psychiatric Disorders** meeting tackled two even more difficult fields: schizophrenia and bipolar disorder (manic depression). The difficulties of doing genetic studies of disorders common in the general population

Banbury Meeting Room

are compounded by the difficulties of accurate diagnosis. Thus, much of the meeting was taken up with discussions of what diagnostic criteria to use and how these might be standardized so that comparable studies can be done in different families in different countries.

Although these meetings contributed to the work on trying to unravel the molecular genetic basis of human inherited disorders, the **DNA-based Diagnosis: From Laboratory to Application** meeting was concerned with the practical applications of that new knowledge. Many tests have now been devised to detect mutations in human genes, and increasing numbers of PCR-based tests are being developed for use in parasitology, microbiology, forensic science, and so on. However, relatively few of these tests have moved from the research laboratory to routine diagnostic laboratories. This meeting brought together those individuals who are developing DNA-based tests with those who have experience in the routine deployment of tests to consider how DNA-based tests can be transferred to routine use cheaply and efficiently.

Sloan Foundation Workshops

This year for the first time we invited both Congressional staff and science journalists to attend the same workshops, rather than segregating the two groups. It was a successful change, leading to further interesting interactions. Our Sloan Foundation Workshops typically cover research in biology that has important social implications, and this year was no exception. The first workshop was on **Aging**. Topics ranged from basic research on the molecular biology and genetics of senescence in cells in tissue culture and in the nematode *Caenor-*

habditis elegans, through Alzheimer's disease, to economic and social consequences of a society made up increasingly of the elderly. The second workshop discussed topics in which political and social considerations play a large part in determining what research can be done and how it can be used. Appropriately called **Biology and Society: Controversial Issues**, the workshop dealt with topics such as using human fetal tissue for transplantation, in vitro fertilization and preimplantation diagnosis, genetic screening, and risk assessment.

Human Genetics and Genome Analysis Workshops

This was the first of what we hope will be a series of workshops intended to introduce the basics of human molecular genetics to nonscientists who are concerned about the human genome projects. Funded by the Department of Energy's Office of Health and Environmental Research (responsible for the DOE's Human Genome Project), the workshops are a joint effort by Banbury Center and the DNA Learning Center. Participants were selected from teachers, journalists, bioethicists, congressional staff, parent groups, and concerned citizens. The program had three components: lectures on the basic science of genetics, experiments at the DNA Learning Center, and talks by invited speakers. Appropriately, the first meeting began with a talk by David Galas, Associate Director of the Office of Health and Environmental Research, on the genome projects. The workshop was very successful, judging by the questionnaires returned by participants after the meeting.

Social Aspects of Science Policy

In October 1981, the Banbury Center hosted an influential meeting entitled **Patenting of Life Forms**. At that time, the first patent (for a bacterium that could metabolize oil) was a little over 1 year old, and both scientists and patent attorneys were trying to understand the implications of that decision for their own fields. In October 1991, the **Intellectual Property and Biotechnology** meeting was held to assess how events have measured up to the expectations and predictions of 10 years ago. There has been an extraordinary proliferation of biotechnology companies, academic institutions are much more sensitive to research of commercial potential, and many scientists have links directly or as consultants to companies. In addition to legal questions that are still unresolved, there was discussion of various socio-scientific questions. For example, there have been fears that the drive for commercial exploitation of research has led to more secretiveness and a reluctance to share data and materials. Fortuitously, the meeting was held just a few weeks after the announcement by the National Institutes of Health that patents had been filed for brain cDNAs.

Baring Brothers/Cold Spring Harbor Laboratory Meeting

Signal transduction—the means by which messages outside the cell are transmitted to the interior of the cell—was especially big news in biotechnology circles in 1991. We thus made the **Signal Transduction** meeting the sixth in the series of meetings sponsored by Baring Brothers for executives of biotechnology companies and investors. As usual, an outstanding group of scientists spoke at the meeting and the participants also spent time with Cold Spring Harbor

Henry Bourne (Baring Bros./CSHL Meeting)

Y. Sugino, J.D. Watson, B.R. Sykes

Laboratory scientists. A highlight of the meeting was the laboratory class, held this year in the teaching laboratories of the Beckman Neuroscience Center. The experiment was simple—isolating DNA from bacteria—but the participants had the privilege of being taught by Nobel laureate Sydney Altman. The scientists who attend Banbury Center meetings in 1992 and thereafter owe Baring Brothers a special vote of thanks. The chairs in the conference room have been renovated so that for the first time in many years participants will be at the correct height for the tables and not be in fear of falling off the chairs!

The William Stamps Farish Fund Meeting

The Farish Fund has given the Center funding for three meetings over a 3-year period. The general subject for the meetings is the genetics of complex human diseases. The first of the meetings was **Molecular Basis of HLA Predisposition**. It has been known for a long time that certain diseases, especially autoimmune diseases like diabetes, are associated with particular forms of genes found in the major histocompatibility locus. The molecular mechanisms underlying these associations are just beginning to be understood, i.e., we are beginning to see how changes in proteins that play an essential role in immune system function can have an effect on many apparently unrelated systems. Participants in the meeting reviewed the latest advances in this field.

Other Meetings

The Banbury Center continued to host a small number of meetings outside the main program. The Joint Informatics Task Force of the Department of Energy's genome project came here in January to review what needs to be done in an area that will prove crucial for the full exploitation of genome data. The Carnegie Council on Ethics and International Affairs held the Sixth Annual U.S.-Japan Agricultural Conference at Banbury Center. Appropriately, the topic for the meeting was a discussion of U.S.-Japan advances in biotechnology. Three other groups held meetings at Banbury Center: the Trustees of Huntington Hospital, the Psychiatry Department at Mt. Sinai, and the Trustees of the Planting Fields Foundation. The first in a new series of Lloyd Harbor seminars was given by Ann Gill of the Cold Spring Harbor Whaling Museum.

Two meetings held at Banbury Center spawned meetings in Grace Auditorium! George Cutting (chairman of LIBA) and I decided to exploit the presence of experts on Lyme disease and breast cancer at Banbury by having meetings for LIBA associates on these topics. So one day in April, four speakers from the meeting on Lyme disease were whisked away to Grace Auditorium for talks and a question and answer session, and 6 months later, we did the same with Mary-Claire King and Marc Lippman for a session on breast cancer.

Meetings Publications

Banbury Center meetings have continued to be a source of books published by Cold Spring Harbor Laboratory Press. Four titles in the new *Current Communications in Cell and Molecular Biology* series appeared, and most notably in the *Banbury Report* series, the book from the meeting, *The Biological Basis for Risk Assessment of Dioxins and Related Compounds*, was published. This was eagerly awaited as the definitive account of a meeting where misinformation following the meeting had been the source of much confusion.

Funding

I have already acknowledged the Alfred P. Sloan Foundation for their support of the Congressional staff and science journalists' workshops. The Foundation has been a staunch supporter of the Banbury Center for many years. The **Human Genetics and Genome Analysis** meetings funded by the Office of Health and Environmental Research, Department of Energy, allow us to expand this program of providing information for nonscientists. We are very grateful to the William Stamps Farish Fund for its 3-year grant. Such support gives us much flexibility in planning ahead, instead of having to scramble for funds each year. It was particularly pleasing that the **Genome of *E. coli*** meeting received funding from all the federal agencies interested in genome research: the National Center for Human Genome Research, the National Science Foundation, the National Library of Medicine, the Office of Health and Environmental Research, and the Department of Energy. The exciting meeting on the **Molecular Genetics and Cell Biology of Marfan Syndrome** was funded by the National Marfan Foundation and by the National Institute of Arthritis and Musculoskeletal and Skin Diseases. Once again, the corporate world was the Banbury Center's principal benefactor, through contributions to individual meetings and by company support of the Cold Spring Harbor Laboratory Corporate Sponsor Program. The legal divisions of five companies contributed to the costs of the meeting on **Intellectual Property and Biotechnology**, and the meeting on the **Molecular Biology of Lyme Disease** was funded by contributions from MetPath, Allen & Hanburys (Glaxo Inc.), Home Infusion, and the National Multiple Sclerosis Society. As usual, our greatest debt is to the members of the Cold Spring Harbor Laboratory Corporate Sponsor Program as funding for no fewer than five of our meetings came from this source.

Acknowledgments

The Banbury Center could not possibly operate with its present intensity without the hard work and enthusiasm of all involved. In particular, our tight schedule puts a tremendous strain on Bea Toliver and Ellie Sidorenko in the Center's office

and on Katya Davey at Robertson House. The importance of their contributions can be seen in the many letters of appreciation that we receive from participants. The groundskeepers, Danny Miller and Andy Sauer, battle leaves in the fall and grass in the summer to make the Banbury estate a very special place for meetings. The Laboratory's housekeeping and catering staff also did a wonderful job coping with the rapid turnover of meetings and the increased numbers of participants at our meetings.

Jan A. Witkowski

Publications

Watson, J.D. , M. Gilman, J.A. Witkowski, and M. Zoller. 1992. *Recombinant DNA*. W.H. Freeman, New York.

Sammis Hall, guest house

MEETINGS

Sloan Foundation Congressional Workshop on Aging

January 24–January 26

ARRANGED BY

J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York

SESSION 1

O.M. Pereira-Smith, Baylor College of Medicine, Houston, Texas: The molecular biology of aging of cells in vitro.
T.E. Johnson, University of Colorado, Boulder: Cell death in vivo and its role in normal development.
P. Davies, Albert Einstein College of Medicine, Bronx, New York: Alzheimer's Disease: Molecular pathology.
R. Tanzi, Massachusetts General Hospital, Boston: The genetics of Alzheimer's Disease.

SESSION 2

R. Suzman, National Institute on Aging, Bethesda, Maryland: The demography of aging populations.
R. Berg, Strong Memorial Hospital, Rochester, New York: Special features of health care for the elderly.
D. Callahan, The Hastings Center, Briarcliff Manor, New York: Provision of health care for the aged.

J. Witkowski, R. Herdman, R. Levinson

Adhesion Molecule Receptors and Disease

March 10–March 13

ARRANGED BY

J. M. Harlan, University of Washington, Seattle
T.A. Springer, Center for Blood Research, Boston, Massachusetts

SESSION 1: The Matrix, Cell Interactions, and Disease

Chairperson: R. Hynes, Massachusetts Institute of Technology, Cambridge

R. Hynes, Massachusetts Institute of Technology, Cambridge: Genetic analyses of cell-matrix adhesion.

M.E. Hemler, Dana-Farber Cancer Institute, Boston, Massachusetts: VLA-2 and VLA-4 in the integrin family: Adhesion receptors of critical importance to inflammation and metastasis.

C.F. Ockenhouse, Walter Reed Army Institute of Research, Washington, D.C.: Molecular basis for adhesion to CD36 and ICAM-1 by *Plasmodium falciparum* malaria-infected erythrocytes.

J. Leong, New England Medical Center, Boston, Massachusetts: Cellular penetration by yersina pseudotuberculosis.

S.-I. Hakomori, The Biomembrane Institute and University of Washington, Seattle: Multiple functional roles of carbohydrates in cell adhesion.

SESSION 2: Adhesion and Inflammation

Chairperson: T.A. Springer, Center for Blood Research, Boston, Massachusetts

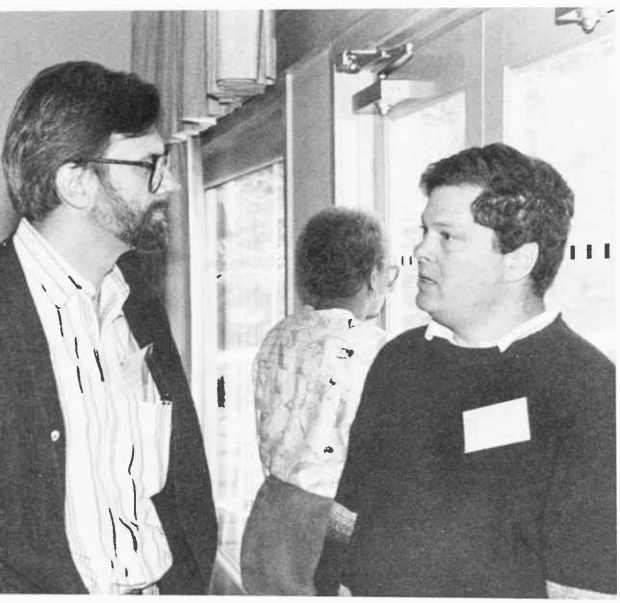
T.A. Springer, Center for Blood Research, Boston, Massachusetts: How many ICAMs?

M.S. Diamond, Center for Blood Research, Boston, Massachusetts: Mac-1 (CD11b/CD18) and its interaction with ICAM-1 (CD54).

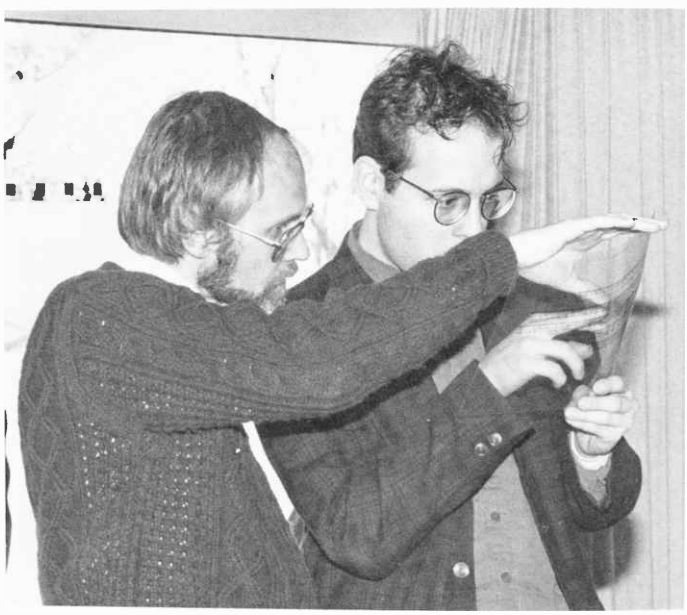
S.D. Wright, The Rockefeller University, New York, New York: Regulation of CD18 function: Activation of ELAM-1, and role of a novel small molecule as an allosteric effector of CD18.

F. Sanchez-Madrid, Hospital de la Princesa, Madrid, Spain: Functional mapping and regulation of VLA-4 adhesion activities.

S. Shaw, National Cancer Institute, Bethesda, Maryland: Adhesion molecules in T-cell differentiation and function.



D. Anderson, M. Diamond



T. Springer, B. Furie

SESSION 3: Adhesion and Inflammation: Inflammation and Selections

Chairperson: B. Furie, Tufts University School of Medicine, Boston, Massachusetts

C. MacKay, Basel Institute for Immunology, Switzerland: Correlations between the physiological recirculation of lymphocytes and expression of adhesion/homing molecules.

B. Furie, Tufts University School of Medicine, Boston, Massachusetts: PADGEM: A receptor that mediates platelet-leukocyte interaction by recognition of a lineage-specific carbohydrate role in thrombosis and inflammation.

R.P. McEver, University of Oklahoma Health Science Center, Oklahoma City: Leukocyte interactions with GMP-140.

G.A. Zimmerman, University of Utah School of Medicine, Salt Lake City: Proadhesive molecules expressed by activated endothelium-binding and -signaling functions.

J.C. Paulson, Cyetel Corporation, La Jolla, California: Comparison of the carbohydrate specificities of ELAM-1 and GMP-140.

SESSION 4: Adhesion and Inflammation: The Immune System

Chairperson: T.F. Tedder, Dana-Farber Cancer Institute, Boston, Massachusetts

R. Lobb, Biogen, Cambridge, Massachusetts: BCAM1 and ELAM1: Recent studies.

T.F. Tedder, Dana-Farber Cancer Institute, Boston, Massachusetts: LAM-1 mediates lymphocyte and neutrophil binding to endothelium in cooperation with other adhesion receptors.

T. Kishimoto, Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, Connecticut: ELAM-1 is a receptor for skin-homing lymphocytes.

U. von Andrian, Pharmacia Experimental Medicine, La Jolla, California: A two-step model of leukocyte-endothelial cell interaction: Different roles for LECAM-1 and the B_2 integrins in vivo.

L.A. Lasky, Genentech, Inc., South San Francisco, California: The homing receptor: A lectin cell adhesion molecule of the immune system.

P.W. Kincade, Oklahoma Medical Research Foundation, Oklahoma City: Adhesion molecules utilized in bone marrow.

SESSION 5: Adhesion Molecules and Therapy

Chairperson: J.M. Harlan, University of Washington, Seattle

J.M. Harlan, University of Washington, Seattle: CD18 monoclonal antibody reduces vascular and tissue injury in acute inflammation.

R.F. Todd III, University of Michigan Medical School, Ann Arbor: β1 integrin expression on human small cell lung cancer cells.

D.C. Anderson, Baylor College of Medicine, Houston, Texas: Role of ICAM-1 in canine myocardial reperfusion injury: Mechanisms of regulation and therapeutic applications.

R. Rothlein, Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, Connecticut: The anti-inflammatory effects of anti-ICAM-1 MAb.

Seven-transmembrane Segment Proteins

March 17–March 20

ARRANGED BY

M.R. Brann, National Institute of Neurological Disorders and Stroke, Bethesda, Maryland
M.G. Caron, Howard Hughes Medical Institute, Duke University Medical Center, Durham, North Carolina
M.R. Hanley, University of California, Davis

SESSION 1

Chairperson: M.R. Hanley, University of California, Davis

R. Henderson, MRC Laboratory of Molecular Biology, Cambridge, United Kingdom: Structure of bacteriorhodopsin.

E.L. Berson, Harvard Medical School, Boston, Massachusetts: Rhodopsin gene mutations in patients with autosomal dominant retinitis pigmentosa.

D.D. Jenness, University of Massachusetts Medical School, Worcester: Factors controlling stability and internalization of the yeast α-factor pheromone receptor.

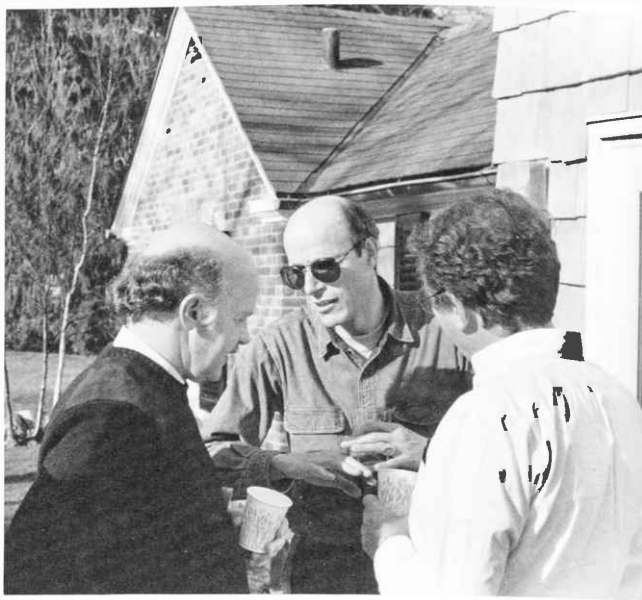
J.A. Kurjan, University of Vermont College of Medicine, Burlington: Role of a G protein in yeast pheromone

response: Effect of mutations in guanine nucleotide binding and carboxy-terminal domains.

L. Marsh, Albert Einstein College of Medicine, Bronx, New York: Determinants of ligand specificity and activation for the yeast α-factor receptor.

J.W. Thorner, University of California, Berkeley: New insights in desensitization: Role of the SST2 gene product in adaptation of the yeast pheromone response pathway.

G. Vassart, R. Sprengle



J. Shine, O. Civelli

SESSION 2

Chairperson: M.G. Caron, Howard Hughes Medical Institute, Duke University Medical Center, Durham, North Carolina

P.N. Adler, University of Virginia, Charlottesville: The frizzled tissue polarity gene of *Drosophila*.

P.N. Devreotes, Johns Hopkins Medical School, Baltimore, Maryland: The cAMP receptor of slime mold.

N.M. Nathanson, University of Washington, Seattle: Function and regulation of muscarinic acetylcholine receptors and G proteins.

E.C. Hulme, MRC National Institute for Medical Research, London, United Kingdom: Muscarinic acetylcholine receptors: Structure-function relationships.

M.R. Brann, National Institute of Neurological Disorders and Stroke, Bethesda, Maryland: Localization of muscarinic receptor subtype gene expression: mRNAs and proteins.

J. Wess, National Institutes of Health, Bethesda, Maryland: Mutational analysis of muscarinic receptors: Identification of ligand binding and effector coupling domains.

SESSION 3

Chairperson: M. R. Brann, National Institute of Neurological Disorders and Stroke, Bethesda, Maryland

R.J. Lefkowitz, Howard Hughes Medical Institute, Duke University Medical Center, Durham, North Carolina: Adrenergic receptors: Structure and regulation.

O. Civelli, Oregon Health Sciences University, Portland: Molecular biology and complexity of dopamine receptors.

J.-C. Schwartz, INSERM, Paris, France: The dopamine D_3 receptor gene and its products: Localization and function.

M.G. Caron, Howard Hughes Medical Institute, Duke University Medical Center, Durham, North Carolina: Catecholamine receptors/structure-function.

G. Vassart, Institut de Recherche Interdisciplinaire en Biologie Humaine, Brussels, Belgium: Identification of adenosine A_1 and A_2 receptors amongst a series of orphan receptors.

S. Nakanishi, Kyoto University Faculty of Medicine, Japan: Molecular characterization of metabotropic glutamate receptors.

E. Mulvihill, ZymoGenetics, Inc., Seattle, Washington: Cloning and expression of the metabotropic glutamate receptor.

SESSION 4

Chairperson: R. J. Lefkowitz, Howard Hughes Medical Institute, Duke University Medical Center, Durham, North Carolina

J. Shine, St. Vincent's Hospital, Sydney, Australia: Molecular cloning and expression of G protein coupled receptors.

M. Brownstein, National Institute of Mental Health, Bethesda, Maryland: Novel G protein coupled receptors.

M.C. Gershengorn, Cornell University Medical College, New York, New York: The TRH receptor.

J.B.C. Findlay, University of Leeds, United Kingdom: Protein chemistry and computer-based approaches to the structure of G-protein-linked receptors.

J. Battey, National Institute of Neurological Disorders and Stroke, Bethesda, Maryland: Bombesin receptors.

D. Richter, University of Hamburg, Germany: Neuropeptide receptors.

T.J. Murphy, Emory University School of Medicine, Atlanta, Georgia: The angiotensin II receptor.

C. Dykes, Glaxo Group Research Limited, Middlesex, United Kingdom: Expression of *MAS* oncogene in *Xenopus* oocytes.

SESSION 5

Chairperson: R. Henderson, MRC Laboratory of Molecular Biology, Cambridge, United Kingdom

R. Cone, Oregon Health Sciences University, Portland: New sites for expression for the glycoprotein hormone receptors: TSH receptor in adipocytes and LH/CG receptor in the thyroid.

R. Sprengel, ZMBH, University of Heidelberg, Germany: Signal transduction at gonadotropin receptors.

E.M. Ross, University of Texas, Dallas: Role of extreme carboxy-terminal regions in determining receptor localization.

A.D. Strosberg, CNRS, Institut Cochin de Genetique Moleculaire, Paris, France: Ligand and G protein binding to B-adrenergic and serotoninergic receptors expressed in *E. coli*.

DNA-based Diagnosis: From Laboratory to Application

March 31–April 3

ARRANGED BY

R.A. Gibbs, Baylor College of Medicine, Houston, Texas
D.W. Yandell, Massachusetts Eye and Ear Infirmary, Boston

Coffee break in Conference Center Library

SESSION 1: DNA Screening Programs

P. Reilly, Shriver Center for Mental Retardation, Waltham, Massachusetts: The CF debate: Do medico-legal forces drive the implementation of new tests?

R.A. Gravel, Montreal Children's Hospital Research Institute, Quebec, Canada: Mutation screening in Tay-Sachs disease.

H.H. Kazazian, Jr., Johns Hopkins Hospital, Baltimore, Maryland: Impact of DNA testing on family diagnosis.

F.K. Fujimura, Nichols Institute, San Juan Capistrano, California: Clinical applications of DNA-based diagnosis.

SESSION 2: New Developments in Instrumentation

L.M. Smith, University of Wisconsin-Madison: High-speed DNA sequencing by horizontal ultra-thin gel electrophoresis with fluorescence detection.

L.J. McBride, Applied Biosystems, Foster City, California: Automated genetic analysis: PCR and analytical fluorescence-based detection.

A. Costa, Beckman Instruments, Palo Alto, California: Robotics in the laboratory.

SESSION 3: Techniques for DNA Screening

D. Nickerson, California Institute of Technology, Pasadena: DNA diagnostics using an oligonucleotide ligation assay.

S.S. Sommer, Mayo Clinic/Foundation, Rochester, Minnesota: An efficient two-tiered diagnostic approach applied to DNA-based testing in hemophilia and familial amyloidotic polyneuropathy.

D.J. Kemp, The Walter and Elizabeth Hall Institute of Medical Research, Victoria, Australia: Capture of PCR products with DNA binding proteins.

S.R. Bouma, Abbott Laboratories, Abbott Park, Illinois: The ligase chain reaction (LCR) in DNA-based diagnostics.

E.R.B. McCabe, Baylor College of Medicine, Houston, Texas: DNA analysis using newborn screening specimens: Evolving applications for dried blood spot technology.

SESSION 4: Applications in Genetic Diseases

D.J. Prockop, Jefferson Medical College, Philadelphia, Pennsylvania: Repetitive sequencing of collagen genes for mutations that can cause common diseases such as osteoarthritis, arterial aneurysms, and osteoporosis.

L.-C. Tsui, The Hospital for Sick Children, Toronto, Ontario, Canada: Identification of mutations in the cystic fibrosis transmembrane conductance regulator gene.

F.F. Chehab, University of California, San Francisco: Molecular diagnostics of cystic fibrosis in a clinical laboratory: A new perspective.

F. Smith, Shriver Center for Mental Retardation, Waltham, Massachusetts: Use of single-strand conformation polymorphism (SSCP) analysis and denaturing gradient gel electrophoresis for screening patient samples for defects in the acid β-glucosidase and proteolipid protein genes.

J.S. Chamberlain, University of Michigan Medical School, Ann Arbor: Diagnosis and carrier detection of DMD via PCR.

L. Ugozzoli, Beckman Research Institute, Duarte, California: Allele-specific PCR: A new application.

D.F. Wirth, Harvard School of Public Health, Boston, Massachusetts: DNA probe diagnosis of parasitic diseases: Use of PCR.

B. Trask, R. Gibbs

SESSION 5: DNA-Based Diagnosis Of Cancer

S.H. Friend, Massachusetts General Hospital Cancer Center, Charlestown: Screening for germ line p53 mutations: Within and outside of L.-Fraumeni families.

D.W. Yandell, Massachusetts Eye and Ear Infirmary, Boston: DNA-based diagnosis of cancer predisposition: Analysis of *RB* and *p53* genes.

K. Hayashi, National Cancer Center Research Institute, Tokyo, Japan: PCR-SSCP analysis: Detection of DNA polymorphisms and mutations in oncogenes, antioncogenes, or genes for hereditary diseases.

B.J. Trask, Lawrence Livermore National Laboratory, California: Chromosome analysis using flow cytometry and fluorescence in situ hybridization.

SESSION 6: Detection of Viruses and Other Pathogens

T.J. White, Roche Diagnostic Research, Alameda, California: Development of PCR-based diagnostic kits and services for HIV-1.

R.A. Gibbs, Baylor College of Medicine, Houston, Texas: Analysis of mixed DNA sequences in genetic and infectious diseases.

B. Weiser, State University of New York at Stony Brook: Measurement of HIV-1 nucleotide sequence diversity among close contacts for epidemiologic investigation.

S.M. Wolinsky, Northwestern University Medical School, Chicago, Illinois: Characterization of HIV-1 sequence variation between mother-infant transmission pairs using PCR-temperature gradient gel electrophoresis (TGGE) and direct PCR cycle sequencing.

D. Shibata, University of Southern California, Los Angeles: Applications of the PCR to routinely obtained fixed tissue.

H.H. Kazazian, Jr., Johns Hopkins Hospital, Baltimore, Maryland: Concluding remarks.

The Molecular Immunobiology of Lyme Disease

April 7–April 10

ARRANGED BY

J.J. Dunn, Brookhaven National Laboratory, Upton, New York
S.E. Schutzer, UMDNJ-New Jersey Medical School, Newark

SESSION 1: Pathogenesis

Chairperson: J.L. Benach, State University of New York at Stony Brook

J.L. Benach, State University of New York at Stony Brook: Diversity in the adhesion process of *Borrelia* to eukaryotic cells.

P.H. Duray, Fox Chase Cancer Center, Philadelphia, Pennsylvania: Mammalian tissue–spirochete interactions.

G. Peltz, Syntex, Palo Alto, California: T cells in the pathogenesis of human Lyme arthritis.

S.W. Barthold, Yale University School of Medicine, New Haven, Connecticut: Lyme borreliosis in the laboratory mouse.

N. Bhardwaj, The Rockefeller University, New York, New York: Dendritic cells in human blood and synovial exudates.

SESSION 2: Neurology

Chairperson: P.K. Coyle, State University of New York at Stony Brook

P.K. Coyle, State University of New York at Stony Brook: Immune complex analysis in *Borrelia burgdorferi* infection.

D.E. Griffin, Johns Hopkins University School of Medicine, Baltimore, Maryland: Immune responses to CNS infections.

S.A. Lukehart, University of Washington School of Medicine, Seattle: Early involvement of the central nervous system by *Treponema pallidum.*

G. Habicht, State University of New York at Stony Brook: Molecular mechanisms of *Borrelia burgdorferi* pathogenicity.

J.C. Garcia-Monco, Hospital de Galdacano, Vizcaya, Spain: Early central nervous system invasion by *Borrelia burgdorferi.*

SESSION 3: Molecular Biology I: Surface Antigens

Chairperson: J.J. Dunn, Brookhaven National Laboratory, Upton, New York

J.J. Dunn, Brookhaven National Laboratory, Upton, New York: High-level expression of *Borrelia burgdorferi* surface proteins.

P.A. Rosa, Rocky Mountain Laboratories, Hamilton, Montana: The molecular basis of (some) outer surface protein variation in *Borrelia burgdorferi*.

J. Radolf, University of Texas, Dallas: Immunobiology of spirochete lipoproteins.

D.H. Persing, Mayo Clinic, Rochester, Minnesota: Ticks, mice, and men: Molecular detection of the Lyme disease spirochete.

I. Saint-Girons, Institut Pasteur, Paris, France: Genome organization of *Borrelia*.

SESSION 4: Immunobiology I: Immune Response

Chairperson: S.E. Schutzer, UMDNJ-New Jersey Medical School, Newark

R.C. Johnson, University of Minnesota, Minneapolis: Immunogenicity of *Borrelia burgdorferi*.

S.E. Schutzer, University of Medicine & Dentistry of New Jersey/New Jersey Medical School, Newark: Autoimmune reactions triggered by *Borrelia burgdorferi* infection, versus persistent infection.

J. Zabriskie, Rockefeller University, New York, New York: Cross-reactive antibody against bacteria.

S.-M. Cheng, Wyeth-Ayerst Research, Radnor, Pennsylvania: Antigen capture PCR.

B. Johnson, Centers for Disease Control, Fort Collins, Colorado: Evaluation of recombinant Ospa as a protective immunogen in tick-challenged hamsters.

E. Fikrig, Yale University School of Medicine, New Haven, Connecticut: Protective immunity in Lyme borreliosis.

D.J. Volkman, State University of New York at Stony Brook: *Borrelia* antigens eliciting early T- and B-cell responses.

U.E. Schaible, Max-Planck-Institut fur Immunbiologie, Freiburg, Germany: A mouse model for *Borrelia burgdorferi* infection: Pathogenesis immune response and protection.

SESSION 5: Special Topics

Chairperson: F.S. Kantor, Yale University School of Medicine, New Haven, Connecticut

Molecular Biology II

J. Hinnebusch, University of Texas, San Antonio: Structural characterization of *Borrelia* linear plasmids.

Immunobiology II-Round Table: Detection of Bb

M. Sand, MetPath Inc., Teterboro, New Jersey: Serological detection of Lyme disease.

M. Golightly, State University of New York at Stony Brook: Comparative studies of antibody detection of *Borrelia burgdorferi*.

NIH Report and Questions to be Researched

B.J. Luft, State University of New York at Stony Brook: Development and use of recombinant proteins for serodiagnosis of Lyme disease.

S.P. Heyse, National Institutes of Health, Bethesda, Maryland: Brief report on the NIAMS Lyme disease research and education programs.

B. Johnson, S. Lukehart, R. Montgomery

The Molecular Genetics and Cell Biology of Marfan Syndrome

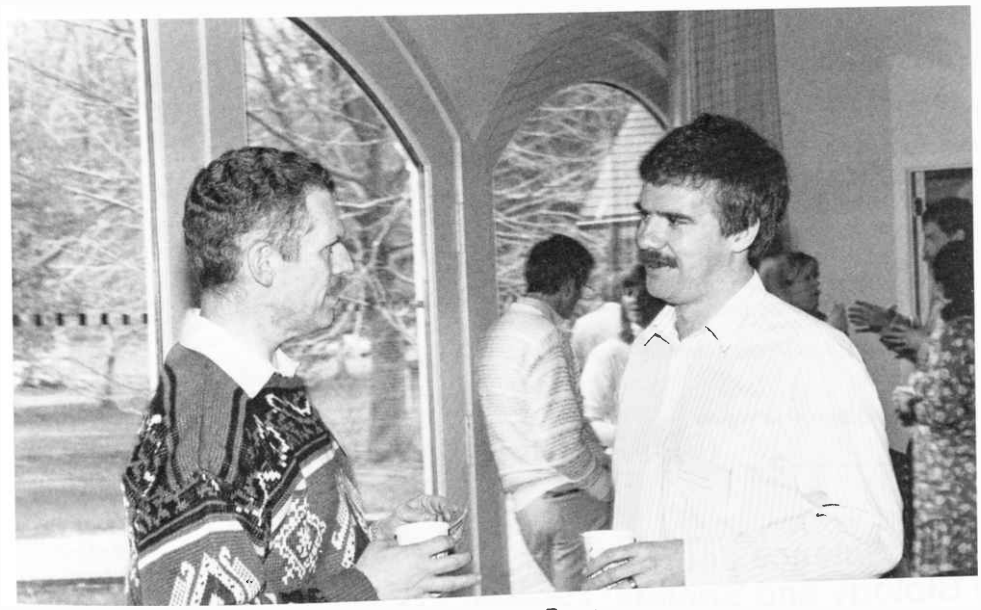
April 19–April 21

ARRANGED BY

F.S. Collins, Howard Hughes Medical Institute, University of Michigan, Ann Arbor
U. Francke, Howard Hughes Medical Institute, Stanford University Medical Center, California
J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York

OPENING SESSION

F.S. Collins, Howard Hughes Medical Institute, University of Michigan, Ann Arbor: Deducing the genetic pathogenesis of MFS: Examples from other disorders.



Meeting participants during coffee break in Conference Room

SESSION 1: Phenotype and Heterogeneity

Chairperson: F.S. Collins, Howard Hughes Medical Institute, University of Michigan, Ann Arbor

R.E. Pyeritz, John Hopkins University School of Medicine, Baltimore, Maryland: The MFS and related disorders: History and nosology.

R.B. Devereux, New York Hospital–Cornell Medical Center, New York, New York: Phenotype and clinical complications of the MFS: The need for definitive (genetic) and intermediate (nongenetic) therapies.

SESSION 2: Linkage Studies

Chairperson: C.A. Francomano, Johns Hopkins Hospital, Baltimore, Maryland

L. Peltonen, National Public Health Institute, Helsinki, Finland: Location of the MFS locus and heterogeneity analysis with markers of 15q.

M. Sarfarazi, University of Connecticut Health Center, Farmington: The linkage map of 15q markers.

H.C. Dietz, The Johns Hopkins Hospital, Baltimore,

Maryland: Linkage relationships between the MFS locus and chromosome 15 markers.

P. Tsipouras, University of Connecticut Health Center, Farmington: Fine mapping of the region surrounding the MFS locus on chromosome 15.

M.W. Kilpatrick, University of Birmingham, United Kingdom: Localization of a gene for MFS by linkage analysis with chromosome 15 markers.

Discussion: Linkage and heterogeneity.

SESSION 3: Elastic Tissue Microfibrils and Fibrillin

Chairperson: P. Byers, University of Washington, Seattle

R.W. Glanville, Shriners Hospital for Crippled Children, Portland, Oregon: Structural studies on fibrillin and microfibril assembly.

L.Y. Sakai, Shriners Hospital for Crippled Children, Portland, Oregon: Biochemical characterization of fibrillin.

C. Maslen, Shriners Hospital for Crippled Children, Portland, Oregon: Cloning and sequence analysis of fibrillin cDNA.

E. Magenis, Oregon Health Sciences University, Portland: Chromosome localization of the fibrillin gene.

M. Godfrey, University of Nebraska Medical Center, Omaha: Immunohistochemical analyses of microfibrils in MFD and other pathological conditions.

D. McGookey, University of Washington, Seattle: MFS: Defective synthesis, secretion, and extracellular matrix formation of fibrillin by cultured dermal fibroblasts.

K. Potter, Washington State University, Pullman: Bovine model for MFS.

Discussion: Fibrillin gene defects in MFS.

SESSION 4: Other Elastic Fiber Genes

Chairperson: B. Sykes, University of Oxford, United Kingdom

R.P. Mecham, The Jewish Hospital of St. Louis, Missouri: The elastic fiber microfibril: Catalyst of elastic fiber.

J. Rosenbloom, University of Pennsylvania, Philadelphia: Molecular cloning of elastic fiber genes.

B. Lee, Mt. Sinai School of Medicine, New York, New York: A candidate gene approach toward characterizing the molecular lesions in MFS.

SESSION 5: Summary and Strategies for Future Research

Chairperson: U. Francke, Howard Hughes Medical Institute, Stanford University Medical Center, California

P. Ciccariello, National Marfan Foundation, Port Washington, New York: The role of NMF.

Sloan Foundation Science Journalists/Congressional Workshop on Biology and Society: Controversial Issues

May 5–May 7

ARRANGED BY

J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York

SESSION 1

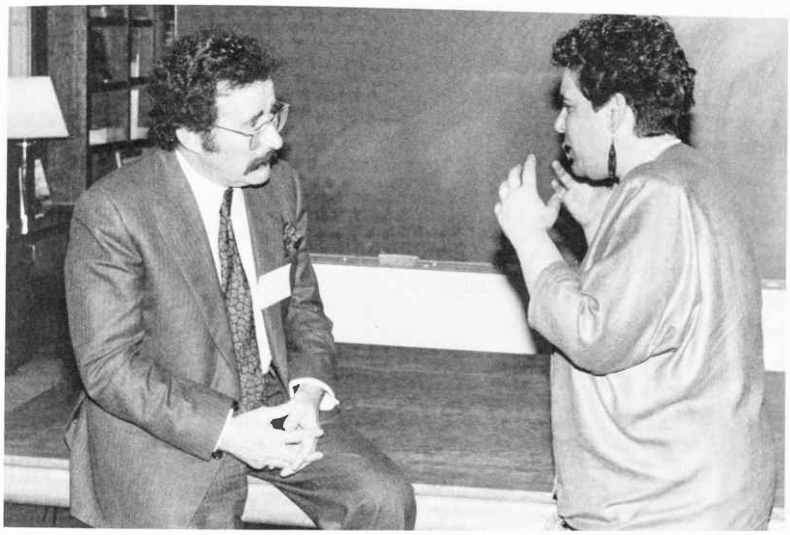
A. Charo, University of Wisconsin Law School, Madison: RU486 and contraceptive research.

R.M.L. Winston, Royal Postgraduate Medical School, Institute of Obstetrics and Gynaecology, London, United Kingdom: Preimplantation genetic diagnosis.

K.J. Lafferty, Childhood Diabetes Center, University of Colorado Health Sciences Center, Denver: Human fetal tissue transplantation.

SESSION 2

D. Vawter, Center for Biomedical Ethics, University of Minnesota, Minneapolis: Ethical and legal aspects of research using human fetal tissue.



R. Winston, A. Charo

SESSION 3

P.T. Rowley, Division of Genetics, University of Rochester Medical Center, New York: Screening populations for genetic mutations: Cystic fibrosis.

W.C. Thompson, Program in Social Ecology, University of California, Irvine: DNA fingerprinting in forensic science: Scientists on the witness stand.

J.D. Graham, Department of Health Policy and Management, Harvard School of Public Health, Boston, Massachusetts: Setting limits for environmental exposure: The use of expert scientific judgment.

Intellectual Property and Biotechnology

October 10–October 13

ARRANGED BY

A.P. Halluin, Fliesler, Dubb, Meyer & Lovejoy, San Francisco, California
J. Maroney, Cold Spring Harbor Laboratory, New York
J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York

OPENING SESSION: Introduction

Chairperson: A.P. Halluin, Fliesler, Dubb, Meyer & Lovejoy, San Francisco, California

A.P. Halluin, Fliesler, Dubb, Meyer & Lovejoy, San Francisco, California: Unanswered questions following the prosecution of Cohen and Boyer patent: The dust has not settled.

N.J. Reimers, Stanford University, California: Managing the Cohen and Boyer licensing program and patent prosecution.

B.I. Rowland, Cooley, Godard, Castro et al., Palo Alto, California: Prosecuting the Cohen and Boyer patent application under the public eye: Pros and cons.

SESSION 1: Prosecuting Patents in the Patent and Trademark Office

Chairperson: J.A. Goldstein, Stern, Kessler, Goldstein and Fox, Washington, D.C.

P. Granahan, Hamilton, Brook, Smith and Reynolds, Lexington, Massachusetts: Establishing patentability over the Prior Art.

K. Murashige, Irell & Manella, Menlo Park, California: Dealing with scope of claim issues and related problems in interference proceedings.

SESSION 2: European Issues

Chairperson: J.F. Haley, Jr., Fish & Neave, New York, New York

Higher Life Forms

R.E. Bizley, Hepworth, Lawrence, Bryer, & Bizley, Harlow, United Kingdom: Developments in life patenting in Europe.

European Prosecution

V. Vossius, Vossius & Partner, Munich, Germany: Strategies for prosecuting European patent applications and oppositions.

SESSION 3: Emerging Issues for Scientists

Chairperson: N. Zinder, The Rockefeller University, New York, New York

Patenting from Scientist's Viewpoint

T.J. White, Roche Diagnostics Research, Alameda, California: Unstated assumptions about scientific publication from industrial laboratories.

N. Zinder, Rockefeller University, New York, New York: Using data from the Human Genome Project.

How Do Academic and Corporate Entities Manage the Licensing Process?

G.M. Gould, Hoffmann-La Roche Inc., Nutley, New Jersey: Licensing biotechnology inventions from the corporate perspective.

L.L. Nelsen, Massachusetts Institute of Technology, Cambridge: Licensing biotechnology inventions from the academic perspective.

G. Frank, K. Mullis, E. Kubasiewicz

SESSION 4: Appealing Patent Decisions

Chairperson: G. Rich, U.S. Court of Appeals for the Federal Circuit, Washington, D.C.

H.C. Wegner, Wegner, Cantor, Mueller & Player, Washington, D.C.: Decisions of the PTO Board of Appeals and Interferences, and the Court of Appeals for the Federal Circuit.

L. Misrock, Pennie & Edmonds, New York: Appeals to the Federal Circuit from the Federal District Courts and the ITC.

A. Lourie, U.S. Court of Appeals for the Federal Circuit, Washington, D.C.: The Federal Circuit's perspective of biotechnology cases.

E.E. Kubasiewicz, Patent and Trademark Office, Arlington, Virginia: PTO's handling and dissemination of Board of Appeals and Interferences and Federal Circuit decisions.

SESSION 5: Managing Patent Litigation

Chairperson: S. Raines, Genentech, Inc., South San Francisco, California

B. Eisen, Genetics Institute, Inc., Cambridge, Massachusetts: Overview in managing patent litigation.

K. Mullis, La Jolla, California: Perspective of the inventor/scientist in patent litigation before a jury.

M. Hall Patel, U.S. District Court Judge for the Northern District of California, San Francisco: Managing complex biotechnology patent litigation from the courts' point of view.

SESSION 6: Overviews

Chairperson: A.P. Halluin, Fliesler, Dubb, Meyer & Lovejoy, San Francisco, California

Patent Office
Legal
Science

The Genome of *E. Coli*

October 16–October 19

ARRANGED BY

F.R. Blattner, University of Wisconsin, Madison
G.M. Church, Harvard Medical School, Boston, Massachusetts
J.H. Miller, University of California, Los Angeles
K.E. Rudd, National Institutes of Health, Bethesda, Maryland
C.L. Smith, University of California, Berkeley

Opening Remarks

J.H. Miller, University of California, Los Angeles

SESSION 1: *E. coli* Sequence

Chairperson: J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York

F.R. Blattner, University of Wisconsin, Madison: Status of the *E. coli* sequence.
D.L. Daniels, University of Wisconsin, Madison: *E. coli* sequencing.
K.I. Isono, Kobe University, Rokkodai, Japan: Genome analysis of *E. coli* K-12: Summary of joint work conducted in Japan.
G.M. Church, Harvard Medical School, Boston, Massachusetts: Automation, multiplex DNA sequencing, and *E. coli* proteins.
K.E. Rudd, National Institutes of Health, Bethesda, Maryland: *E. coli* genome data bases: EcoMap, EcoSeq, and EcoGene.

SESSION 2: Data Bases (A)

Chairperson: K.E. Rudd, National Institutes of Health, Bethesda, Maryland

M. Kroger, Justus-Liebig-Universitat Giessen, Germany: Collection of *E. coli* DNA sequence data; structure and use of the ECD data bank; detection of sequencing or other errors; comparison of different strains.
S. Henikoff, Howard Hughes Medical Institute, Hutchinson Cancer Research Center, Seattle, Washington: Protein blocks as aids to sequence interpretation.
R. VanBogelen, University of Michigan Medical School, Ann Arbor: Genome-linked protein data base of *E. coli.*
M.R. Riley, Marine Biological Laboratory, Woods Hole, Massachusetts: A data base of *E. coli* metabolism connected to the underlying genes.
M. Berlyn, Yale University, New Haven, Connecticut: Genes, products, functions, maps: A subset of the *E. coli* Genetic Stock Center data base.

SESSION 3: Data Bases (B)

Chairperson: C.L. Smith, University of California, Berkeley

K.E. Sanderson, University of Calgary, Alberta, Canada: Data management at the *Salmonella* Genetic Stock Center.
J.C. Wootton, National Institutes of Health, Bethesda, Maryland: NCBI data bases and sequence interpretation.
D.W. Mount, University of Arizona, Tucson: Some short and long-range planning considerations for an E. coli data base.
C.L. Smith, University of California, Berkeley: The application of logic programming to genomic analysis.

K. Rudd, G. Church, M. Kroger, D. Daniels, R. Gesteland

SESSION 4: Organization of the Genome

Chairperson: F.R. Blattner, University of Wisconsin, Madison

J.R. Roth, University of Utah, Salt Lake City: Genetic stability and plasticity of the bacterial genetic map.
G. Ferro-Luzzi Ames, University of California, Berkeley: Tandem chromosomal duplications: A role for REP sequences.
P.M. Sharp, Trinity College, Dublin, Ireland: DNA sequence variability in *E. coli.*
G.D. Stormo, University of Colorado, Boulder: Using the *E. coli* sequence to learn about biology.
S. Gottesman, National Cancer Institute, Bethesda, Maryland: A new cryptic prophage in *E. coli.*
D.E. Berg, Washington University Medical School, St. Louis, Missouri: Transposon and PCR-based methods for efficient DNA sequencing. Large-scale reverse genetic analysis of the *E. coli* genome.

SESSION 5: The Future

Chairperson: J.H. Miller, University of California, Los Angles

This session was devoted to additional unannounced talks about the issues raised at the meeting and the planning for the next meeting, a larger meeting of 150–200 to be held 1 year hence.

Molecular Genetics of Breast Cancer

November 10–November 13

ARRANGED BY

M.-C. King, University of California, Berkeley
D. Slamon, University of California, Los Angeles

SESSION 1: Expression of Oncogenes and Growth Factor Receptors in Breast Cancers

Chairperson: D. Slamon, University of California, Los Angeles

D. Slamon, University of California, Los Angeles: HER2/neu amplification and breast cancer metastasis.
M.F. Press, University of Southern California, Los Angeles: HER2/neu expression and risk of recurrence in node-negative breast cancers.
A. Chan, National Cancer Institute, Bethesda, Maryland: Expression cDNA cloning of genes important in mitogenic signaling pathways.
W.J. Gullick, Hammersmith Hospital, London, United Kingdom: Expression of the ERBB3 protein in normal and malignant tissues.
A.L. Harris, Churchill Hospital, Oxford, United Kingdom: Expression of epidemal growth factor receptors in breast cancer.
S.A.W. Fuqua, University of Texas, San Antonio: Variants of estrogen receptor RNA in breast tumors.

SESSION 2: p53, Protein Kinases, nm23, and Stromelysin

Chairperson: D. Slamon, University of California, Los Angeles

G. Casey, University of California, Irvine: p53 mutations and breast cancer.
E.Y.-H. Lee, University of Texas Health Science Center, San Antonio: Suppressing neoplasia by replacing *RB* and *p53* genes in breast cancer cells.
E. Liu, University of North Carolina at Chapel Hill: Protein kinases in human breast cancer.
P. Basset, Institute of Biological Chemistry, Strasbourg, France: Stromelysin and breast cancer metastasis.
M.E. Lippman, Georgetown University Medical Center, Washington, D.C.: Growth factor control of malignant progression in human breast cancer.
H.M. Shepard, Genentech, Inc., San Francisco, California: Monoclonal antibody therapy of human cancer. Taking the *HER2* proto-oncogene to the clinic.

SESSION 3: Gene Mapping of Breast Cancer in Families

Chairperson: M.-C. King, University of California, Berkeley

M.-C. King, University of California, Berkeley: Closing in on a breast cancer gene on chromosome 17q.

G.M. Lenoir, International Agency for Research on Cancer, Lyon, France: Closing in on a breast cancer gene on chromosome 17q.

N.K. Spurr, Imperial Cancer Research Fund, Herts, United Kingdom: ICRF studies on the genetic analysis of familial breast cancer.

B.J. Ponder, University of Cambridge, United Kingdom: Closing in on a breast cancer gene on chromosome 17q.

D.T. Bishop, Imperial Cancer Research Fund, Leeds, United Kingdom: Genetic heterogeneity and breast cancer.

Discussion: Critical recombinants from informative pedigrees.

SESSION 4: Mapping Breast Cancer Genes in Tumors

Chairperson: M.-C. King, University of California, Berkeley

P. Devilee, Sylvius Laboratories, Leiden, The Netherlands: Deletion mapping in primary tumors as a tool to identify tumor suppressor.

R. Callahan, National Cancer Institute, Bethesda, Maryland: Mutations in breast cancer.

G. Peters, Imperial Cancer Research Fund, London, United Kingdom: Amplification of chromosome 11q13 and cyclin D1 in breast cancer.

H.S. Smith, Geraldine Brush Cancer Research Institute, San Francisco, California: Tumor suppressor genes and breast cancer progression.

Discussion: Consensus regions of interest on chromosome 17 and elsewhere.

SESSION 5: Tools for Genetic Analysis

Chairperson: N.J. Risch, Yale University School of Medicine, New Haven, Connecticut

Physical and Genetic Mapping of Chromosome 17q

E. Solomon, Imperial Cancer Research Fund, London, United Kingdom: Physical and genetic mapping of early onset breast cancer region on 17q.

R.L. White, Howard Hughes Medical Institute, University of Utah Medical Center, Salt Lake City: Genetic mapping of chromosome 17.

F.S. Collins, Howard Hughes Medical Institute, University of Michigan, Ann Arbor: Genetic and physical mapping of 17q21-q23.

Application of Molecular Genetics to Early Detection

D.L. Page, Vanderbilt University Medical Center North, Nashville, Tennessee: Precursor lesions of human breast cancer.

P. Steeg, National Cancer Institute, Bethesda, Maryland: Genetic alterations associated with breast cancer aggressiveness.

General Discussion: How can results from molecular genetics be applied to early diagnosis of breast tumors generally (not only in families)?

G. Casey, J. Witkowski, B. Ponder, R. Callahan

Genetics of Psychiatric Disorders

November 17–November 20

ARRANGED BY

K. Berg, National Institute of Mental Health, Bethesda, Maryland
S.M. Paul, National Institute of Mental Health, Bethesda, Maryland
D.G. Kirch, National Institute of Mental Health, Rockville, Maryland

SESSION 1: Bipolar Disorder

Chairperson: J.A. Egeland, University of Miami North Psychiatric Research Office, Hershey, Pennsylvania

E.S. Gershon, National Institute of Mental Health, Bethesda, Maryland: A systematic genomic scan for linkage to manic-depressive illness: Current results.
W.F. Byerley, University of Utah School of Medicine, Salt Lake City: Genomic mapping of Utah bipolar pedigrees.
M. Baron, New York State Psychiatric Institute, New York: Uncertainties in linkage studies of bipolar affective disorder.
N. Barden, Laval University Hospital, Quebec, Canada: Linkage studies of bipolar disorders in a very large pedigree derived from a Quebec isolate.
E.I. Ginns, National Institute of Mental Health, Bethesda, Maryland: Update on the search for DNA markers linked to manic depressive illness in the Old Order Amish.

Discussants

T. Reich, Jewish Hospital of St. Louis, Missouri
E. Thompson, University of Washington, Seattle
D. L. Pauls, Yale University School of Medicine, New Haven, Connecticut

SESSION 2: Schizophrenia

Chairperson: I.I. Gottesman, University of Virginia, Charlottesville

K.S. Kendler, Medical College of Virginia, Richmond: A case-control family study and linkage study of schizophrenia in Ireland.
C.A. Kaufmann, New York State Psychiatric Institute, New York: Diagnostic interview for genetic studies: A polydiagnostic, multidimensional instrument.
J. Mallet, CNRS, Gif-sur-Yvette, France: Schizophrenia and the pseudoautosomal region.
H.M.D. Gurling, University College and Middlesex School of Medicine, London, United Kingdom: Increasing the efficiency of detection of heterogeneity of linkage or false positive results in psychiatric genetics.
M.J. Owen, University of Wales College of Medicine, Cardiff, United Kingdom: Chromosome 11q and schizophrenia.
L.E. DeLisi, State University of New York, Stony Brook: X and Y chromosome linkage in schizophrenia: The evidence for and against.

Discussants

S.S. Kety, National Institute of Mental Health, Bethesda, Maryland
J. Ott, New York State Psychiatric Institute, New York
D. R. Cox, University of California, San Francisco

T. Reich, J. Ott

SESSION 3: Markers and Mapping

Chairperson: J.R. Kelsoe, University of California, San Diego

A. Chakravarti, University of Pittsburgh, Pennsylvania: Candidate gene association studies in schizophrenia.
C. Gilliam, New York State Psychiatric Institute, New York: Genome-wide search for linkage to bipolar disorder using mini- and micro-satellite markers.
J.L. Kennedy, Clarke Institute, Toronto, Canada: Antibody selection of candidate genes in psychiatric disorders.
D.R. Cox, University of California, San Francisco: Sorting the haystack: A sequential approach to identify bipolar disorder genes by using genetic linkage analysis.

Discussants

R. Freedman, University of Colorado, Denver
T. Keith, Collaborative Research Inc., Bedford, Massachusetts

SESSION 4: Quantitative Analysis

Chairperson: N.J. Risch, Yale University School of Medicine, New Haven, Connecticut

J. Ott, New York State Psychiatric Institute, New York: Linkage analyses with psychiatric traits under one-locus and two-locus models.
B. K. Suarez, Washington University School of Medicine, St. Louis, Missouri: Detecting loci for oligogenic traits via linkage analysis.
E. Thompson, University of Washington, Seattle: Linkage and segregation analysis for the quantitative indicators of complex traits.

Discussants

R.C. Elston, Louisiana State University Medical Center, New Orleans.
H.M.D. Gurling, University College and Middlesex School of Medicine, London, United Kingdom
R. Plomin, Pennsylvania State University, University Park

SESSION 5: Overview and Strategies for Future Research

Discussants

T. Keith, Collaborative Research Inc., Bedford, Massachusetts
R. C. Elston, Louisiana State University Medical Center, New Orleans
C. Van Broeckhoven, University of Antwerp, Belgium
N.S. Wexler, Columbia University College of Physicians & Surgeons, New York
D.A. Regier, National Institute of Mental Health, Rockville, Maryland
S.S. Kety, National Institute of Mental Health, Bethesda, Maryland
A.I. Leshner, National Institute of Mental Health, Rockville, Maryland

L. DeLisi, I. Gottesman

Receptor-mediated Virus Entry into Cells

November 24–November 27

ARRANGED BY

R.A. Weiss, Institute of Cancer Research, London, United Kingdom
E. Wimmer, State University of New York at Stony Brook

Opening Remarks

E. Wimmer, State University at New York at Stony Brook: Viruses and viral receptors.

SESSION 1: Glycoprotein-mediated Endocytosis, Polarized Cells

Chairperson: B. Fields, Harvard Medical School, Boston, Massachusetts

J.J. Skehel, National Institute for Medical Research, London, United Kingdom: Receptor binding and membrane fusion by influenza hemagglutinin.

D.C. Wiley, Howard Hughes Medical Institute, Harvard University, Cambridge, Massachusetts: Binding of influenza virus to cells: A second binding site, synthetic inhibitors.

A. Helenius, Yale University School of Medicine, New Haven, Connecticut: Uncoating and intracellular targeting during virus entry.

S. Tucker, University of Alabama at Birmingham: Interaction of viruses with polarized epithelial cells.

SESSION 2: Retroviruses

Chairperson: P.G. Spear, Northwestern University, Chicago, Illinois

R.A. Weiss, Institute of Cancer Research, London, United Kingdom: Entry of HIV into cells.

S.C. Harrison, Howard Hughes Medical Institute, Harvard University, Cambridge, Massachusetts: Structure of CD4 domains.

J. Gavalchin, State University of New York at Syracuse: A monoclonal antibody, 34-23, blocks HTLV-I binding and infection.

J. Cunningham, Harvard Medical School, Boston, Massachusetts: Mouse ecotropic retrovirus receptor: Cellular function and role in infection.

D. Kabat, Oregon Health Sciences University, Portland: Basic amino acid transport by the receptor for ecotropic murine retroviruses.

B. O'Hara, Lederle Laboratories, Pearl River, New York: Regions of GLVR1 required for infection by gibbon ape leukemia virus.

J.A.T. Young, University of California, San Francisco, School of Medicine: Isolation and characterization of avian DNA sequences that confer susceptibility to subgroup A-ALV infection upon mammalian cells.

K. Holmes

SESSION 3: Corona- and Sindbisvirus

Chairperson: E. Wimmer, State University of New York at Stony Brook

K.V. Holmes, Uniformed Services University of the Health Sciences, Bethesda, Maryland: Coronavirus receptors.

J.H. Strauss, California Institute of Technology, Pasadena: Identification of a cellular receptor for sindbis virus.

SESSION 4: Picornaviruses

Chairperson: J.J. Skehel, National Institute for Medical Research, London, United Kingdom

R.L Crowell, Hahnemann University School of Medicine, Philadelphia, Pennsylvania: Characteristics of the group B cox-sackie virus receptors.

J.M. Hogle, Harvard Medical School, Boston, Massachusetts: Conformational changes in poliovirus associated with cell entry.

V. Racaniello, Columbia University, New York, New York: Poliovirus-receptor interaction: Role in entry and pathogenesis.

S. Koike, The Tokyo Metropolitan Institute of Medical Science, Japan: Poliovirus receptor: Structure and function.

J.M. Greve, Miles Research Center, West Haven, Connecticut: The interaction and consequences of ICAM-1 interaction with rhinovirus in vitro and in vivo.

T.A. Springer, Center for Blood Research, Boston, Massachusetts: ICAM-1 as a rhinovirus receptor: Structural features important in virus entry.

D. Blaas, University of Vienna, Austria: The rhinovirus minor group receptor.

SESSION 5: Herpesviruses

Chairperson: J.M. White, University of California, San Francisco

B. Roizman, University of Chicago, Illinois: Herpes simplex virus glycoproteins interact with more than one cellular receptor and restrict the entry of superinfecting virus into infected cells.

P.G. Spear, Northwestern University, Chicago, Illinois: Virion-cell interactions required for the binding of herpes simplex virus to cells and for infection of the cells.

N.R. Cooper, Scripps Research Institute, La Jolla, California: Human herpes virus ligands and receptors.

SESSION 6: HBV

A.R. Neurath, The New York Blood Center, New York: HBV: HBV-cell receptor interactions mediated by the pre-S1 region of the virus envelope protein.

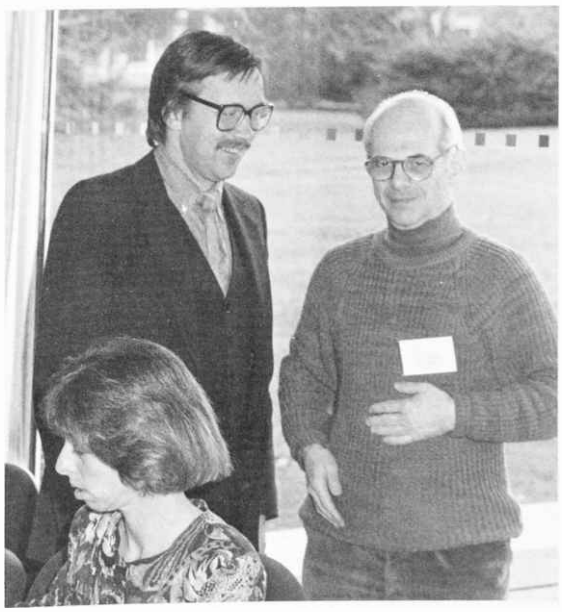
H. Schaller, University of Heidelberg, Germany: Early steps in hepadnavirus infection: Mechanism of virus uptake and participation of *env* proteins.

SESSION 7: Reovirus

Chairperson: R.L. Crowell, Hahnemann University School of Medicine, Philadelphia, Pennsylvania

B. Fields, Harvard Medical School, Boston, Massachusetts: Entry of reovirus into cells: Role of ISVPs.

P.W.K. Lee, University of Calgary Health Sciences Centre, Alberta, Canada: Structure-function relationships of the reovirus cell-attachment protein sigma 1.



J. Gavalchin, V. Zakis, A. Neurath

SESSION 8: Polyomavirus

R.A. Consigli, Kansas State University, Manhattan: Early events of polyomavirus infection: Adsorption, penetration, uncoating, and nuclear entry.

SESSION 9: Inhibitors

J.M. White, University of California, San Francisco: Fusion mechanism of the influenza hemagglutinin: Toward rational drug design.

F J. Dutko, Sterling Research Group, Rensselaer, New York: Antiviral drugs that inhibit rhinovirus-receptor interactions and virus uncoating.

The William Stamps Farish Fund Conference on the Molecular Basis of HLA Predisposition

December 1–December 4

ARRANGED BY

J. Bell, John Radcliffe Hospital, Oxford, United Kingdom
J.L. Strominger, Harvard University, Cambridge, Massachusetts

Introduction

J. Bell, John Radcliffe Hospital, United Kingdom.

SESSION 1: MHC Structure/Function and Peptide Binding

Chairperson: J. Trowsdale, Imperial Cancer Research Fund, London, United Kingdom

D. Madden, Harvard University, Cambridge, Massachusetts: Structural views of peptide binding to HLA B27.
T. Elliott, Institute for Molecular Medicine, Oxford, United Kingdom: Role of peptides in MHC class I assembly.
G. Ruberti, Stanford University School of Medicine, California: Presentation of antigen by mixed isotope class II molecules in normal H-2^d mice.
D.C. Wraith, Cambridge University, United Kingdom: Molecular characterization of MHC and T-cell interactions with a dominant autoantigenic epitope: Biological significance and implications for disease.

SESSION 2: MHC Disease Genetics

Chairperson: J. Bell, John Radcliffe Hospital, Oxford, United Kingdom

A.V.S. Hill, John Radcliffe Hospital, Oxford, United Kingdom: HLA associations with malaria: A route to an effective vaccine?
G.T. Nepom, Virginia Mason Research Center, Seattle, Washington and Ann Begovich, Cetus Corporation, Emeryville, California: Probing beneath the surface: Genetic control of class II allelic variation.
H.A. Erlich, Cetus Corporation, Emeryville, California: Role of individual residues and of haplotypes in HLA disease predisposition.
P. Parham, Stanford University, California: Unusual class I HLA molecules of American Indians: Role in disease.
A.B. Rickinson, University of Birmingham, United Kingdom: HLA polymorphism and target antigen choice in the Epstein-Barr virus system.
A.J. McMichael, John Radcliffe Hospital, Oxford, United Kingdom: HIV escape mutants in vivo.

SESSION 3: Transporters and Proteases

Chairperson: P. Parham, Stanford University, California

J.J. Monaco, Medical College of Virginia/Virginia Commonwealth University, Richmond: H-2 linked transporter (HAM1 and HAM2) and proteasome (LMP) genes.
J. Trowsdale, Imperial Cancer Research Fund, London, United Kingdom: A cluster of transporter and protease genes in the HLA class II region that may have a role in antigen processing.
A.J. McMichael, Institute of Molecular Medicine, Oxford, United Kingdom: MHC-linked factor(s) affecting epitopes presented by class I.

SESSION 4: Diabetes and Target Antigens

Chairperson: A.J. McMichael, John Radcliffe Hospital, Oxford, United Kingdom

L. Wicker, Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey: Influence of non-MHC-linked diabetogenic genes in the NOD mouse.
J.-F. Bach, Hospital Necker, Paris, France: Cellular level of MHC and non-MHC gene expression in type-1 diabetes.
J. Bell, John Radcliffe Hospital, Oxford, United Kingdom: An HLA DR4-dependent diabetes susceptibility gene on chromosome 11.

S. Baekkeskov, University of California, San Francisco: Role of MHC-class I antigen expression in presentation and targeting of autoantigen.
N. Willcox, John Radcliffe Hospital, Oxford, United Kingdom: Autoimmune T cells and HLA restriction in myasthenia gravis: First footsteps.
S. Reeders, Yale University School of Medicine, New Haven, Connecticut: Preliminary studies on the molecular characterization of the Goodpasture antigen.

SESSION 5: TCR Repertoire

Chairperson: D.C. Wraith, Cambridge University, United Kingdom

J. Silver, North Shore University Hospital, Manhasset, New York: Influence of HLA genes on the human T-cell receptor repertoire.
X. Paliard, Howard Hughes Medical Institute Research Laboratories, Denver, Colorado: Interaction of EBV with human T cells.
J. Oksenberg, Stanford University Medical Center, California: TCR usage in MS brain plaques.
J. Bell, John Radcliffe Hospital, Oxford, United Kingdom: T-cell receptor repertoire in man.

U.S. Department of Energy Workshop on Human Genetics and Genome Analysis

December 8–December 11

ARRANGED BY

M. Bloom, DNA Learning Center, Cold Spring Harbor Laboratory, New York
D. Micklos, DNA Learning Center, Cold Spring Harbor Laboratory, New York
J. A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York

SESSION 1

D. Galas, U.S. Department of Energy, Washington, D.C.: Origins and impacts of the Human Genome Project.

SESSION 2

D. Micklos, DNA Learning Center, Cold Spring Harbor Laboratory, New York: Mendelian genetics and linkage.
J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York: DNA, restriction enzymes, and molecular cloning.

SESSION 3

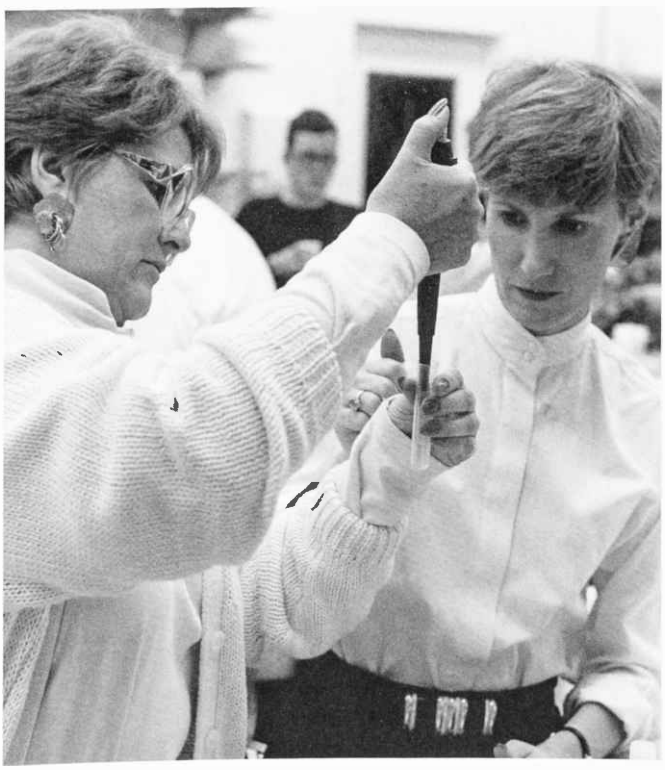
D. Micklos, DNA Learning Center, Cold Spring Harbor Laboratory, New York: Laboratory: Measurements, micropipetting, and sterile techniques: DNA restriction analysis and restriction mapping.

SESSION 4

P. Reilly, Shriver Center for Mental Retardation, Waltham, Massachusetts: The Eugenics movement.

SESSION 5

M. Bloom, DNA Learning Center, Cold Spring Harbor



J. Edwards, M. Peck

Laboratory, New York: Construction and screening of human gene libraries.

J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York: DNA polymorphisms and linkage analysis.

SESSION 6

D. Micklos, DNA Learning Center, Cold Spring Harbor Laboratory, New York: Laboratory: Transformation of *E. coli* with plasmid DNA.

M. Bloom, DNA Learning Center, Cold Spring Harbor Laboratory, New York: Laboratory: Human DNA fingerprinting by polymerase chain reaction.

SESSION 7

M. Wallace, University of Florida Hillis Miller Health Center, Gainesville: Cloning human disease genes: Neurofibromatosis 1.

SESSION 8

D. Micklos, DNA Learning Center, Cold Spring Harbor Laboratory, New York: Laboratory results: Transformation of *E. coli* with plasmid DNA.

M. Bloom, DNA Learning Center, Cold Spring Harbor Laboratory, New York: Laboratory: DNA fingerprinting by polymerase chain reaction.

SESSION 9

C. Gilliam, New York State Psychiatric Institute, New York: Searching for genes for mental disorders.

SESSION 10

K. Culver, National Institutes of Health, Bethesda, Maryland: The first human gene therapy trials.

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The Molecular and Cellular Biology of the Yeast Saccharomyces
Vol. 1: *Genome Dynamics, Protein Synthesis, and Energetics*
J. Broach, J. Pringle, and E. Jones (eds.)

Banbury Report Series

Biological Basis for Risk Assessment of Dioxins and Related Compounds
(Banbury Report 35)
M. Gallo, R. Scheuplein, and K. van der Heijden (eds.)

Current Communications in Cell and Molecular Biology Series

Vol. 1: *Electrophoresis of Large DNA Molecules*
E. Lai and B. Birren (eds.)

Vol. 2: *Cellular and Molecular Aspects of Fiber Carcinogenesis*
C. Harris, J. Lechner, and B. Brinkley (eds.)

Vol. 3: *Apoptosis: The Molecular Basis of Cell Death*
D. Tomei and F. Cope (eds.)

Vol. 4: *Animal Applications of Research in Mammalian Development*
R. Pedersen, A. McLaren, and N. First (eds.)

Cancer Surveys Series

Vol. 10: *Cancer, HIV and AIDS*
V. Beral, H. Jaffe and R. Weiss (eds.)

Vol. 11: *Prostate Cancer*
J. Isaacs (ed.)

Genome Analysis Series

Vol. 1: *Genetic and Physical Mapping*
K. Davies and S. Tilghman (eds.)

Vol. 2: *Gene Expression and Its Control*
K. Davies and S. Tilghman (eds.)

Vol. 3: *Genes and Phenotypes*
K. Davies and S. Tilghman (eds.)

Journals

Genes & Development (Volume 5, numbers 1–13)

Cancer Cells: A Monthly Review (Volume 3, numbers 1–12)

PCR Methods and Applications (Volume 1, numbers 1–2)

Other

CSHL Annual Report 1990

Abstract/program books for 14 CSHL meetings

COLD SPRING HARBOR LABORATORY PRESS

After the fine financial performance of the previous year, the Press began 1991 with high hopes of similar success. However, like other science publishers, we found we had entered a difficult period in which sales slowed in all of our major markets. Faced with diminished grant support, many scientists told us frankly that they were opting to pay for experiments, not books, and some authors had to delay work on projects that we had hoped would be 1991 publications.

Nevertheless, 15 new book titles were published. The most spectacular of these, indeed the most striking book ever published at the Laboratory, was *Houses for Science* by Elizabeth Watson. A gorgeously illustrated pictorial history of the Laboratory from its foundation to the present, the book grew out of the author's expertise in restoration and close involvement with the refurbishment and expansion of the Laboratory in the past two decades. The book is a beautiful and intriguing introduction to the institution and was an instant success among staff, friends, and neighbors when published in November, just in time for the gift-giving season. Assisted by Liz Watson's unstinted round of signings and interviews, our promotion to libraries and bookstores in the local and metropolitan area, and good reviews, sales of the book approached 1000 copies by the end of the year.

Turning the author's manuscript into a 351-page book with over 300 photographs and other illustrations was an extraordinarily complex task. Many people made vital contributions to this process, but the key figures were Andrew Garn, the principal photographer, Emily Harste, the designer, and Nancy Ford, the Press's Managing Editor, for whom the book was a personal publishing challenge. Its beauty is an eloquent tribute to their success.

Three New Book Series Are Launched

All of the new book titles in 1991 confirmed once again the commitment to the highest publishing standards shown by the staff of the Editorial Production Department, coordinated by Dorothy Brown and Annette Kirk. These books included the first volumes in three new series. *Genome Analysis* is a timely information source for the growing community of scientists who map genes in humans and other species. The editors, Kay Davies of Oxford University and Shirley Tilghman at Princeton, began the series in January with an excellent volume on genetic and physical mapping methods that won praise for its content and design. A book on gene expression followed in mid-year, and in December, a third volume highlighted successes in the isolation of human disease genes.

Much time was spent preparing the re-launch of the series *Cancer Surveys*, to which we acquired publishing rights in 1990. Careful coordination was needed between our editorial, production, and marketing activities and the work of the editor L.M. Franks and his staff at ICRF in London. This resulted, in November, in a handsomely designed volume on cancer related to AIDS, followed in December by a volume of prostate cancer. This series has a more clinical orientation than any of the many previous Cold Spring Harbor publications in the cancer field.

Another new book series, *Current Communications in Cell and Molecular Biology*, began with the publication of four volumes. Each had a different theme linked by a common thread: the dramatic impact of recombinant DNA technology. Volumes were published on DNA electrophoresis, fiber carcinogenesis, and mammalian developmental biology, but the most striking success was *Apoptosis*, a book on cell death and its role in development, neurodegeneration, and aspects of immune function. With its origins in a formative meeting at the Banbury Center, it is an invaluable summary of a fast-moving field and is already being revised.

The foundation of our book-publishing program remains our best-selling laboratory manuals and the annual Symposium series. *Molecular Cloning*, *Antibodies*, and *Manipulating the Mouse Embryo* continued their remarkable dominance of the technical literature in their fields, and an updated version of the course manual in yeast genetics had strong sales. This year's Symposium volume, number 55, *The Brain*, was the first neuroscience book to be published at Cold Spring Harbor Laboratory for several years. It was warmly welcomed as an up-to-date review with an unusually broad editorial sweep, its attraction enhanced by the initial low price of the paperback edition.

The Symposium apart, the value of meetings proceedings volumes in the eyes of scientists has sunk dramatically in recent years. However, as part of the Laboratory's centennial celebrations in 1990, Ed Harlow, Tom Curran, Frank McCormick, and Joan Brugge organized a remarkable meeting that provided, in a week, a panoramic view of human cancer research. At the organizers' urging, nearly all of the celebrated contributors to the meeting subsequently wrote review articles on the topics they discussed. These were published in December as *Origins of Human Cancer: A Comprehensive Review*, a notable 900-page book that has proved attractive to both research scientists and clinicians.

One Journal Closes and Another Begins

The convergence of laboratory and clinic in oncology is rooted in the growing conviction that insights into the molecular origins of cancer do assist diagnosis and may improve treatment of many kinds of tumors. The wish to spread awareness of these advances was the ambitious goal of the journal we began in 1989, *Cancer Cells: A Monthly Review*. In 1991, the journal continued to publish a wide range of good-quality articles skillfully edited to appeal to a broad spectrum of professional interests in cancer research. However, its distinctive voice had to struggle to be heard among half a dozen new journals clamoring for the attention of the same research community. Neither the dedicated efforts of the editors nor our marketing activities produced the hoped for increase in the journal's circulation in its second full year of publication, resulting in another significant financial loss. The risk of continuing losses in future years was clearly high. After careful review, the painful decision was made to discontinue publication of the journal with the December 1991 issue. We remain committed to publishing for the cancer research community and discussions began immediately about ways of building upon the foundation provided by *Cancer Cells*. Paula Kiberstis, who founded the journal, moved to Washington, D.C., to become a senior editor of the *Journal of the National Cancer Institute*, and Catriona Simpson, the assistant editor, transferred to work on our books.

Elsewhere in our journal publishing program, there was excellent news. The

first issue of our new journal *PCR Methods and Applications* was published in August, after a well-planned publicity campaign that attracted papers as well as subscriptions. A second issue of the quarterly followed in November. The journal offers a mixture of commissioned reviews, technical tips, and peer-reviewed original research papers. Our designer, Jim Suddaby, created an innovative cover for the journal and an attractive interior layout. The editor, Judy Cuddihy, assembled a fine collection of commissioned articles for these issues, which signaled the journal's intention of covering all kinds of amplification-based technologies. The invaluable assistance of the associate editors Rick Myers, Eric Green, David Bentley, and Richard Gibbs, the editorial board members, and many ad hoc reviewers ensured that the quality of the original research published in the journal was high from the start. The journal's sponsors, Perkin-Elmer Cetus Instruments, provided a large database of PCR users to whom the journal could be promoted and purchased multiple subscriptions that gave the journal's circulation a solid foundation. The first issues were well received by scientists worldwide and subscriptions rose rapidly through the end of the year.

The journal was also greeted warmly by companies marketing DNA detection products, and Nancy Kuhle was able to place a substantial amount of advertising in each issue, allowing us to keep the initial subscription price attractively low.

The success of this launch did not overshadow the continuing progress of *Genes & Development*. In its fifth year of publication, the journal grew in size, submission rate, and subscriptions. The number of papers submitted to the editorial offices rose to over 600 and the acceptance rate remained at the previous year's level of 36%. The number of editorial pages in the average issue increased by 9%. The critical judgment of editors Terri Grodzicker and Nick Hastie and their advisors ensured that the quality of the journal's editorial content remained high. There was a satisfactory 13% increase in overall subscription growth. The journal now has subscribers in 40 countries, predominantly in the United States, Japan, Canada, the United Kingdom, and Germany, and expansion was seen in all these major markets.

Our satisfaction in the journal's performance was underlined by the results of the 1990 citation analysis from The Institute for Scientific Information, which showed that the journal had the highest impact factor of any monthly journal in biology. By this measure, *Genes & Development* was once again the top journal in genetics and in developmental biology. A pleasing corollary to this growing reputation was the substantial increase in demand for advertising space in its pages.

New Books Are Initiated and Books for Children Are a Great Success

The year's acquisition activities initiated a number of diverse publishing projects, most of which will come to fruition next year or beyond. Because the reputation of the Press is founded so securely on high-quality technical books, we were delighted to be able to form talented editorial teams to work on several important new manuals. In addition, the organizers of well-established Cold Spring Harbor Laboratory courses in *Schizosaccharomyces pombe* biology, neural gene cloning, and plant molecular biology were persuaded to construct manuals of the methods they have taught. The series of monographs revived in 1989 will continue with volumes now being assembled on RNA, *Arabidopsis*, and reverse transcriptase. The complex history of molecular biology and genetics, neglected in

publishing terms, will be illuminated by three books commissioned in 1991 on yeast genetics, transposable elements, and protein synthesis. A new edition of the long out-of-print classic *Phage and the Origins of Molecular Biology* with newly added material will be published in 1992. Another book with a historical slant was commissioned from Tom Silhavy and Jonathan Beckwith. Based on the prokaryote genetics courses they teach at Princeton and Harvard, it is a course text on genetics that relies on the discussion and analysis of landmark research papers.

The 1991 projects included two novelties for the Press. The eminent biologist Ron Laskey occasionally performs witty songs at conferences about life in the laboratory, and in September, he was persuaded to give an hour-long concert in Grace Auditorium during the DNA Replication meeting. Our audiocassette of his concert, *Songs for Cynical Scientists,* proved to be an immediate hit. Another striking success was a series of award-winning books for children imported from HarperCollins, U.K., for exclusive distribution in the United States. *Cell Wars*, *Cells Are Us*, and *DNA Is Here To Stay* by Fran Balkwill and Mic Rolph explain the workings of cells and genes simply and clearly, assisted by colorful graphics. Our professional audience, delighted by this novel way of explaining their work, bought the books by the thousands. We were delighted to be able to extend the Laboratory's educational mission in a new direction.

Staff Changes

We entered 1991 with a new organizational structure and an additional facility at Plainview that houses the Business Operations Department. The staff of this department had to cope not only with separation into new and very different surroundings, but also with the advent in January of a more complex computer system for order processing and fulfillment. Conversion to the new system put the staff under great strain, but they responded willingly to the challenge and the department was soon fully functional. By December, the benefits in customer service and marketing analysis were already clear. The smooth orchestration of the move and conversion was an enormous credit to the department's head, Charlaine Apsel, and the staff. Toward the end of the year, Charlie announced her intention to leave the Laboratory after 11 years of service in customer service, financial, and marketing capacities. Her knowledge and expertise were invaluable, but we were fortunate to have in Guy Keyes, promoted from Warehouse Manager to Fulfillment and Distribution Manager, someone who knew the Press well and brought to it a commitment to improved sales and excellent customer service. Responsibility for monitoring and reporting the financial affairs of the Press was devolved to the new post of Finance Coordinator.

The position of Marketing Manager created by the reorganization was filled in March by Ingrid Benirschke, who had gained much relevant experience with Springer Verlag in Manhattan. She quickly impressed us with her efficiency and enthusiasm. The seven conference exhibits she attended between April and December immersed her rapidly in our publications list, and by the end of the year, she had created the first of the new series of attractive subject-specific catalogs with which we intend to exploit the targeted marketing and analytic capacities of our new customer database.

The Marketing Department also encompasses the campus bookstore in the basement of Grace Auditorium, managed by Connie Halloran. During the winter

months, the store was refurbished to make maximum use of its small space. It reopened during the first laboratory course season, restocked with a greater range of books of general interest to scientists, an expanded range of CSHL-branded clothing, and sundry items useful for conference attendees. Both visitors and the Laboratory's staff applauded the new approach, and this enthusiasm was reflected in the bookstore's increased revenues.

A Year of Consolidation Follows

The past few years have been a time of extraordinary change in publishing at Cold Spring Harbor Laboratory, and no aspect of it remains untouched. New technology is an enormous influence. Our decision to undertake in-house typesetting has led to certain efficiencies in editorial production, and we are exploring the possible complementary benefits of electronic page design. Most of the manuscripts we receive from authors are now on disk. The new computer system for order processing offers many opportunities for better marketing and sales management. As we move ahead with these innovations, we are fortunate to have a dedicated staff willing to accept the new and capable of making it work.

The greatest single change has been the advent of journal publishing. Doing this well requires a dedicated, forward-looking editorial staff, innovation in marketing, advertising sales, production, and customer service, and significant investment. But most importantly, to justify a new journal, there has to be a clearly defined, unmet need. We remain alert for such opportunities. We are also aware of the possibilities offered by electronic media. In the meantime, however, for the first time in several years, we look forward to the next 12 months with no new journal launch in progress. 1992 is planned as a year of consolidation, for improving the success of existing publications, expanding our successful book list still further, improving overall cost-effectiveness, and maintaining our hard-won reputation for high-quality publishing at Cold Spring Harbor.

John R. Inglis


DNA LEARNING CENTER
DNA LEARNING CENTER
33

DNA LEARNING CENTER

David A. Micklos, Director
Mark V. Bloom, Assistant Director
Susan M. Lauter, Designer
Margaret E. Henderson, Education Manager
Sandra H. Ordway, Administrative Assistant
Jane P. Conigliaro, Middle School Specialist

It has now been seven years since we began introducing precollege students and teachers to the intellectual challenge of molecular genetics. Our goal has always been to give students and teachers a view of "the cutting edge" of biological research and a base of knowledge that allows them to follow new developments on their own. With its assemblage of young enthusiastic researchers and visiting scientists from all corners of the globe, Cold Spring Harbor Laboratory is an ideal place from which to look into the future of biology. We, at the DNA Learning Center (DNALC), endeavor to exploit this unique vantage point to help direct the future of biology education. We have clung stubbornly to the notion that students should always be given the chance to experience the fun of biology—which is doing experiments. So, much of our effort has been devoted to finding safe ways for students to successfully perform experiments that closely parallel those done by research biologists.

When we initiated our teacher-training activities in 1985, we were one of only a very few research institutions in the nation that had made a substantive commitment to help precollege educators bring biology teaching into the gene age. At that time, even reasonable people could dismiss our emphasis on genetics as too narrow or appropriate only for the upper echelon of advanced students. However, there is now growing awareness that genetic literacy is an essential element of basic health education and cultural literacy for *all* young people.

There can be no doubt that the Human Genome Project has accelerated the pace of research that is propelling our culture into a brave new world, where DNA diagnosis can detect serious disease well in advance of symptoms, where defective genes can be replaced by healthy ones, where scantron machines identify individuals by reading the bar code of their DNA profiles, and where criminals cannot afford to leave even a few of their own cells at a crime scene. It promises to be a world of triumph over genetic disease and crime, but also one fraught with new questions of personal and social responsibility:

- How will individuals deal with the knowledge that they harbor genes that predispose them to crippling or painful diseases?
- Should screening for certain genetic diseases or conditions be mandatory?
- Who should have access to personal genetic profiles?
- Will employers and insurance companies be permitted to use genetic data to exclude individuals from jobs or insurance?
- Will prospective parents be permitted to pick and choose among in vitro fertilized embryos for those with the "best" genes?

DNALC staff David Micklos, Jane Conigliaro, Sandra Ordway, Margaret Henderson, Mark Bloom, and Susan Lauter in front of new exhibit, *Eye on the Prizes*. Jane and Mark display plans for the DNA Learning Center addition.

The Growth and Problems of Genetics Education

When we began our efforts in genetic literacy, the Education Directorate of the National Science Foundation (NSF) was just arising, phoenix-like, after its disbandment under the Reagan administration in 1982–1984. (The NSF Education Directorate traditionally is the largest single funder of science education in the U.S.) There was little money to go around, and we were thankful in 1986 to receive the first of several NSF grants for our teacher-training programs. By the end of the decade, funding for the Education Directorate had risen to its highest level in history ($278 million). Funding for NSF, as well as other federal funding agencies, is likely to remain at high levels throughout the 1990s, given the charge of the President's *America 2000* Program, "By the Year 2000, U.S. students will be first in the world in science and mathematics achievement." Private foundations have also initiated major science education initiatives, including the Howard Hughes Medical Institute, the Pew Memorial Trust, and the Johnson Foundation. Genetics education has been among the beneficiaries of the dramatic increase in funding, both federal and private.

By 1991, the world of biotechnology and genetics education had become sufficiently large that the NSF convened a conference for leaders in the field, which filled a conference facility at the University of Wisconsin at Madison. It was gratifying to see the large number of creative educators now involved in biotechnology/genetics education—quite a few of these educators had been trained through the DNALC's *DNA Science* Workshops, had used our *DNA Science* text,

and had in some way modeled their programs after ours. The sense of shared accomplishment and consensus on key issues was amazing. There was unanimous agreement that biotechnology is not just a "flash in the pan" topic, but an opportunity to integrate concepts from biology, chemistry, and physics, as well as the personal and social implications of new technologies. This is consistent with a "whole learning" approach that relates learning to the life and culture of the individual.

A number of project leaders had been surprised to find that efforts targeted at one level of the educational system had unforeseen effects at a higher level. This "trickle-up" effect is caused by the elevated expectations of students who have been exposed to novel instruction and who then challenge higher-level teachers to update their instructional methods. Thus, the familiarity of graduating high school seniors with gene manipulation methods challenges college faculty to offer more entailed experiments with DNA in their introductory courses. Likewise, a growing number of middle school teachers who now allow their students to transform bacteria with a new gene challenge high school teachers to do even better experiments. This trickle-up effect is contrary to the traditional notion that educational innovation trickles down from higher levels. It suggests that focusing instructional change at the elementary level can have a multiplier effect, which, ultimately, ramifies throughout middle school, high school, and college. Furthermore, student and teacher enthusiasm, flexible scheduling, and lack of standardized curricula greatly simplify instructional change at the elementary level. Thus, with encouragement from the NSF, educators are now placing increased emphasis on upgrading elementary science instruction.

An undercurrent of tension, however, pervaded the meeting. Participants agreed that funding agencies' emphasis on *model* programs had created a patchwork of competing and often redundant programs, whose survival hangs on a nearly constant battle for grants. No foundation or agency, including NSF, has addressed the problem of how to provide continuing funding to mature programs whose usefulness as models has been established. Lacking long-term support, the very best investigators must devote inordinate effort to developing specific grant proposals and administering an intricate web of interlocking programs. Their rare communicative talents could be better employed writing new curricula, rather than writing still another grant.

The Need for Human Genome Education Centers

Many voices now argue that American biology education is in urgent need of major reform. The task of rebuilding the *biological education system* might be well served by strategies that have made America's *biological research system* without peer in the world. Mechanisms and infrastructures that support innovation in the biology laboratory might also support innovation in the biology classroom. NSF and other funders of biology education have, indeed, followed the lead of their research counterparts by providing most support to *individual investigators for well-defined projects of approximately 3 years duration.* However, well-supported, multidisciplinary research centers are another important element of biological research in the United States and include the Human Genome Research Centers funded by the National Institutes of Health (NIH) and the Department of Energy, NIH Comprehensive Cancer Centers and Program Project

Grants, and the Howard Hughes Medical Institutes. This approach acknowledges that innovation arises from a critical mass of highly motivated individuals. Such large-scale core support for exemplary centers is essentially absent from biology education today. (NSF and other federal agencies have provided major block grants to state education authorities, but these cannot be viewed as parallels to independent research centers.)

The educational challenges of the Human Genome Project present an ideal test for a centers approach in biology education. Imagine the potential impact of creating eight regional Human Genome Educational Centers—stable, well-equipped environments in which multidisciplinary teams of biologists, educators, designers, and computer programmers can devote their creative energies entirely to the problems of public genetics education. Genome Education Centers would be sited at innovative institutions with established educational outreach programs, expertise in genetics education, and strong linkages to research biology. If each center was funded for a renewable term of 5 years at a level of $500,000–750,000 per year, the total annual cost of the centers program would be less than 3% of the combined annual NIH-DOE budget for the Human Genome Project. The meeting at the University of Wisconsin made clear that the nucleus institutions for regional centers and an infrastructure for collaboration between them, in fact, already exists.

Plans for a Major Capital Development Program

A 1991 planning grant from the E.S. Webster Foundation allowed us to complete the concept design phase of a major capital development program, which would firmly establish the DNALC as a comprehensive prototype of such a Genome Education Center. An architectural study was conducted by Centerbrook Associates, who have been responsible for all of the major design work at the Laboratory for more than a decade. Floor plans and elevations of a 3500-square-foot *BioMedia* addition to the south of the building, redevelopment of the existing building, and a site plan for improved parking and visitor access were completed in time for the November meeting of the Board of Trustees.

The term *BioMedia* engenders our goal to explore ways to link together experimental, computer, and audiovisual resources to encourage understanding of biological concepts. The addition will include a genetics laboratory, multimedia computer laboratory, student research library, and a much needed atrium/lunchroom. The laboratory facilities will be adjacent to the existing *Bio2000* Laboratory and will include a redeveloped research/prep lab for instructional support and student research. The new and redeveloped facilities will allow students to move between biochemical experiments, microscope observations, and parallel computer experiences that illustrate molecular events. Library and laboratory resources will allow students to search the literature and work independently on specialized projects. Redevelopment of the original auditorium will create a 96-seat *Cellarium* for specialized video presentations and seminars. The new facility will also provide a much needed meeting space for the many small museums and educational organizations in the Cold Spring Harbor–Huntington area.

The capital campaign was quietly launched by year's end with lead grants of $250,000 from the Stone Foundation and $100,000 from the Weezie Foundation.

The Stone grant will outfit the *BioMedia* addition with specialized laboratory and computer equipment, including some items that we have for years only dreamed of affording. The laboratory equipment will be used for new student experiments in cell culture and microscopy and to support advanced research projects by student interns from neighboring school districts. The computer equipment will allow us to initiate a program of computer multimedia field trips to complement experiment field trips that are currently available. The Weezie grant will allow us to complete construction of a new permanent exhibit, *Exploring the Human Genome*, which will be located in a remodeled gallery in the existing building.

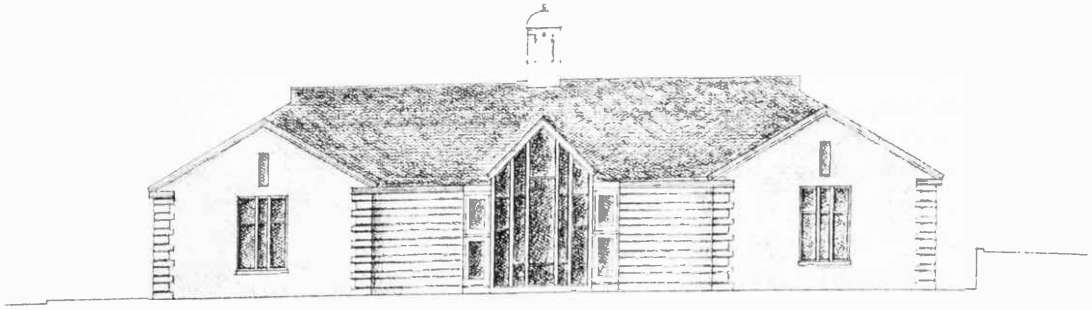
BioMedia Laboratory Development

With support from the New York State Legislature and the William Randolph Hearst Foundation, designer Susan Lauter has organized an efficient multimedia computing laboratory occupying the former offices on the main level. The *BioMedia* Laboratory is currently equipped with three Macintosh II computers, a top-of-the-line Quadra 900, and supporting audiovisual equipment. The Quadra will be the central file server of an integrated multimedia *network* of 12–15 computer stations, which will be installed in the student computer laboratory of the proposed *BioMedia* addition.

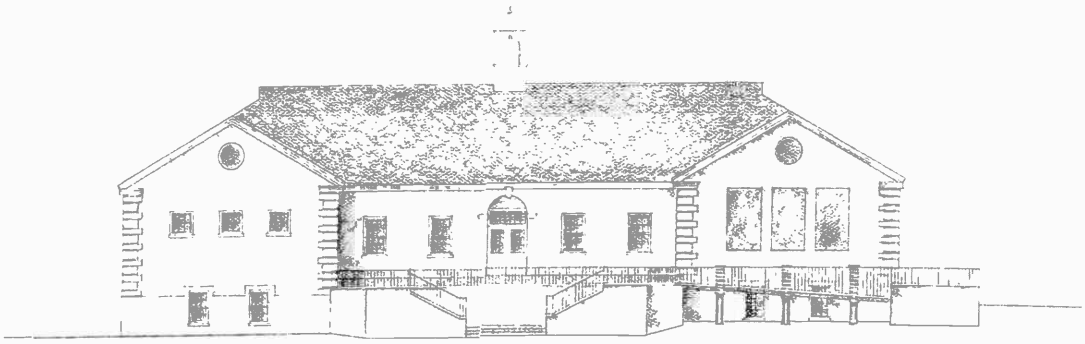
With strong support from our computer-savvy interns, Sue completed our first interactive computer program, *DNA Detective*, which features real-life cases involving DNA fingerprints. The program was presented to the museum world in October at the annual meeting of the Association of Science-Technology Centers in Louisville, Kentucky. *DNA Detective* is the first of eight multimedia computer programs scheduled for development as part of the forthcoming exhibit, *Exploring the Human Genome*.

Our ultimate objective is to configure the enlarged *BioMedia* Laboratory as a prototype node of a *BioMedia Network* to demonstrate the use of a central server to distribute multimedia programs to schools around the country. We intend to seek additional funding to purchase and maintain a multiprocessor and to install a fiber optic backbone and connections to public optical trunk lines. In this system, one central processor would respond to routine keyboard commands from clients' personal computers to retrieve files from the central storage disk; another processor is available to perform computationally intensive activities, such as manipulations of video files and three-dimensional rotations. Although a central multiprocessor is expensive, it would provide the computing power to allow simple receiving computers at client schools around the country to access and manipulate multimedia files maintained at the DNALC.

The *BioMedia* Laboratory also provides an unprecedented opportunity for students and biology educators to work coordinately with Cold Spring Harbor scientists as they develop a prototype computational system for genetic research. Cold Spring Harbor Laboratory is part of a newly formed consortium, funded by the External Research Program of Digital Equipment Corporation (DEC), which includes the California Institute of Technology, San Diego Supercomputer Center, University of Michigan, Massachusetts Institute of Technology, and Johns Hopkins University. The objective of the consortium is to develop "A Unified Interactive Environment for Scientific Collaboration" that will allow biologists to manage and interface effectively with the vast amounts of DNA and protein sequence data generated by human genome research. Laboratory staff scientist Thomas Marr, who helped conceive the DEC consortium, serves as *ad*



SOUTH ELEVATION



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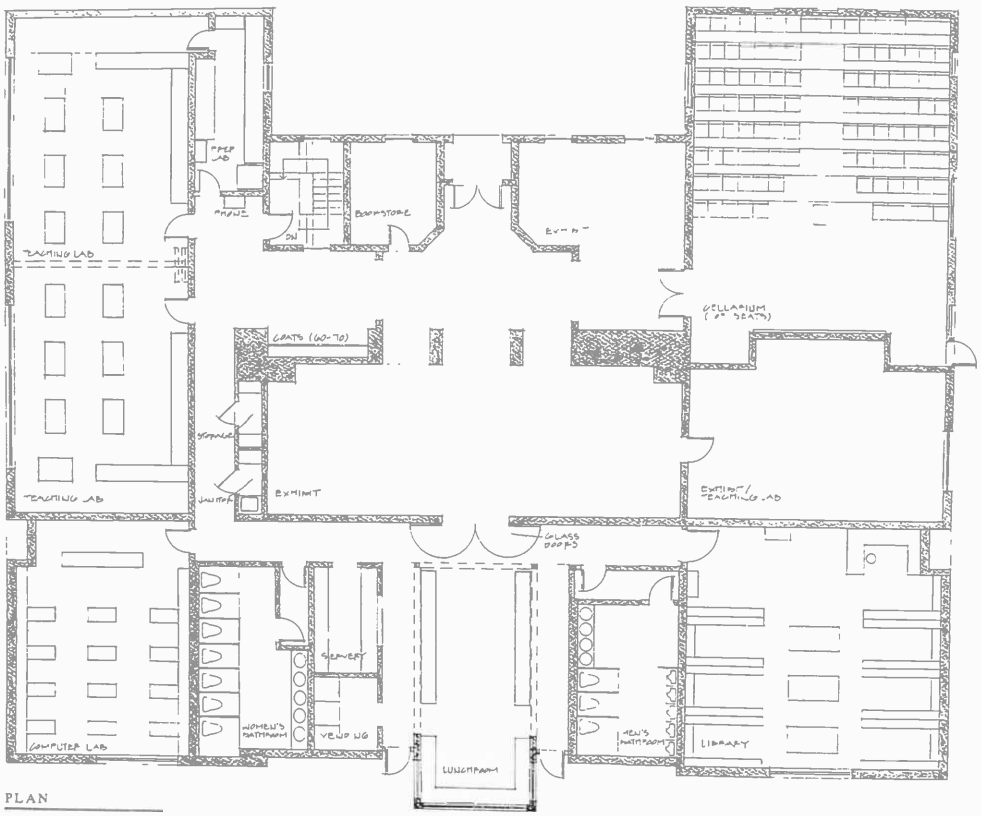
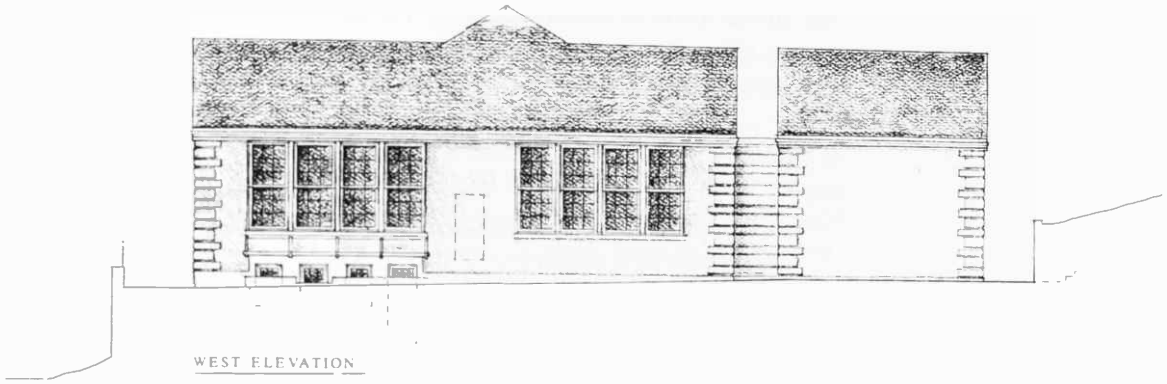


PLAN

Plans developed by Centerbrook Associates for the *BioMedia* addition to the DNA Learning Center.



WEST ELEVATION

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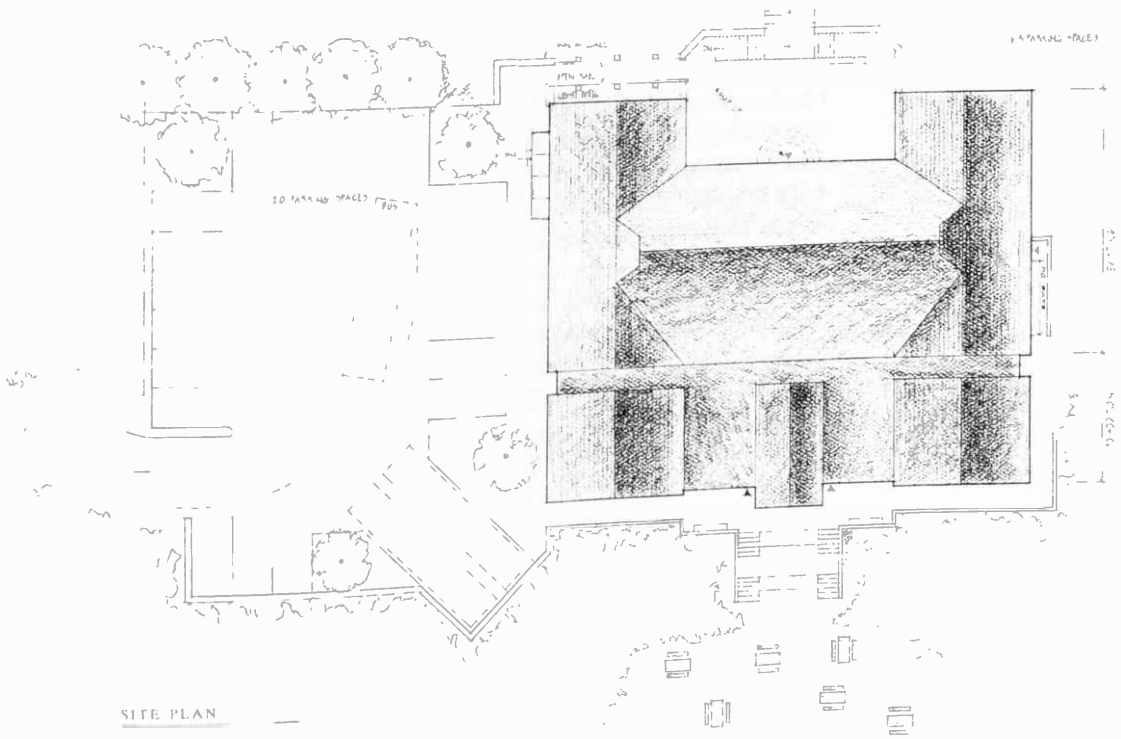


SITE PLAN

hoc adviser to the *BioMedia* Laboratory. He can facilitate a direct flow of expertise, allowing us to apply solutions to problems of biological computing to educational computing:

- How to construct a "user friendly" computing network that allows information to be accessed at low cost by individuals in all parts of the country.
- How to allow transparent interfacing with different types of computers/operating systems (e.g., IBM vs. Mac).
- How to distribute computationally intensive activities among a number of regional "nodes."
- How to provide for real-time flow of computationally intensive activities between a central multiprocessor and personal computers.

Routinizing Human DNA Fingerprinting for Educational Purposes

In 1989, Mark Bloom began developing an experiment that would allow students to make their own DNA fingerprints using the automated procedure of polymerase chain reaction (PCR). We thought that this would be an ideal way to "personalize" gene technology and to illustrate the uses of individual DNA variations (polymorphisms) in forensic biology, identity testing, and disease diagnosis. In principle, the experiment is brilliantly safe and simple. Cheek cells are obtained by rinsing the mouth with a mild salt solution. The cells are collected by centrifugation, mixed with an agent to bind impurities, and boiled for several minutes to liberate DNA. A sample of this crude DNA extract is added to a "cocktail" of reagents that identifies and amplifies a specific region of DNA on chromosome 1. The amplified DNA is then separated by electrophoresis and stained with a dye that reveals a simple "bar code" consisting of one or two bands. However, routinizing the experiment so that it could be reproduced by novices proved daunting. The amplification procedure relies on pure biochemistry involving ten key components, each of which must be present in strictly prescribed amounts. Mark's experimental system worked brilliantly at first, but then crashed unexplainedly. In the course of more than 1 year of experiments, Mark tested and retested every component of the system, finally uncovering the culprit that had spoiled the results. In the process, he had become an authority on DNA amplification of this type.

Thanks to Mark's tenacity, the human DNA fingerprinting experiment has been successfully performed by members of diverse groups, including middle school through college faculty, business leaders, museum personnel, ethicians, and administrators from government agencies. At the end of the year, the experiment became part of the regular laboratory "menu" available for high school laboratory field trips. Comprehension of the experiment is much improved through an animated computer program, developed in our *BioMedia* Laboratory, that illustrates the complex biochemistry of the polymerase chain reaction. The program is available in a computer kiosk in the *Bio2000* Laboratory, which is linked to a new closed-circuit TV system. Thus, students can visualize the chemical reactions that are taking place in their test tubes. The DNALC is, to our knowledge, the only place in the world where DNA fingerprints are produced by nonscientists on a routine basis; we estimate that 1000–1500 students will perform this experiment annually.

Corporate Advisory Board Formed

Competition in the global market increasingly demands access to a number of emerging technologies. Gone are the days when a region could exist under a single government-supported industry. Most agree that the long-term economic health of Long Island depends on finding growth industry to replace an aged industrial infrastructure based on military aircraft. Many now look to biotechnology as a "great hope" for the Island's future. The preexisting infrastructure to support such an industry is often cited—a concentration of major research facilities with biotechnical expertise (Cold Spring Harbor Laboratory, Brookhaven National Laboratory, Cornell/North Shore Medical Center, and the new Picower Institute), a state-supported biotechnology center and industrial incubator at SUNY Stony Brook, and a number of existing start-up companies.

Less often considered is the educational infrastructure that primes technological development and community support. Since 1985, the DNALC of Cold Spring Harbor Laboratory has precipitated a quiet revolution within Long Island school systems. More than 300 faculty have been trained to bring cutting-edge biotechnology experiments into primary, secondary, and college classrooms. More than 4000 area students visit the DNALC each year to perform state-of-the-art experiments—many of which are not available to students elsewhere in the nation. Thanks to the partnership between DNALC staff and local educators, Long Island school systems rank alongside those of San Francisco as the nation's leaders in biotechnological education at the precollege level. The DNALC continues to build, at the grass-roots level, an educated constituency that supports the growth of high technology on Long Island. Moreover, it contributes to the academic excellence that can attract and retain the intellectual work force needed to drive Long Island's effort to retool itself for the gene age.

The biotechnology industry has acknowledged the DNALC as an innovative force in promoting biotechnological development. Biotechnology companies have supported its activities since 1986. A training program conducted by the DNALC was the kickoff event and continuing model for the education programs of the North Carolina Biotechnology Center, which is widely regarded for its effective promotion of the Research Triangle area. The Biotechnology Center at Stony Brook has supported the DNALC as its major contribution to public education and is now developing a "sister" learning center to serve teachers and students from eastern Suffolk County. Unfortunately, too few Long Islanders are aware of the DNALC and its role in the long-term economic health of the region. Thus, the Laboratory's Development Department was charged to work with us to establish a Corporate Advisory Board with the following objectives:

- To educate business leaders and the Long Island community about the importance of quality science education in maintaining Long Island's technological base, developing future talent, and attracting high-caliber employees.
- To identify opportunities for industry/DNALC partnerships to improve the quality of Long Island science and technology education.
- To provide expertise in strategic planning, marketing, and other business practices that will help ensure the DNALC's long-term success.
- To provide a sustainable level of annual core funding for DNALC programs from the Long Island business community—primarily to expand access to local students and teachers.

Corporate Advisory Board members brought high-level expertise and represented a range of businesses: Michael Aboff, President, Aboff's, Inc.; Mark Abrahams, President, Cybex; Thomas J. Calabrese, Vice President and General Manager-Long Island, New York Telephone; Vincent Carosella,President, Jetson Direct Mail Services; Marybeth Chritie, Community Affairs Manager, Cablevision; Robert E. Diller, Vice President-Marketing, Brinkmann Instruments; Douglas Fox, President, Newsday; D. Kent Gale, Chief Executive Officer, Daniel Gale Agency; Arthur D. Herman, Chairman, A.D. Herman Construction; Richard K. Koehn, Director, Center for Bio technology, SUNY, Stony Brook; Lilo Leeds, Chair, CMP Publications; Joseph McDonnell, Vice President, LILCO; Robert R. McMillan, McMillan, Rather, Bennett & Rigano, P.C.; Francis Roberts, Superintendent, Cold Spring Harbor Central School District; Paul A. Vermylen, Jr., Vice President-Finance, Meenan Oil Company, Inc.; Michael Vittorio, Vice President, Chase Manhattan Bank; and Lawrence Waldman, Partner, KMPG Peat Marwick.

Local Programs Expand Despite the Recession

It is a tribute to the foresightedness of school teachers and administrators that programs for local schools continued to flourish despite severe budget cutbacks. Membership in the Cold Spring Harbor Curriculum Study, for which there is an

Great Moments in DNA Science lecturers Lynda Sherlock, Scott Henderson, and Raymond O'Keefe.

annual fee, actually increased to 28 districts in 1991. Curriculum Study schools receive free or reduced admission to all DNALC programs, spaces in student/teacher workshops, and equipment purchase options. The *Bio2000* Laboratory operated at peak capacity, with 3900 students representing 75 high schools participating in hands-on experiments on DNA restriction analysis, bacterial transformation, and human DNA fingerprinting. The *Great Moments in DNA Science* series, which has been held annually since 1985, attracted 600 students and teachers to seminars on a range of topics:

- DNA Typing in Forensic Case Work, Lynda Sherlock, Suffolk County Crime Laboratory
- Clinical Applications of Human Gene Therapy, Elizabeth Fenjves, SUNY at Stony Brook
- 3-D Organization of the Cell Nucleus, Scott Henderson and Raymond O'Keefe, CSHL
- Telomerase and Aging, Carol Grieder, CSHL

A new grant from the William Randolph Hearst Foundation and continued support from the Center for Biotechnology at SUNY Stony Brook provided the impetus for us to greatly enlarge our enrichment activities for middle school students. Supported by these grants, Jane Conigliaro joined the staff in the spring and immediately organized a mailing to advertise a series of 5-day summer camps for gifted fourth to seventh graders. Calling on our network of teachers at collaborating schools on Long Island, we had hoped to fill four *Fun With DNA* Camps. Despite the late date and limited number of schools contacted, response was nearly overwhelming. Six *Fun With DNA* Camps were filled within several weeks of the announcement, drawing the participation of 150 students from 45 Long Island schools.

Fun With DNA focuses on science as a way of making sense of the world and solving problems. Students learn a system to expand creative and critical thinking skills while mastering basic principles of genetics and gene manipulation. Categorizing mutations in the fruit fly *Drosophila*, analyzing kernel characteristics in Indian corn, and compiling a ministudy of classmates' traits illustrate principles of variation and Mendelian genetics. Constructing models of biological structures ("baggie" cells and popsicle stick DNA) is paralleled by observing cells under the microscope and extracting DNA from bacterial cells. The relationship between genes and visible characteristics is discovered by adding a gene for antibiotic resistance to an antibiotic-sensitive strain of bacteria. In the culminating experiment, students construct a "DNA fingerprint" by cutting virus DNA with restriction enzymes and sorting the resulting fragments by size in an electric field. Current newspaper and magazine articles are used extensively to evaluate the use of DNA fingerprints in law and medicine, and as a basis for debate on societal issues of biotechnology. The highlight of each week was the final "parent participation day," when the student teams demonstrated experiments and explained to their parents what they had learned.

Following the great success of the the summer program, Jane organized a new program of laboratory field trips for middle school students. Modeled after the existing high school field trip program, *Genetic FUNdamentals* offers a "menu" of five genetics laboratories, each of which has been rigorously tested over a 2-year period during student and teacher workshops. Laboratories are of-

Fun With DNA participant, Stacey Mon, demonstrates a DNA extraction on Parent Participation Day.

fered Mondays and Fridays at the DNALC; special arrangements can be made for in-school instruction by DNALC staff. Teachers may select one laboratory as stand-alone enrichment or build a unit composed of several experiences. Participating teachers receive a planning packet to prepare students for the laboratory experience, including current articles, vocabulary list, pre- and post-activities, and topics for follow-up discussion. Fifty laboratories were booked for the 1991–92 school year, with an estimated 1000 students from 32 different schools participating. The new program increased student laboratory participation at the DNALC by 25%.

Minority and Special Programs

We always stand ready to assist graduates of our training courses in implementing DNA manipulation laboratories. However, we take special interest in collaborations to help upgrade biology teaching at minority institutions. This commitment dates to our earlier collaboration with the Josiah Macy, Jr., Foundation to set up molecular genetics laboratory programs at four minority high schools in the urban northeast—A. Phillip Randolph (Harlem), Clara Barton (Brooklyn), Dewitt Clinton (The Bronx), and Hill House School (New Haven)—and a Navajo Indian high school in Arizona. We also organized a mobile laboratory program to reach rural schools in Alabama. In 1991, we collaborated with Ventures in Education (the foundation spinoff of the Macy minority programs) to help set up a molecular genetics laboratory at the John McDonogh High School in New Orleans. We also collaborated with the Abell Foundation to set up model teaching laboratories at Baltimore City College High School and Baltimore Polytechnic Institute. In August 1991, Margaret Henderson traveled to Claverack State Prison to help instruct a laboratory illustrating principles of DNA fingerprinting to juvenile offenders. The program was arranged by Lynn Lee, a middle school teacher who participated in our NSF training program.

During the last several years, we have devoted a substantial portion of our NSF funds for teacher training in rural and historically minority regions of the south and southwest. For example, high school workshops have been held in Columbia and Charleston, South Carolina; Columbus, Mississippi; Murray, Kentucky; San Antonio, Texas; Pensacola, Florida; and Harvey, Louisiana. Additional workshops will serve rural populations in Kentucky, Arkansas, and the intermountain west in 1992. Morehouse College, in Atlanta, was the site of a college workshop in 1991 and Howard University will be a host school in 1992. NSF-sponsored workshops, at both the high school and college levels, will be held in Puerto Rico in 1992.

The DNALC's curriculum development and nationwide teacher training programs are funded through major federal and foundation grants; however, enrichment activities for local students are largely supported through fees charged to participating schools and/or parents. Although fees are waived in hardship cases, this funding dichotomy has limited participation by minority and disadvantaged populations in our own "back yard." Thus, late in 1991, we established a Special Programs Fund as a means to redress this situation.

Our experience thus far has highlighted the difficulties of introducing innovation in disadvantaged school settings—especially the need to overcome teachers' "science phobias" and to provide intensive support and follow-up. We will have to commit considerable staff resources if we are serious about providing enrichment opportunities to deserving schools in our local area. Therefore, the Special Programs Fund will initially be used to provide start-up support for minority *Fun With DNA* summer camps and an *Intensive Enrichment Program*. Through this program, we will essentially "adopt" several deserving school systems in the metro-New York area to demonstrate the vertical integration of the DNALC's existing laboratory curricula at several levels of instruction.

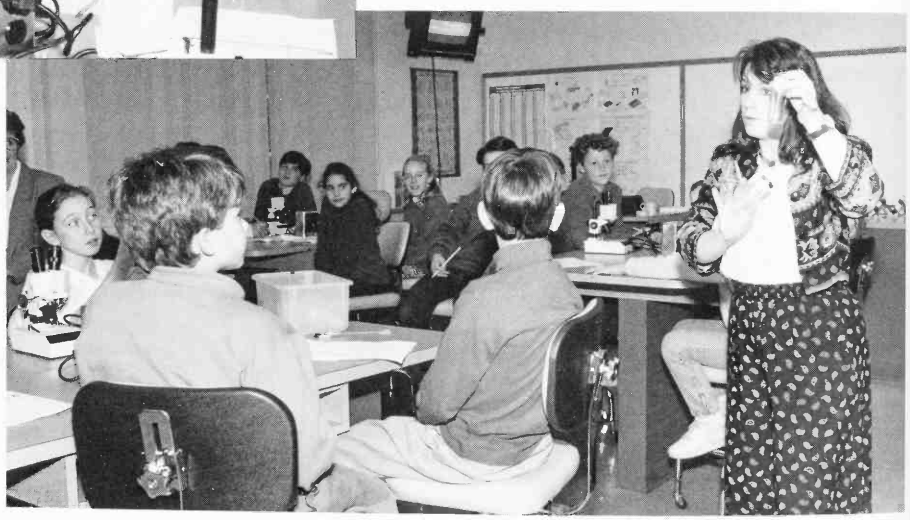
A Hectic Summer Schedule

Summer is traditionally reserved for teacher training workshops, and 1991 was our most rigorous to date. A hectic schedule of two college faculty workshops, eight high school faculty workshops, and 11 middle school faculty workshops was compounded by the addition of six *Fun With DNA* Camps for students. The organization of four different types of workshops in eight states is an extremely complicated task—mailing several thousand announcements; screening several hundred applications; shipping, setting up, and repacking approximately 500 pounds of reagents and equipment; motel reservations, paying participant stipends, etc. It still amazes us that (in a pinch) we can arrive at a location on Monday at 7:30 am, meet with our local organizer (often for the first time face-to-face!), unload a van-load of equipment, have a molecular biology laboratory functioning by 10:00 a.m. and still have time for a cup of coffee!

Developed in 1985 and supported by the NSF since 1987, the *DNA Science* Workshop is our best known workshop. The 5-day program, designed for advanced high school teachers, introduces modern methods for producing and analyzing recombinant DNA molecules, including microbial culture, gel electrophoresis, DNA restriction analysis, DNA ligation, and plasmid transformation and purification. In keeping with our recent geographical focus in the south and southwest, workshops were taught in Miami, Tampa, and Pensacola, Florida; Columbus, Mississippi; and Houston and San Antonio, Texas. These workshops—plus additional ones at the DNALC and in Montgomery County,

Maryland—drew 170 participants. Collaborators in California, Florida, and Wisconsin taught the *DNA Science* curriculum to 235 participants at ten additional workshops. Our records show that since 1985, the *DNA Science* curriculum has been the basis of at least 110 teacher-training workshops, organized by the DNALC and other education agencies, which have been taken by 2300 biology educators in 30 states.

The 4-day *Exploring Human Genetics* Workshop, developed under an NSF grant in 1990, prepares middle school instructors to introduce an experience-based unit on Mendelian and human genetics. The activities in this program are the basis of the *Fun With DNA* Camp and *Genetic FUNdamentals* field trips for middle school students. With strong support from Margaret Henderson, a cohort of New York State teachers trained in summer 1990 introduced new genetics activities during the 1990–1991 school year. Then, the lead teachers coordinated with Margaret to organize eight "second-round" workshops in summer 1991, which reached an additional 80 teachers in regions around the state. The lead teachers incorporated into the second-round workshop insights and resources drawn from their own classroom experiences during the prior school year. Several went on to share their experiences at professional conferences through-



Jane Conigliaro prepares middle school students to observe fruit flies with dissecting microscopes during a *Genetic Fundamentals* class.

out the state. A similar level of enthusiasm was evidenced by a second cohort of lead teachers trained in Maryland in summer 1991. Hopefully, the success of the New York and Maryland programs will demonstrate that this type of training can be replicated elsewhere in the nation.

Developed in 1989, the 10-day *Advanced DNA Science* Workshop introduces college faculty to many of the modern methods for analyzing complex genomes, including restriction mapping, gene library construction and screening, Southern hybridization, and polymerase chain reaction. The *Advanced DNA Science* curriculum articulates with and extends the concepts introduced in *DNA Science*. Structured to follow directly from an introduction to principles of biology, the entire laboratory series can form the basis of a first course in a molecular biology sequence, or appropriate sets of laboratories can be integrated as modules in many types of courses, including introductory biology, genetics, microbiology, and biochemistry. In summer 1991, 46 faculty from 24 states participated in NSF-sponsored *Advanced DNA Science* Workshops held at Morehouse College, Atlanta, and the University of California, San Francisco. As the San Francisco workshop was concluding, we were pleased to learn that the Fund for Improvement of Post-secondary Education, of the U.S. Department of Education, had approved a 2-year grant that will allow us to expand the college workshop program to four sites in summer 1992. After incorporating feedback from the summer 1992 workshops, the laboratories will be formally published later in the year as *Advanced DNA Science: An Introduction to Methods of Genome Analysis*.

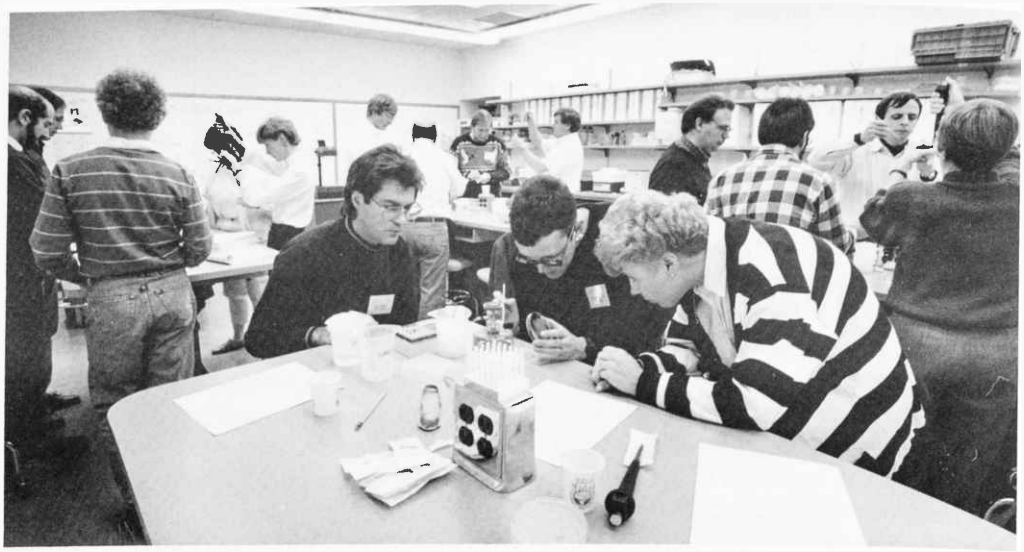
College intern, Amy Phillips, prepares a colony hybridization; this lab is included in the new *Advanced DNA Science* curriculum.

New DOE Workshop for Opinion Leaders and Public Policy Makers

Communications and sociological research suggest that information campaigns have an indirect effect on public opinions and behavior. Information often flows to the public in two steps: "Opinion leaders" assess information from a variety of sources and form attitudes about issues. These well-informed individuals, in turn, influence the opinion and behaviors of people around them. Thus, information campaigns aimed at a relatively small number of opinion leaders may provide a cost-effective means to reach large segments of the general public.

With this model in mind, we collaborated with Jan Witkowski of the Laboratory's Banbury Center to design a new workshop for influential non-scientists who interface with human genetics research and society. We were very pleased when our program, *Human Genetic and Genome Analysis: A Practical Workshop for Opinion Leaders and Public Policy Makers*, received 2-year funding from the Department of Energy under its new program, Ethical, Legal, and Social Implications of the Human Genome Initiative. The first workshop was held in December, 1991 and drew together a heterogeneous mixture of congressional staff and government administrators, officers and staff in health-related foundations, genetic rights activists, science journalists, and staff at science museums.

The workshop is composed of three components that juxtapose the theory, practice, applications, and implications of human gene manipulation. Concept seminars, presented by Banbury and DNALC staff, introduce key principles that underpin human genome analysis, including Mendelian genetics, the molecular basis of inheritance, gene mapping and cloning, and DNA diagnosis. These topics were made tangible through laboratory sessions where participants construct a restriction map, transform *Escherichia coli* with an ampicillin resistance gene, and make their own DNA fingerprint using PCR. Feature seminars, presented by working scientists, provide first-person insight into the research process and the ethical dilemmas of human genetics research:



David Micklos (left foreground) monitors a bacterial transformation during "Human Genetics and Genome Analysis," a DOE-sponsored workshop for opinion leaders and public policy makers.

- Origins and Impacts of the Human Genome Project, David Galas, U.S. Department of Energy
- The Eugenics Movement, Philip Reilly, Shriver Center for Mental Retardation
- Cloning Human Disease Genes: Neurofibromatosis 1, Margaret Wallace, University of Florida Health Sciences Center
- The First Human Gene Therapy Trials, Ken Culver, National Institutes of Health
- Searching for Genes for Mental Disorders, Conrad Gilliam, Columbia University

International Collaborations: Ending the Cold War and "Warm Spring Harbor"

Following up on a collaboration initiated in 1990 with the Shemyakin Institute of Bioorganic Chemistry, Mark Bloom returned to Moscow in February to co-instruct a *DNA Science* Workshop with three Shemyakin staff members. The week-long workshop was attended by 12 students and 6 teachers selected for their science and English ability from four Moscow area high schools. Professor Konstantine Skryabin, Vice-Director of the Engelhardt Institute of Molecular Biology, opened the workshop with a seminar on the origins and implications of DNA manipulation technology. Participants used the *DNA Science* lab/text supplemented with Russian translations of the laboratory protocols, with Dr. Bloom and Dr. Nikolai Zvonok presenting laboratory briefings that alternated English with Russian translation. This team-teaching approach, coupled with meticulous preparation, yielded experimental results on par with those routinely achieved in the United States.

Efforts to continue the collaboration were delayed by the dramatic events that dissolved the former Soviet Union. As the year ended, we received word from

Professor V.T. Ivanov, Director of the Shemyakin Institute, that funds to continue our exchange were available through the newly formed Russian Academy of Sciences. With this letter of support in hand, we submitted a request to NSF for a 3-year Russian/American program in Undergraduate Biomolecular Manipulation.

The year also brought opportunity for a new collaboration with Marcello Siniscalco. A human geneticist of the first order, Marcello has based his life's research on the island of Sardinia, where his early studies provided evidence that the gene for the blood disorder thalassemia provides protection from malignant malaria. In the 1950s, he showed that thalassemia was restricted primarily to populations living in lowland areas of Sardinia, where malaria had been prevalent. Our friendship with Marcello dates to 1985, when, on sabbatical at Cold Spring Harbor Laboratory, he and his family lived next door to Dave Micklos. Over the years, Marcello had watched with keen interest the development of the DNA Learning Center.

Several years ago, Marcello left his research position at Memorial Sloan Kettering Cancer Center to assume responsibility for the development of a new genetics research institute on the Sardinian coast—the Porto Conte Research and Training Laboratories, which will open in 1992. Marcello's intent is to model his new institution after Cold Spring Harbor Laboratory, and, due to its location on the warm waters of the Mediterranean Sea, he refers to the Porto Conte facility as "Warm Spring Harbor." He envisioned public genetics education as an integral part of the new institute and set about to gather support for a museum of science modeled after the DNA Learning Center.

Marcello invited Dave to Sardinia to participate in a week of "Disseminating Scientific Culture," March 18–22, sponsored by the Italian Ministry of Scientific and Technological Research. Although events were held at universities and science museums throughout the country, the Sardinian event was the largest and most publicized in Italy. Participants included Nobel laureate Rita Levi Montalcini and Piero Angela, a television science reporter whose phenomenal celebrity is comparable to Walter Cronkite in his heyday. The need for DNA literacy took on national scope during a 50-minute program on Italian network television, moderated by Piero Angela and Marcello Siniscalco, which included a 15-minute documentary on the DNA Learning Center.

The proposed collaboration gained substance in December, when Marcello received a major grant from the Italian Ministry of University, Scientific and Technological Research, to develop plans for a science museum at Porto Conte. Marcello, in turn, subcontracted the DNALC to produce a mini-exhibit to be displayed in Sassari, Sardinia as a focal point of celebrations conducted there during the Italian national "Week of Science," in April. The "genetic video arcade" will include four interactive computer modules, which will play several multimedia programs developed with Hearst support–*DNA Detective*, *DNA Diagnosis*, and *Gene Therapy*. Following presentation during the Italian Week of Science, the video arcade will be installed at the Porto Conte Research and Training Laboratories in anticipation of its own museum. Marcello's efforts, together with the Italian translation of *DNA Science* by the Piccin New Library, will likely establish Italy as a European model for public genetics education.

Staff and Interns

In early spring, we recruited a new part-time staff member, Jane Conigliaro, to take responsibility for expanding our programs for middle school students. With

degrees in elementary and special education, Jane has consulted for six Long Island school districts to design multidisciplinary programs for intellectually gifted elementary and middle school students. In addition to her position here, Jane juggles a consultancy for Roslyn Public Schools, an adjunct faculty position at Long Island University's C.W. Post campus, and a private practice in the remediation of learning disabilities. Jane was among the first local educators to use the DNALC as an enrichment opportunity for young students. She also served on the advisory committee for the original *Fun With DNA* student workshop in 1988 and the NSF training program for middle school teachers. Her interest in educating the "whole child" and unique ability to facilitate associative learning is an inspiration to the staff, as well as to the many teachers for which she is a model educator.

A number of middle and high school faculty assisted with summer programs, getting a "micro-teaching" experience and a behind the scenes look at how our workshops run. *DNA Science* Workshop alumnus Ellen Mayo, of Douglas Freeman High School in Richmond, traveled with us to instruct workshops in Miami and Tampa. A number of faculty assisted with instruction of the *Fun With DNA* Camps: Barbara Cullen, Huntington SEARCH Gifted Program; Gerri Faivre, East Woods School; Peggy Frisina; Karen Gough, Half Hollow Hills High School; Diane Jedlicka, Roslyn OMNI Gifted Program; James Lauter, Huntington Elementary School; Adele Nicefero, Hicksville Academic Enrichment Program; and Nancy Van Vranken, St. Bernard's School.

A small group of high school interns supply "behind the scenes" support that allow us to provide high-quality laboratory experiences to more than 4000 precollege students per year. Before departing for her first year at SUNY, Geneseo, senior intern Amy Phillips pretested experiments for the *Advanced DNA Science* curriculum. She became so proficient with these advanced techniques that she functioned as sole laboratory aide for two 10-day summer workshops for college faculty—a task previously performed by undergraduate and graduate interns. Mark Staudinger, a junior at Cold Spring Harbor High School, functioned without flaw as laboratory aide at *DNA Science* Workshops held in Houston, San Antonio, and the DNALC. Nicole Nicefero, a senior at Cold Spring Harbor High School, functioned as a mentor to younger students participating in five *Fun With DNA* Workshops. New interns Maggie Choi, a sophomore at Syosset High School and Charles Rexer, a senior at Manhasset High School, assumed responsibility for the *Bio2000* Laboratory, but also found time for independent research projects.

With the opening of the *BioMedia* Laboratory, interns also have the opportunity to apply their computer abilities to the problems of biology education. Intern involvement in multimedia development began during a summer stay by Claudio Siniscalco, the son of our Italian collaborator, Marcello. A freshman at Westminster School in London, Claudio completed casework for the initial program, *DNA Detective*. David Hollman, a junior at Cold Spring Harbor High School, completed a program that accesses a laser disk containing video demonstrations of student laboratory techniques. The program is now used in the *Bio2000* Laboratory, allowing our instructors to call up, at the touch of a button, appropriate video clips that serve as visual references to assist students as they perform experiments.

Publication

Bloom, M., G. Freyer, and D. Micklos. *Advanced DNA Science An Introduction to Methods of Genome Analysis.* (In preparation.)

Sites of Major 4–8 Day Workshops 1985–1991

ALABAMA	University of Alabama, Tuscaloosa	1987,1988,1989,1990
ARIZONA	Tuba City High School	1988
CALIFORNIA	University of California, Davis	1986
	San Francisco State, San Francisco	1991
CONNECTICUT	Choate Rosemary Hall, Wallingford	1987
FLORIDA	N. Miami Beach Senior High School, Miami	1991
	University of W. Florida, Pensacola	1991
	Armwood Senior High School, Tampa	1991
GEORGIA	Fernbank, Inc., Atlanta	1989
	Morehouse College, Atlanta	1991
HAWAII	Kamehameha Secondary School, Honolulu	1990
ILLINOIS	Argonne National Laboratory, Chicago	1986, 1987
INDIANA	Butler University, Indianapolis	1987
IOWA	Drake University, Des Moines	1987
KENTUCKY	Murray State University	1988
LOUISIANA	Jefferson Parish Public Schools, Harvey	1990
MANITOBA	Red River Community College, Winnipeg	1989
MARYLAND	Annapolis Senior High School	1989
	McDonogh School, Baltimore	1988
	Montgomery County Public Schools	1990,1991
	St. John's College, Annapolis	1991
MASSACHUSETTS	Beverly High School	1986
	Dover-Sherborn High School, Dover	1989
	Randolph High School	1988
	Winsor School, Boston	1987
MICHIGAN	Athens High School, Troy	1989
MISSISSIPPI	Mississippi School for Math & Science, Columbus	1990,1991
MISSOURI	Washington University, St. Louis	1989
NEW HAMPSHIRE	St. Paul's School, Concord	1986,1987
NEW YORK	Albany High School	1987
	Bronx High School of Science	1987
	Cold Spring Harbor High School	1985,1987
	DeWitt Middle School, Ithaca	1991
	DNA Learning Center, High School Workshop	1988(3),1989(2), 1990(2), 1991
	DNA Learning Center, College Workshop	1990
	DNA Learning Center, Middle School Workshop	1990,1991
	Fostertown School, Newburgh	1991
	Huntington High School	1986
	Irvington High School	1986
	Junior High School 263, Brooklyn	1991
	Lindenhurst Junior High School	1991
	Orchard Park School, Orchard Park	1991
	Plainview-Old Bethpage Middle School, Plainview	1991
	State University at Purchase	1989
	State University at Stony Brook	1987,1988,1989,1990
	Titusville Middle School, Poughkeepsie	1991
	Wheatley School, Old Westbury	1985
NORTH CAROLINA	North Carolina School of Science, Durham	1987

OHIO	Case Western Reserve University, Cleveland	1990
	Cleveland Clinic	1987
	North Westerville High School, Westerville	1990
PENNSYLVANIA	Duquesne University, Pittsburgh	1988
	Germantown Academy, Fort Washington	1988
SOUTH CAROLINA	Medical University of South Carolina, Charleston	1988
	University of South Carolina, Columbia	1988
TEXAS	J.J. Pearce High School, Richardson	1990
	Langham Creek High School, Houston	1991
	Taft High School, San Antonio	1991
VERMONT	University of Vermont, Burlington	1989
VIRGINIA	Jefferson School of Science, Alexandria	1987
	Mathematics and Science Center, Richmond	1990
WEST VIRGINIA	Bethany College	1989
WISCONSIN	Marquette University, Milwaukee	1986,1987
	University of Wisconsin, Madison	1988,1989
WYOMING	University of Wyoming, Laramie	1991

1991 Workshops, Meetings, and Collaborations

January 10	Meeting, Association of American Colleges, Washington, D.C.
January 25	Congressional Workshop, DNA Learning Center, Cold Spring Harbor, New York
January 25–26	Follow-up High School Workshop, National Science Foundation, Kamehameha Secondary School, Honolulu, Hawaii
January 29–Feb. 11	High School Workshop, Shemyakin Institute, Moscow, Russia
February 2–3	Follow-up High School Workshop, National Science Foundation, Jefferson Parish Public Schools, Harvey, Louisiana
February 26	Seminar, University of Massachusetts, Amherst, Massachusetts
March 2	Middle School Follow-up Workshop, National Science Foundation, Albany, New York
March 8–10	Meeting, Math and Science Schools, Columbus, Mississippi
March 14	Great Moments in DNA Science, Curriculum Study, Cold Spring Harbor, New York
March 18–22	Italian Week at Science, Sassari, Sardinia
March 26–31	NSTA Meeting, Houston, Texas
April 4	Great Moments in DNA Science, Curriculum Study, Cold Spring Harbor, New York
April 11	Great Moments in DNA Science, Curriculum Study, Cold Spring Harbor, New York
April 11–13	College Workshop, Bates College, Lewiston, Maine
April 15–17	Collaboration with Terri Woodin of University of Nevada, Reno
April 17	Great Moments in DNA Science, Curriculum Study, Cold Spring Harbor, New York
April 22	Abell Foundation Meeting, Baltimore, Maryland
June 3–4	Collaboration with Raynard Sanders and Michelle Brierre of New Orleans, Louisiana
June 10–14	High School Workshop, National Science Foundation, University of West Pensacola, Florida
June 12–14	ABLE Workshop, Laramie, Wyoming
June 17–21	High School Workshop, National Science Foundation, Armwood Senior High School, Tampa, Florida
June 24–26	Second-round Middle School Workshop, National Science Foundation, Lindenhurst, New York
June 24–28	High School Workshop, National Science Foundation, N. Miami Beach Senior High Schools, Miami, Florida
	High School Workshop, National Science Foundation, Mississippi School for Math and Sciences, Columbus, Mississippi
June 25–27	Second-round Middle School Workshop, National Science Foundation, Poughkeepsie, New York
June 27–July 1	Second-round Middle School Workshop, National Science Foundation, DNA Learning Center, Cold Spring Harbor, New York
July 8–10	Second-round Middle School Workshop, National Science Foundation, Ithaca, New York
July 8–12	Fun With DNA, DNA Learning Center, Cold Spring Harbor, New York
July 8–19	College Workshop, National Science Foundation, Morehouse College, Atlanta, Georgia
July 10–12	Second-round Middle School Workshop, National Science Foundation, Orchard Park, New York
July 15–19	Fun With DNA, DNA Learning Center, Cold Spring Harbor, New York
July 22–26	High School Workshop, Montgomery County, Burtonsville, Maryland
	Fun With DNA, DNA Learning Center, Cold Spring Harbor, New York
July 29–August 1	Middle School Workshop, National Science Foundation, Annapolis, Maryland
July 29–August 2	Fun With DNA, DNA Learning Center, Cold Spring Harbor, New York

August 5–9	Workshop, National Science Foundation, Taft High School, San Antonio, Texas
	Fun With DNA, DNA Learning Center, Cold Spring Harbor, New York
August 5–16	College Workshop, National Science Foundation, University of California, San Francisco, California
August 12–14	Second-round Middle School Workshop, National Science Foundation, Delmar, New York
August 12–16	High School Workshop, National Science Foundation, Langham Creek High School, Houston, Texas
	Fun With DNA, DNA Learning Center, Cold Spring Harbor, New York
August 14–16	Second-round Middle School Workshop, National Science Foundation, Newburgh, New York
August 26–28	Second-round Middle School Workshop, National Science Foundation, Germantown, New York
August 26–30	High School Workshop, Curriculum Study, DNA Learning Center, Cold Spring Harbor, New York
August 27–29	Second-round Middle School Workshop, Brooklyn, New York
August 28–30	Second-round Middle School Workshop, Plainview, New York
September 19	Physicians' Continuing Education Program, Huntington Hospital, Huntington, New York
September 23–24	Carolina Biological Supply Co., Burlington, North Carolina
October 1	New Jersey Science Workshop
October 8–9	Project Share, Middle School Workshop, Killingworth, Connecticut
October 18–21	ASTC, Louisville, Kentucky
October 23	Rutgers University Lecture, New Jersey
October 26–27	Follow-up High School Workshop, National Science Foundation, University of West Florida, Pensacola, Florida
October 28–30	Meeting, Madison, Wisconsin
November 1	NRC Meeting, Washington, D.C.
November 2–3	DOE Grant Meeting, Washington, D.C.
November 3–23	WHO Grantee, Antonio Jacalne, Manila, Phillippines
November 5–6	Middle School Workshop, Centerbrook, Connecticut
November 9–10	Follow-up High School Workshop, National Science Foundation, Langham Creek High School, Houston, Texas
November 14	Genome Presentation, Baltimore, Maryland
November I5	Lecture, United States Military Academy, West Point, New York
November 15	Site Visit, Raynard Sanders of New Orleans, Louisiana
November 15–16	Follow-up High School Workshop, Montgomery County, Maryland
December 7–8	Follow-up High School Workshop, National Science Foundation, Mississippi School for Math and Science, Columbus, Mississippi
December 8–11	DOE Workshop, Cold Spring Harbor, New York
December 9–11	Proposal Development Workshop, Washington, D.C.
December 14–15	Follow-up High School Workshop, National Science Foundation, Taft High School, San Antonio, Texas

EDUCATIONAL ACTIVITIES

The academic program at Cold Spring Harbor Laboratory comprises a wide-ranging series of postgraduate laboratory and lecture courses, workshops, large meetings, and a summer research program for undergraduates. The program now extends from a spring session of courses early in April through a fall session of courses ending early in November. Hundreds of visiting scientists participate in the courses as students, instructors, assistants, and lecturers.

Several new courses and meetings were begun this year and new facilities were used for the first time. The Howard Hughes Medical Institute (HHMI) teaching laboratories in the Beckman Neuroscience Center were inaugurated this summer when three courses moved up the hill from James Laboratory: Molecular Embryology of the Mouse, Advanced Molecular Cloning, and Cloning of Neural Genes. The greatly expanded space and state-of-the-art facilities were immediately hugely popular with all of the instructors and students. Next year, the Neurobiology Laboratory courses that are now held in Jones Laboratory will move to a second HHMI teaching laboratory. A new neurobiology laboratory course, Imaging Structure and Function in the Nervous System, was offered this year, taught by Larry Katz and Richard Lewis. In addition, two new courses were offered in the fall session: Analysis and Genetic Manipulation of YACs, taught by Georges Carle, Eric Green, and Rodney Rothstein, and Computational Genomics, taught by Elbert Branscomb, Nate Goodman, Eric Lander, and Tom Marr. In total, the Laboratory was host to 15 laboratory courses in molecular genetics and neurobiology during the spring, summer, and fall sessions (instructors and students are listed in the following pages) and four 1–2-week summer neurobiology lecture courses held at the Banbury Center.

The courses are supported by, and would not be possible without, a series of grants from federal and private sources. The summer molecular genetics courses have been supported for many years by grants from the National Institutes of Health and the National Science Foundation, and a grant from the National Institute of Mental Health has supported several of the neurobiology courses. However, it has been a large education grant (renewed again this year) from HHMI that has provided stable support for the neurobiology program and has allowed the Laboratory to begin its series of spring and fall courses. The Laboratory also received an award from the Klingenstein Fund for the support of neurobiology courses. As has been the case for several years, the Grass Foundation provided funds for scholarships for students in neurobiology courses.

Although conferences at the Laboratory started with a single Symposium in 1933 and have since grown to 14 meetings per year covering a wide variety of topics, the Symposium continues to be a highlight. This year's Symposium, The Cell Cycle, organized by David Beach, Bruce Stillman, and James Watson, was, in fact, oversubscribed; more than 450 scientists gathered to discuss their work in this rapidly moving area of research. Several of the meetings, in addition to the Symposium, were filled to capacity as every seat (and sometimes parts of the floor) in Grace Auditorium was occupied. These meetings included RNA Processing, RNA Tumor Viruses, and the Cancer Cells meeting on Regulation of Eukaryotic Transcription. The success of the meetings really depends on all of the scientists who serve as organizers (listed in the following pages) and on the enthusiastic participation of all of the visiting scientists. A highlight of the meeting season in 1991 was the opening of Dolan Hall for the accommodation of visiting scientists. These facilities were very much needed and elicited enthusiastic comment.

The fact that so many scientists can participate in the courses and meetings is due, in great measure, to the skill and hard work of the staff in the Meetings Office; Barbara Ward, Diane Tighe, Micki McBride, Laurie Myers, Eileen Paetz, Marge Stellabotte, and Nancy Weeks. The audiovisual staff, headed by Herb Parsons, has become well known to visiting scientists who are continually amazed at their ability to juggle thousands of slides and overhead projections with such efficiency. The course instructors have come to depend on the skills of Cliff Sutkevich in setting up and maintaining all of the necessary equipment and supplies. The success of the academic program depends, to a great extent, on the work and assistance of Laboratory staff from all areas—from scientists who give lectures (and sometimes teach) in the courses, to those in the Purchasing and Grants Offices, to staff in the Buildings and Grounds Department and Blackford Hall.

While graduate students, postdoctoral fellows, and faculty participate in the courses and meetings, the Undergraduate Research Program (URP) provides an opportunity for college undergraduates to spend 10 weeks during the summer at the Laboratory. The program, headed by Winship Herr, allows students to do research in the laboratories of staff scientists.

Terri Grodzicker
Assistant Director for Academic Affairs

Postgraduate Courses

The summer program of Postgraduate Courses at Cold Spring Harbor Laboratory is aimed at meeting the special need for training in interdisciplinary subjects that are either so new or so specialized that they are not adequately treated by universities. Our aim is to provide intensive study in the most recent developments and techniques in these subjects and to prepare students to enter directly into research in a particular area. To ensure up-to-date coverage of current research work, we bring together a workshop staff from many laboratories around the world and supplement this staff with a series of seminar speakers.

Protein Purification and Characterization

April 8–April 22

INSTRUCTORS

Erickson, Bruce, Ph.D., University of North Carolina, Chapel Hill
Kadonaga, James, Ph.D., University of California, San Diego
Marshak, Daniel, Ph.D., Cold Spring Harbor Laboratory, New York
Smith, John, M.D., Ph.D., Merck Sharpe & Dohme Research Labs, Rahway, New Jersey

ASSISTANTS

Kerrigan, Leslie, University of California, San Diego
Vazquez, Greg, University of North Carolina, Chapel Hill
Williams, Kevin, Massachusetts General Hospital, Boston

This course was intended for scientists who are not familiar with techniques of protein isolation and characterization. It was a rigorous program that included laboratory work all day and a lecture with discussion session every evening. Each student became familiar with each of the major techniques in protein purification by actually performing four separate isolations including (1) a regulatory protein from muscle tissue; (2) a sequence-specific, DNA-binding protein; (3) a recombinant protein overexpressed in *E. coli;* and (4) a membrane-bound receptor. A variety of bulk fractionation, electrophoretic, and chromatographic techniques were employed including precipitation by salts, pH, and ionic polymers; ion exchange, gel filtration, hydrophobic interaction, and reverse-phase chromatography; lectin affinity, oligonucleotide affinity, and immunoaffinity chromatography; polyacrylamide gel electrophoresis, two-dimensional gel electrophoresis, and electroblotting; and high-performance liquid chromatography. Methods of protein characterization were utilized including immunological and biochemical assays, peptide mapping, amino acid analysis, protein sequencing, and mass spectrometry. Emphasis was placed on strategies of protein purification and characterization rather than on automated instrumental analysis. Guest lecturers discussed protein structure, modifications of proteins, methodologies for protein purification and characterization, chemical synthesis of peptides, and applications of protein biochemistry to cell and molecular biology.

PARTICIPANTS

Asselin, C., Ph.D., University of Sherbrooke, Canada
Attie, A., Ph.D., University of Wisconsin, Madison
Catala, F., Ph.D., Institut Pasteur, Paris, France
El Ridi, R., Ph.D., Cairo University, Egypt
Grant, S., M.D., Columbia University, New York, New York
Howe, M., Ph.D., University of Tennessee, Memphis
Jenison, S., M.D., Fred Hutchinson Cancer Center, Seattle, Washington
Kiebler, M., B.S., Institute of Physiological Chemistry, Munich, Germany
Koh, G., M.D., Cornell Medical College, New York, New York
Marshall, C., Ph.D., University of Pennsylvania, Philadelphia
Neckelmann, S., Ph.D., Emory University, Atlanta, Georgia
Riva, M., Ph.D., CEN Saclay, Gif-sur-Yvette, France
Rubin, B., B.A., Cornell University, New York, New York
St-Arnaud, R., Ph.D., Shriners Hospital, Montreal, Canada
Stebbins-Boaz, B., Ph.D., Worcester Foundation, Shrewsbury, Massachusetts
Upton, C., Ph.D., University of Alberta, Edmonton, Canada

SEMINARS

Aebersold, R., University of British Columbia. Protein sequence analysis.
Chamow, S., Genentech, Inc. CD4 immunoadhesins as anti-HIV therapeutics.
Erickson, B., University of North Carolina. Peptide synthesis.
Garrels, J., Cold Spring Harbor Laboratory. Two-dimensional electrophoresis: Methods and analysis.
Hart, G., Johns Hopkins University. Glycosylation of proteins.
Kadonaga, J., University of California, San Diego. DNA-binding proteins.
Kelly, J., Texas A & M University. Spectroscopic techniques for studying protein structure.
Marshak, D., Cold Spring Harbor Laboratory. 1. Purification of proteins, Part II. 2. Mass spectroscopic methods.
Pace, N., Texas A & M University. Protein denaturation.
Paterson, Y., University of Pennsylvania. Anti-peptide antibodies.
Rose, G., Pennsylvania State University. Protein structure.
Smith, J., Merck Sharp & Dohme Research Laboratories. Purification of proteins, Part I.
Smith, J. and Marshak, D., Cold Spring Harbor Laboratory. Translational modification of proteins.
Wiktorowicz, J., Applied Biosystems. Protein and DNA isolation and characterization.

Cloning and Analysis of Large DNA Molecules

April 8–April 22

INSTRUCTORS

Birren, Bruce, Ph.D., California Institute of Technology, Pasadena
Klapholz, Sue, Ph.D., Cell Genesys Inc., Foster City, California
Shepherd, Nancy, Ph.D., DuPont Merck Pharmaceutical Co., Wilmington, Delaware

ASSISTANTS

Campbell, Michael, Stanford University, California
Henderson, Nancy, DuPont Merck Pharmaceutical Co., Wilmington, Delaware
Pfrogner, Beverly, DuPont Merck Pharmaceutical Co., Wilmington, Delaware
Wang, Yu Kang, California Institute of Technology, Pasadena

This course covered the theory and practice of manipulating and cloning high-molecular-weight DNA. Lectures and laboratory work dealt with the use of bacteriophage P1 and yeast artificial chromosome (YAC) cloning systems, the isolation and manipulation of high-molecular-weight DNA from mammalian cells for cloning (including the size selection of >200-kb DNA fragments), and the analysis of high-molecular-weight DNA by pulse field gel (PFG) separation techniques. P1 and YAC recombinant DNA molecules were produced, introduced into cells (*E. coli* and yeast, respectively), and reisolated after appropriate clone selection and colony screening procedures. A variety of size standards for pulsed-field gel

electrophoresis were prepared, and gels were run to compare the DNA separation capabilities of the common PFG techniques. Students gained experience with physical mapping of YAC inserts and high-molecular-weight genomic DNA. Lectures by outside speakers on topics of current interest supplemented the laboratory work.

PARTICIPANTS

Amler, L., B.A., Deutsches Krebsforschungszentrum, Heidelberg, Germany
Beard, H., B.S., USDA Plant Molecular Biology Laboratory, Beltsville, Maryland
Cairns, P., Ph.D., Marie Curie Institute, Oxted, England
Chiu, S., Ph.D., Hong Kong Baptist College, Kowloon
Cohn, M., M.S., Wallenberg Laboratory, Lund, Sweden
Fioretos, T., M.D., University Hospital, Lund, Sweden
Gardiner, J., M.A., University of Missouri, Columbia
Levesque, R., Ph.D., University Laval, Quebec, Canada
Melville, S., Ph.D., University of Cambridge, England
Morzaria, S., Ph.D., ILRAD, Nairobi, Kenya
Ng, S., Ph.D., University of California, Santa Barbara
Ozelius, L., B.S., Massachusetts General Hospital East, Charlestown
Stahl, J., M.D., Walter & Eliza Hall Institute, Victoria, Australia
Tawheed, A., M.S., Institut Pasteur, Paris, France
Wagner, K., Ph.D., Institute for Medical Biology & Human Genetics, Graz, Austria

SEMINARS

Burke, D., Princeton University. YAC cloning.
Cox, D., University of California, San Francisco. Use of radiation hybrid mapping in the analysis of large genomes.
Gemmill, R., Eleanor Roosevelt Institute. Pulsed-field gel mapping of human chromosomes.
Hieter, P., Johns Hopkins Medical School. Yeast artificial chromosomes: Promises kept and pending.
Lai, E. , University of North Carolina, Chapel Hill. New methods in electrophoresis and restriction fragment analysis.
Pierce, J., DuPont Merck Pharmaceutical Co. Cloning in the P1-positive selection system.
Skorupski, K., DuPont Merck Pharmaceutical Co. The P1 in vitro packaging reaction: Proteins involved in cleavage of the *pac* site.
Smith, D., Collaborative Research Inc. PFGE and YAC cloning.
Smoller, D., Washington University School of Medicine. Large DNA cloning of the *Drosophila* genome.

Advanced Bacterial Genetics

June 7–June 27

INSTRUCTORS

Maloy, Stanley, Ph.D., University of Illinois, Urbana-Champaign
Stewart, Valley, Ph.D., Cornell University, Ithaca, New York
Taylor, Ronald, Ph.D., University of Tennessee, Memphis

ASSISTANTS

Chen, Li-Mei, University of Illinois, Urbana-Champaign
Peek, Joel, University of Tennessee, Memphis
Rabin, Ross, Cornell University, Ithaca, New York

This laboratory course demonstrated genetic approaches that can be used to analyze biological processes and their regulation, as well as detailed structure/function relationships of genes. Techniques covered include isolation, complementation, and mapping of mutations; use of transposable genetic elements;

construction of operon and gene fusions; cloning of DNA; restriction endonuclease mapping; Southern blotting; polymerase chain reaction; site-directed mutagenesis; and DNA sequencing. The course consisted of a set of experiments incorporating most of these techniques in the genetic analysis of diverse bacterial and bacteriophage species. These experiments were supplemented with lectures and discussions. The aim was to develop in students the ability to design a successful genetic approach to any biological problem.

PARTICIPANTS

Alpuche, C., M.D., Massachusetts General Hospital, Boston
Behlau, I., M.D., Massachusetts General Hospital, Boston
Brown, A., B.S., Albert Einstein College of Medicine, Bronx, New York
Budrene, E., Ph.D., Harvard University, Cambridge, Massachusetts
Eliasson, A., B.A., Uppsala University, Sweden
Forsberg, D., Ph.D., University of Guelph, Canada
Granger, D., M.D., Duke University, Durham, North Carolina
Hedderich, R., Ph.D., Phillips Universitat, Marburg, Germany
Kiessling, L., Ph.D., California Institute of Technology, Pasadena
Maldonado, R., B.A., University of Seville, Spain
Moreillon, P., M.D., Rockefeller University, New York, New York
Poole, R., Ph.D., King's College, London, England
Regnier, P., Ph.D., Institut de Biologie Physico-Chimique, Paris, France
Smith, D., Ph.D., Case Western Reserve University, Cleveland, Ohio
Stein, J., M.S., Scripps Institution of Oceanography, La Jolla, California
Thune, R., Ph.D., Louisiana State University, Baton Rouge

SEMINARS

Manoil, C., University of Washington. 1. Membrane protein localization: Using gene fusions to make a complicated process a little simpler. 2. Genetics of F factor conjugation in *Escherichia coli.*
Roth, J., University of Utah. 1. Genetic analysis of chromosome rearrangements. 2. Regulation of vitamin B_{12} synthesis in *Salmonella typhimurium.*
Silhavy, T., Princeton University. 1. SDI and protein secretion. 2. Osmoregulation of porin synthesis in *Escherichia coli.*
Weinstock, G., University of Texas. Molecular genetics of shipping fever.

Molecular Embryology of the Mouse

June 7–June 27

INSTRUCTORS

Lovell-Badge, Robin, Ph.D., National Institute for Medical Research, London, England
Parada, Luis F., Ph.D., Basic Research Program, NCI-FCRDC, Frederick, Maryland

CO-INSTRUCTORS

Mann, Jeff, Beckman Research Institute, Duarte, California
Rastan, Sohaila, Clinical Research Centre, Middlesex, England

ASSISTANTS

Conlon, Frank, Columbia University, New York, New York
Nichols, Jenny, University of Edinburgh, Scotland
Reid, Susan, Basic Research Program, NCI-FCRDC, Frederick, Maryland

This course was designed for molecular biologists, biochemists, and cell biologists interested in applying their expertise to the study of mouse embryonic development. Lecture and laboratory components of the course emphasized both the practical aspects of working with mouse embryos and the conceptual basis of current embryonic research. The following procedures and their potential applications were described: isolation and culture of germ cells and preimplantation and postimplantation embryos, embryo transfer, establishment and genetic manipulation of embryo-derived stem cell lines, germ layer separation, chimera formation, nuclear transplantation, microinjection of DNA into eggs, microinjection of cell lineage tracers, in situ hybridization and immunohistochemistry. Guest lecturers discussed current research in the field.

PARTICIPANTS

Collo, G., M.D. Scripps Clinic & Research Foundation, La Jolla, California
Cordes, S., Ph.D. Stanford University, California
Covarrubias, L., Ph.D. UNAM, Cuernavaca, Mexico
Crossley, P., Ph.D., Imperial College of Science, Technology & Medicine, London, England
Ikuta, K., Ph.D., Stanford University, California
Labosky, P., B.A. Wesleyan University, Middletown, Connecticut
Noakes, P., Ph.D., Washington University, St. Louis, Missouri
Pearson-White, S., Ph.D., University of Virginia, Charlottesville
Siracusa, L., Ph.D., Thomas Jefferson University, Philadelphia, Pennsylvania
Swain, A., Ph.D., Tufts University, Boston, Massachusetts
Talarico, D., Ph.D., New York University, New York, New York
Tecott, L., Ph.D., University of California, San Francisco
Wilson-Gunn, S., Ph.D., University of Michigan, Ann Arbor
Zack, D., Ph.D., Johns Hopkins Medical School, Baltimore, Maryland

SEMINARS

Bradley, A., Baylor College of Medicine. Homologous recombination.
Bronner-Fraser, M., University of California, Irvine. Neural crest cell migration and differentiation.
Copeland, N., NCI-FCRDC, Frederick, Maryland. Insertional mutagenesis and oncogene activation by retroviruses.
Curran, T., Roche Institute. *fos* and *jun*: Inducible proto-oncogene transcription factors.
Hastie, N., Western General Hospital. Wilm's tumour—Anaeridia and mouse model systems.
Herr, W., Cold Spring Harbor Laboratory. *Pou* genes: Transcriptional regulation.
Hogan, B., Vanderbilt University Medical School. 1. Extraembryonic membranes. 2. TGF-β-like proteins.
Jessell, T., Columbia University. Vertebrate nervous system development.
Joyner, A., Mt. Sinai Hospital, Toronto. Role of *engrailed* genes in murine development.
Krumlauf, R., National Institute of Medical Research. *HOX* genes.
Lovell-Badge, R., National Institute for Medical Research. 1. Germ cells and sex determination I. 2. Germ cells and sex determination II.
McMahon, A., Roche Institute of Molecular Biology. *Wnt* genes.
Noden, D., Cornell University. Craniofacial development.
Papaioannou, G., Tufts University. Chimeras in development.
Parada, L., NCI-FCRDC, Frederick, Maryland. Cellular proto-oncogenes in development.
Rastan, S., Clinical Research Centre. The X-inactivation region.
Rinchik, E., Oak Ridge National Laboratory. Genetic resources.
Robertson, E., Columbia University. ES cells; biology and uses.
Rossant, J., Mt. Sinai Hospital, Toronto. Preimplantation development.
Solter, D., Max Planck Institute. Genomic imprinting.
Strickland, S., SUNY at Stony Brook. Maternal RNA and the mouse oocyte.
Tam, P., University of Sydney. 1. Postimplantation development: Analysis of cell lineage and tissue fate. 2. The allocation of cells to the paraxial mosoderm during somitogenesis
Wassarman, P., Roche Institute of Molecular Biology. Fertilization.
Wilkinson, D., National Institute for Medical Research. Segmentation in the hindbrain.
Williams, D., Children's Hospital, Boston. Hematopoiesis.

Molecular Approaches to Ion Channel Expression and Function

June 7–June 27

INSTRUCTORS

Goldin, Al, M.D., Ph.D., University of California, Irvine
Hausdorff, Sharon, Ph.D., Brandeis University, Waltham, Massachusetts
Snutch, Terry, Ph.D., University of British Columbia, Vancouver, Canada
Yang, Xian-Cheng, Ph.D., California Institute of Technology, Pasadena

ASSISTANTS

Dubel, Stefan, University of British Columbia, Vancouver, Canada
Gilbert, Mary, University of British Columbia, Vancouver, Canada

Application of the techniques of molecular biology to neurobiology has provided novel approaches and a new level of sophistication to the examination of many neurobiological problems. This intensive laboratory/lecture course was designed to introduce students to the application of model systems in which to express cloned neurotransmitter receptors and voltage-gated ion channels. The course covered the following topics: preparation of RNA transcripts in vitro and microinjection in *Xenopus* oocytes; characterization of exogenous receptors and ion channels in oocytes using two-microelectrode voltage clamping and patch clamping; transient expression of voltage-gated ion channels in mammalian cells using vaccinia virus vectors; transient expression of voltage-gated ion channels in insect cells using baculovirus vectors; characterization of exogenous ion channels in mammalian and insect cells using patch clamping; theory, and analysis of ionic currents.

PARTICIPANTS

Deitcher, D., B.S., Harvard Medical School, Boston, Massachusetts
Furman, I., M.A., Hebrew University, Jerusalem, Israel
Gelli, A., B.S., University of Toronto, Canada
Ketchum, K., Ph.D., Yale University, New Haven, Connecticut
Lin, D., M.D., Yale University, New Haven, Connecticut
Moll, C., M.D., Salk Institute, La Jolla, California
Moss, G., Ph.D., Yale Medical School, New Haven, Connecticut
Rogart, R., Ph.D., University of Chicago, Illinois
Valera, S., M.S., University of Geneva, Switzerland
Zhao, B., M.S., Columbia University, New York, New York

SEMINARS

Enyeart, J., Ohio State University. Calcium channels in secretory cells: Excitation transcription coupling.
Hsu, H., California Institute of Technology. Channels and receptors expressed in mammalian cells infected with recombinant vaccinia virus.
Joho, R., Baylor College of Medicine. Voltage-gated K channels: Expression cloning and structure-function analysis.
Leonard, J., University of Illinois, Chicago. Expression and modulation of excitatory amino acid receptors.
MacKinnon, R., Harvard Medical School. A look into the pore of K channels.
Mandel, G., SUNY at Stony Brook. Sodium channel expression in the nervous system.
Margiotta, J., Mt. Sinai School of Medicine. Regulation of AChR expression.
Ribera, A., University of Colorado. Developmental regulation of neuronal excitability.
White, M., University of Pennsylvania Medical School. Feeling your way around the AChR.

Molecular Neurobiology: Brain Development and Function

June 15–June 30

INSTRUCTORS

McKay, Ronald, Ph.D., Massachusetts Institute of Technology, Cambridge
Patrick, James, Ph.D., Baylor College of Medicine, Houston, Texas
Reichardt, Louis, Ph.D., University of California, San Francisco
Schwarz, Thomas, Ph.D., Stanford University, California

This lecture course presented both basic concepts and currently exciting research problems in molecular neurobiology. It focused on approaches and methods now used to study the development and function of the nervous system. Topics covered included gene expression, receptor structure and function, ion channel cloning, second messenger systems, learning, sensory transduction, behavioral genetics, neural induction, cell lineage, immortal cell lines, cell adhesion, oncogenes, and neurite outgrowth. The course provided the opportunity to discuss this rapidly expanding research area with invited lecturers. Individuals from a wide variety of backgrounds (graduate students to faculty) were encouraged to apply.

PARTICIPANTS

Bohner, A., B.S., Stanford University, California
Cagan, R., Ph.D., University of California, Los Angeles
Conneely, O., Ph.D., Baylor College of Medicine, Houston, Texas
Dahlstrand, J., B.S., Karolinska Institute, Stockholm, Sweden
Garner, A., B.A., Case Western Reserve University, Cleveland, Ohio
Gordon, P., Ph.D., Tufts University, Boston, Massachusetts
Harkness, P., B.A., Medical Research Centre, Cambridge, England

Hata, Y., M.D., University of Tokyo, Japan
Hernandez, M., M.S., University of Geneva, Switzerland
Huntsman, M., B.S., University of California, Irvine
Illing, N., Ph.D., University of Cape Town, South Africa
Kirchhof, B., B.A., Freie University, Berlin, Germany
Nye, S., Ph.D., Regeneron Pharmaceuticals, Tarrytown, New York
Okano, H., M.D., Rockefeller University, New York, New York
Rappaport, D., Ph.D., University of Sydney, Australia
Rejas, M., Ph.D., Instituto Cajal, Madrid, Spain
Ronnett, G., Ph.D., Johns Hopkins University, Baltimore, Marland
Seitanidou, A., B.A., Pasteur Institute, Paris, France
Skoglund, P., B.A., University of California, San Diego
Suri, C., B.S., Tufts University, Boston, Massachusetts
Tang, C., Ph.D., University of Pennsylvania, Philadelphia
Ultsch, A., M.S., University of Heidelberg, Germany

SEMINARS

Bastiani, M., University of Utah. Development of the nervous system.
Beach, D., Cold Spring Harbor Laboratory. Control of cell growth.
Chalfie, M., Columbia University. *C. elegans* neurobiology.
Curran, T., Roche Institute. Immediate early gene expression.
Greenberg, M., Harvard Medical School. c-*fos* regulation in neurons.
Hall, J., Brandeis University. *Drosophila* behavior.
Hayes, T., Massachusetts Institute of Technology. Regulation of cell growth.
Hiromi, Y., Princeton University. Neuronal determination in *Drosophila*.
Howard, J., University of Washington. Biophysics of hair cells.
Jessell, T., Columbia University. Neuronal commitment in chick.
Katz, L., Duke University. Cortical development.
Kobilka, B., Stanford University. Serpentine receptors.
Kuwada, J., University of Michigan. Axon guidance in zebrafish.
Lillien, L., Harvard Medical School. Cell differentiation and lineage.
Logothetis, N., Baylor College of Medicine. Primate visual system.
Madison, D., Stanford University. Long-term potentiation.
McKay, R., Massachusetts Institute of Technology. Mammalian neuronal differentiation.
Myers, R., University of California, San Francisco. Genetics of Huntington's disease.
Patrick, J., Baylor College of Medicine. Ligand gated ion channels.
Reichardt, L., University of California, San Francisco. Chemistry of axon growth.
Schwarz, T., Stanford University. Voltage gated ion channels.
Siegelbaum, S., Columbia University. Ion channel modification.
Stryker, M., University of California, San Francisco. Visual cortex development and plasticity.
Sweat, J., Baylor College of Medicine. Second messager systems and LTP.
Tank, D., Bell Laboratories. Neurotransmitter release/imaging.
Ts'o, D., Rockefeller University. Mammalian visual cortex.
Weinmaster, G., Salk Institute. Schwann cell differentiation.
Yancopoulous, G., Regeneron Pharmaceuticals. Nerve growth factors.

Neurobiology of *Drosophila*

July 1–July 21

INSTRUCTORS

Hartenstein, Volker, Ph.D., University of California, Los Angeles
Ready, Don, Ph.D., Purdue University, West Lafayette, Indiana

ASSISTANTS

Green, Patricia, University of California, Los Angeles
Wolff, Tanya, Purdue University, West Lafayette, Indiana

This laboratory/lecture course was a series of in-depth discussions with researchers active in genetic, physiological, cellular, molecular, and behavioral studies of *Drosophila* neurobiology. It was organized around a core of seminars, extensive informal discussion, and lab work. Lecturers often brought original preparations for viewing and discussion and directed lab exercises and experiments in their areas of special interest. The course was intended for researchers at all levels who want to use *Drosophila* as an experimental system for studying neurobiology. The course covered basic electrophysiological techniques, as applied to mutants with altered ion channels or sensory physiology. In addition, the course familiarized students with various preparations, including the embryonic nervous system, imaginal discs, and adult nervous system.

Topics included *Shaker* and the biophysics and diversity of potassium channels, the genetics and molecular biology of excitability, the control of neurogenesis and neuronal diversity, embryonic and postembryonic development of the CNS and PNS, axonal pathfinding, development of the nervous system, mesoderm and muscle development, eye and optic lobe development, olfaction, learning, and the neural control of flight.

PARTICIPANTS

Chang, H., M.D., University of California, Los Angeles
Cobb, M., Ph.D., CNRS, Gif-sur-Yvette, France
DiAntonio, A., M.S., Stanford University, California
Fernandes, J., M.S., Tata Institute, Bombay, India
Glaesener-Cipollone, G., Ph.D., University of Wurzburg, Germany
McNabb, S., Ph.D., University of Cambridge, England
Miller, C., M.D., University of Southern California, Los Angeles
Prokop, A., M.S., University of Mainz, Germany
Schmucker, D., M.S., Institute of Genetics, Munich, Germany
Vosshall, L., B.A., Rockefeller University, New York, New York
Wang, E., B.S., Purdue University, West Lafayette, Indiana
Yang, X., Ph.D., National University of Singapore

SEMINARS

Bate, M., University of Cambridge. Muscle patterning in the fly.
Bieber, A., Purdue University. Axonal guidance in *Drosophila*.
Campos-Ortega, J., University of Koln. Neurogenesis.
Doe, C., University of Illinois. CNS II: Neuronal fate.
Ganetzky, B., University of Wisconsin. Genetics of membrane excitability.
Hardie, R., University of Cambridge. Channels and signal transduction in the fly visual system.
Hartenstein, V., University of California. Introduction to development.
Martinez-Arias, A., University of Cambridge. Pattern formation in the *Drosophila* embryo.
Ready, D., Purdue University. Compound eye morphogenesis.
Schwarz, T., Stanford University. Ion channels and *Shaker*
Strausfeld, N., University of Arizona. Neuroanatomy of the adult brain.
Taghert, P., Washington University. Stomatogastric nervous system and peptide transmitters.
Truman, J., University of Washington. Development of adult CNS.
Tully, T., Brandeis University. Learning/Behavior.
Wu, C.-F., University of Iowa. Neuronal plasticity and excitability in *Drosophila*.
Young, M., Rockefeller University. *per* and circadian rhythms.
Zuker, C., University of California, San Diego. Molecular biology of signal transduction.

Molecular Cloning of Neural Genes

July 1–July 21

INSTRUCTORS

Boulter, James, Ph.D., Salk Institute, San Diego, California
Eberwine, James, Ph.D., University of Pennsylvania, Philadelphia
Evinger, Marian, Ph.D., SUNY at Stony Brook, New York

CO-INSTRUCTOR

Inman, Irene, Stanford University, California

ASSISTANTS

Cao, Yan, University of Pennsylvania, Philadelphia
Lai, Cary, Salk Institute, San Diego, California

This intensive laboratory/lecture course was intended to provide scientists with a basic knowledge of the use of the techniques of molecular biology in examining questions of neurobiological significance. Particular emphasis was placed on using and discussing methods for circumventing the special problems posed in the study of the nervous system; for example, examination of low abundance mRNAs in extremely heterogeneous cell populations. The laboratory work included mRNA quantitation methods (nuclease protection, etc.), preparation of hybridization probes, library construction (λ ZAP and IST procedure), plaque screening techniques (probe hybridization, antibody interaction), DNA sequencing, PCR amplification, RNA amplification, and DNA-mediated gene transfer. A major portion of the course was devoted to in situ hybridization and in situ transcription technologies. The lecture series, presented by invited speakers, focused on emerging techniques and how they may be applied to the study of the nervous system.

PARTICIPANTS

Brown, J., B.A., University of California, San Francisco
Buxbaum, J., Ph.D., Rockefeller University, New York, New York
Dawson, T., Ph.D., Johns Hopkins University, Baltimore, Maryland
Fishman, R., Ph.D., Columbia University College of Physicians & Surgeons, New York, New York
Gershon, M., M.D., Columbia University College of Physicians & Surgeons, New York, New York
Gray, G., B.A., Washington University Medical School, St. Louis, Missouri
Herman, J.-P., Ph.D., Washington University Medical School, St. Louis, Missouri
Jacobson, L., Ph.D., Stanford University, California
McKeon, R., Ph.D., Case Western Reserve University, Cleveland, Ohio
Navaratnam, D., Ph.D., University Department of Pharmacology, Oxford, England
Nitabach, M., B.A., Columbia University, New York, New York
Sahin, M., B.S., Yale University, New Haven, Connecticut
Snyder, D., Ph.D., Duke University Medical Center, Durham, North Carolina
Soha, J., Ph.D., E.K. Shriver Center, Waltham, Massachusetts
Sullivan, J., Ph.D., Salk Institute, San Diego, California
Yucel, Y., Ph.D., University of British Columbia, Vancouver

SEMINARS

Brugge, J., University of Pennsylvania. Signal transduction through *src*-related tyrosine protein kinases in neural cells.

Cepko, C., Harvard Medical School. Lineage analysis of the vertebrate CNS.

Chao, M., Cornell University Medical Center. Mechanism of action of neurotrophic factors.

Curran, T., Roche Institute of Molecular Biology. *fos* and *jun*: Inducible proto-oncogene transcription factors.

Lemke, G., Salk Institute. Transcriptional control in glial differentiation and myelination.

Merlie, J., Washington University Medical School. Regulation of synapse formation and acetylcholine receptor gene expression in muscle.

Nef, P., Salk Institute. A molecular biological approach to understanding olfactory functions.

Quinn, C., Massachusetts Institute of Technology. Genes related to learning and memory in *Drosophila*.

Reichardt, L., University of California, San Francisco. Growth factor regulation of CNS gene expression.

Stevens, C., Salk Institute. Site-directed mutagenesis and the molecular basis of gating in nicotinic acetylcholine receptors.

Molecular and Developmental Biology of Plants

July 1–July 21

INSTRUCTORS

Klessig, Daniel, Ph.D., Rutgers University, Piscataway, New Jersey
Maliga, Pal, Ph.D., Rutgers University, Piscataway, New Jersey
Varner, Joseph, Ph.D., Washington University, St. Louis, Missouri

ASSISTANTS

Cutt, John, Rutgers University, Piscataway, New Jersey
Dixon, Dave, Rutgers University, Piscataway, New Jersey
Gibson, Susan, Michigan State University, East Lansing

Hajdukiewicz, Peter, Rutgers University, Piscataway, New Jersey
Kanevski, Ivan, Rutgers University, Piscataway, New Jersey
Staub, Jeffrey, Rutgers University, Piscataway, New Jersey
Svab, Zora, Rutgers University, Piscataway, New Jersey
Ye, Z.-H., Washington University, St. Louis, Missouri

This course provided an intensive overview of current topics and techniques in plant biology, with an emphasis on molecular and developmental biology and genetics. It was designed for scientists with experience in molecular techniques who are working, or wish to work, with plant systems. The course consisted of a rigorous lecture series, a hands-on laboratory, and informal discussions. Guest speakers provided both an in-depth discussion of their work and an overview of their specialty. The laboratory sessions covered established and novel techniques in plant biology including transformation via *Agrobacterium*, electroporation, and the particle gun, assays for transient gene expression, microinjection, tissue printing, and in situ detection of RNA and protein, reconstitution of protein transport, in vivo and in vitro analysis of transcription factors, genome mapping using yeast artificial chromosomes, and cytogenetics.

PARTICIPANTS

Allison, L., Ph.D., CNRS, Gif-sur-Yvette, France
Atkinson, R., M.S. University of Auckland, New Zealand
Desiderio, A., B.S., Universita Degli Studi di Roma, Italy
Douma, A., Ph.D., State University of Leiden, The Netherlands
Haub, O., B.S., Columbia University College of Physicians & Surgeons, New York, New York
Hayes, R., Ph.D., Imperial College of Science, Technology & Medicine, London, England
Hershkovitz, M., Ph.D., University of California, Davis
Jacobs, J., M.S., University of Nijmegen, The Netherlands
Ohm, H., Ph.D., Purdue University, West Lafayette, Indiana
Orosz, L., Ph.D., Institute of Molecular Genetics, Godollo, Hungary
Perez Flores, L., Ph.D., Universidad Autonoma, Mexico City, Mexico
Reddy, A., Ph.D., Washington State University, Pullman
Reuveni, M., Ph.D., Purdue University, West Lafayette, Indiana
Savka, M., M.S., University of Illinois, Urbana
Taschner, P., Ph.D., Leiden University, The Netherlands
Tavladoraki, P., Ph.D., E.N.E.A., Rome, Italy

SEMINARS

Beachy, R., Scripps Research Institute. Molecular biology of plant RNA viruses.
Cashmore, A., University of Pennsylvania. Control of tissue-specific gene expression in plants.
Clarke, A., University of Melbourne. Self-incompatibility in flowering plants.
Federoff, N., Carnegie Institution of Washington. Maize transposable elements.
Goodman, H., Massachusetts General Hospital. The *Arabidopsis* genome.
Gruissem, W., University of California, Berkeley. Function of RNA-binding proteins in regulation of mRNA stability.
Hanson, M., Cornell University, Ithaca. Molecular biology of plant mitochondria: Cytoplasmic male sterility.
Jones, A., University of North Carolina. Signal transduction in plants.
Keegstra, K., University of Wisconsin. Protein targeting in plants.
Klessig, D., Rutgers University. Pathogenesis-related proteins in plants.
Kondorosi, A., CNRS, Gif-sur-Yvette. Rhizobium-plant symbiotic interactions.
Lucas, W., University of California, Davis. Application of microinjection to the study of plasmodesmatal transport.
Maliga, P., Rutgers University. Transgenic plants.
Meyerowitz, E., California Institute of Technology. Genes regulating flower development in *Arabidopsis*.
Mullet, J., Texas A & M University. Chloroplast development and gene expression.
Nester, E., University of Washington, Seattle. *Agrobacterium*-mediated transformation of higher plants.
Ow, D., Plant Gene Expression Center. Application of a prokaryotic site-specific recombination system in plants.
Phillips, R., University of Minnesota. Genomic rearrangements induced by tissue culture.
Poethig, S., University of Pennsylvania. Developmental patterns in plants.
Rochaix, J.-D., University of Geneva. Molecular biology of chloroplasts.
Somerville, C., Michigan State University. Biochemical genetics in *Arabidopsis*.
Varner, J., Washington University. The plant cell wall.
Vierstra, R., University of Wisconsin. Control of phytochrome turnover.

Molecular Genetic Analysis of Human Neurological Disease

July 2–July 9

INSTRUCTORS

Breakefield, Xandra, Ph.D., Massachusetts General Hospital, Charlestown and Harvard Medical School, Boston

Evans, Glen, Ph.D., Salk Institute, San Diego, California

Gusella, James, Ph.D., Massachusetts General Hospital, Charlestown and Harvard Medical School, Boston

This intensive lecture/discussion course was designed to provide a comprehensive overview of strategic approaches and state-of-the-art technology for the analysis of diseases of the nervous system. The course focused on the analysis and understanding of human disease at the molecular level emphasizing approaches to gene localization and cloning, as well as gene expression, molecular physiology, and the creation and use of animal models. Topics included genetic linkage and transmission genetics; the identification and isolation of disease genes; the molecular biology and physiology of genes expressed in the brain; multigene families encoding receptors and ion channel and their role in diseases; animal models for neurological diseases using transgenics and stem cell chimeras manipulated using homologous recombination paradigms for neurological disorders such as Huntington's disease, Duchenne's muscular dystrophy and Fragile X syndrome; gene transfer and expression in the nervous system; neural expression of proto-oncogenes and neurotropic viruses;

mitochondrial genetics and inherited epilepsies; genes active in neural regeneration and programmed cell death. This course was intended for students with backgrounds in neurology, molecular biology, or medical genetics and those investigators interested in the molecular dissection of neurological disorders.

PARTICIPANTS

Andersen, J., Ph.D., Massachusetts General Hospital, Charlestown
Andreason, G., Ph.D., Salk Institute, San Diego, California
Astarloa, R., Ph.D., Fundacion Jimenez Diaz, Madrid, Spain
Brown, A., M.D., Vanderbilt University, Nashville, Tennessee
Chernak, J., Ph.D., National Institute on Aging, Baltimore, Maryland
Duyao, M., B.A., Boston University School of Medicine, Massachusetts
Gravel, C., Ph.D., Clinical Research Institute, Montreal, Canada
Hashimoto, L., B.S., University of Western Ontario, London, Canada
Henske, E., M.D., Massachusetts General Hospital, Boston
Konig, G., M.S., University of Heidelberg, Germany
Kristensen, P., M.D., Novo Nordisk, Bagsvard, Denmark
Lathe, R., M.S., University of Edinburgh, Scotland
Lee, S., Ph.D., New York Blood Center, New York, New York
Lin, C., Ph.D., Massachusetts General Hospital, Charlestown
Maurer, S., Ph.D., Salk Institute, La Jolla, California
Mirow, A., M.D., University of California, San Diego
Reyes Lezama, M., M.S., Instituto Mexicano de Psiquiatria, Xochimilco
Riba-Ramirez, L., M.S., Massachusetts Institute of Technology, Cambridge
St. Clair, D., Ph.D., Western General Hospital, Edinburgh, Scotland
Taylor, S., Ph.D., Massachusetts General Hospital, Charlestown
Yao, K., Ph.D., Brandeis University, Waltham, Massachusetts

SEMINARS

Breakefield, X., Massachusetts General Hospital. Gene transfer using viral vectors.
Cannon, S., Massachusetts General Hospital. Na channel in hyperkalemic periodic paralysis.
Cepko, C., Harvard Medical School. Cell lineage in the developing nervous system.
Civelli, O., Vollum Institute. Dopaminergic receptors.
Curran, T., Roche Institute of Molecular Biology. Role of oncogenic transcription factors, *fos* and *jun*, in stimulus-transcription coupling in the brain.
Evans, G., Salk Institute. 1. Strategies for scanning the human genome. 2. DNA repair syndromes.
Gravel, R., Montreal Children's Hospital. Mutational analysis of Tay-Sachs.
Green, E., Washington University School of Medicine. YACs as cloning vehicles.
Gusella, J., Massachusetts General Hospital. Linkage analysis in Huntington's disease.
Heinemann, S., Salk Institute. Receptors: The glutamate receptor family and structure and function.
Hoffman, E., University of Pittsburgh. Dystrophin in muscular dystrophy.
Korsmeyer, S., Washington University School of Medicine. Sequences involved in translocations.
Lemke, G., Salk Institute. Myelination mutations and myelin mutants.
Mansour, S., University of Utah. Creating mouse mutants: Homologous recombination.
McKay, R., Massachusetts Institute of Technology. Gene delivery to the nervous system: Grafting genetically modified cells.
Moyzis, R., Los Alamos National Laboratory. Repeat elements.
O'Connell, P., University of Utah. Neurofibromatosis type 1.
Popko, B., University of North Carolina. Creating mouse mutants: Transgenics.
Prusiner, S., University of California, San Francisco. Neurodegenerative diseases: Prions.
Rosenfeld, M., University of California, San Diego. Homeobox genes in development.
Sapienza, C., Ludwig Institute for Cancer Research. Imprinting.
Sutcliffe, G., Research Institute of Scripps Clinic. Identifying mouse disease genes.
Wallace, D., Emory University. Mitochondrial defects.
Willard, H., Stanford University School of Medicine. X-inactivation.

The Cell and Molecular Biology of Learning and Memory

July 14–July 28

INSTRUCTORS

Byrne, Jack, Ph.D., University of Texas Medical School, Houston
Kandel, Eric, Ph.D., Columbia University College of Physicians & Surgeons, New York, New York
Pearson, Keir, Ph.D., University of Alberta, Canada
Squire, Larry, Ph.D., University of California, San Diego

This lecture course provided an introduction to cell and molecular biological approaches to learning and memory. It was suited for graduate students in molecular biology, neurobiology, and psychology as well as research workers who are interested in an introduction to this new field. The course covered topics ranging from behavioral considerations of learning and memory to gene regulation in the nervous system. The lectures provided an intensive coverage of six selected areas: (1) an introduction to modern behavioral studies of learning and memory; (2) an overview of the cell biology of neuronal plasticity and second messenger systems; (3) the regulation of gene expression; (4) cellular and molecular mechanisms of simple forms of learning and memory in invertebrates and vertebrates; (5) cellular and molecular mechanisms of long-term potentiation in various regions of the mammalian brain; and (6) neural approaches to human learning and its abnormalities.

PARTICIPANTS

Alvarez-Royo, P., M.S., University of California, San Diego
Bringuier, V., M.S., Universite Paris-Sud, France
Brulet, P., Ph.D., Unite de Genetique Cellulaire, Paris, France
Buonomano, D., B.S., University of Texas, Houston
Cai, H., M.S., University of California, Los Angeles
Castro-Alamancos, M., M.S., Instituto Cajal, Madrid, Spain
Davidson, J., B.S., Queen's University, Belfast, Ireland
Durand, G., M.S., Albert Einstein College of Medicine, New York, New York
Galliot, B., Ph.D., Center for Molecular Biology, Heidelberg, Germany
Greenspan, R., Ph.D., Roche Institute of Molecular Biology, Nutley, New Jersey
Li, X.-C. Ph.D., Columbia University, New York, New York
Nick, T., B.S., Yale University, New Haven, Connecticut
Paulsen, O., M.D., University of Oslo, Norway
Pieroni, J., Ph.D., University of Texas, Houston
Roder, J., Ph.D., Mt. Sinai Hospital, Toronto, Canada
Saron, C., B.A., Albert Einstein College of Medicine, New York, New York
Smith, B., Ph.D., Ohio State University, Columbus
Torroja, L., B.S., Instituto Cajal, Madrid, Spain
Valenzuelo, D., Ph.D., Massachusetts General Hospital, Charlestown
Wang, X., B.S., University of Illinois, Urbana
Wiel, D., B.A., University of Oregon, Eugene
Zimmerly, S., B.A., Yale University, New Haven, Connecticut

SEMINARS

Byrne, J., University of Texas, Houston. 1. Overview of membranes and synaptic transmission. 2. Learning in *Aplysia III*. Classical conditioning.
Clark, G., Princeton University. Cellular mechanisms of associative learning in *Hermissenda*.
Dash, P., University of Texas, Houston. Cloning of genes important to learning II.
Davis, R., Baylor College of Medicine. Genetic approaches to study associative learning in *Drosophila*.
Eichenbaum, H., University of North Carolina. Role of the hippocampus and hippocampal long-term potentiation in learning.
Ganetzky, B., University of Wisconsin. Introduction to the study of genes and behavior.
Gould, J., Princeton University. Ethological approaches to learning.
Greenough, W., University of Illinois. Morphological correlates of learning and experience.
Holland, P., Duke University. Introduction to learning theory.
Kandel, E., Columbia University. 1. Introduction to the cellular study of learning. 2. Learning in *Aplysia I*. Habituation. 3. Learning in *Aplysia II*. Sensitization.
Kenyon, C., University of California, San Francisco. Genes and the behavior of *C. elegans*.
Lisberger, S., University of California, San Francisco. Plasticity in the vestibulo-occular reflex.
Malinow, R., University of Iowa. Long-term potentiation II.
Merzenich, M., University of California, San Francisco. Cortical plasticity and learning.
Nicoll, R., University of California, San Francisco. Long-term potentiation I.
Nottebohm, F., Rockefeller University. Bird-song learning.
Pfaffinger, P., Baylor College of Medicine. Cloning of genes important to learning I.
Posner, M., University of Oregon. Neuropsychology of cognition.
Schulman, H., Stanford University. Overview of second messenger systems and their role in learning and memory.
Squire, L., University of California, San Diego. 1. Memory in nonhuman primates. 2. Human memory and disorders of memory.
Stock, A., Center for Advanced Biotechnology and Medicine. Genes and the behavior of bacteria.
Thompson, R., University of Southern California. Classical conditioning of the nictitating membrane response.

Advanced Molecular Cloning and Expression of Eukaryotic Genes

July 23–August 12

INSTRUCTORS

Kingston, Robert, Ph.D., Harvard Medical School, Boston, Massachusetts
Learned, Marc, Ph.D., University of California, Davis
Myers, Richard, Ph.D., University of California, San Francisco
Robbins, Alan, Ph.D., DuPont Merck Pharmaceutical Co., Wilmington, Delaware

ASSISTANTS

Mak, Arkady, University of California, San Francisco
Stuve, Laura, University of California, San Francisco

This course focused on how to manipulate cloned eukaryotic genes to probe questions on their structure, expression, and function. As a model system, they examined *cis*- and *trans*-acting components involved in the regulation of eukaryotic gene expression. Students learned the theoretical and practical aspects of constructing genomic and cDNA libraries. Expression libraries from various organisms were screened with recognition site probes for specific DNA-binding proteins. A variety of transfection techniques were used to introduce cloned DNA molecules that had been manipulated in vitro into eukaryotic cells in culture. Mutants were generated by oligo-directed and random mutagenesis procedures and characterized by DNA sequencing. The expression pattern of wild-type and mutant DNAs were analyzed by S1 nuclease, RNase protection, and enzymatic assays. Techniques and theory for detecting and characterizing the interaction between regulatory DNA sequences and *trans*-acting protein factors were presented. Guest lecturers discussed present problems in eukaryotic molecular biology as well as technical approaches to their solutions. Experience with basic recombinant DNA techniques was a prerequisite for admission to the course.

PARTICIPANTS

Adams, J., Ph.D., Brigham & Women's Hospital, Boston, Massachusetts

Aird, W., M.D., Brigham & Women's Hospital, Boston, Massachusetts

Boyd, J., Ph.D., University of California, Davis

Briegel, K., B.S., Institute of Molecular Pathology, Vienna, Austria

Chung, C., Ph.D., Seoul National University, Korea

Datson, N., M.S., Leiden University, The Netherlands

Fernandez, A., B.S., M.D. Anderson Cancer Center, Hous-

ton, Texas
Harley, V., Ph.D., Imperial Cancer Research Fund, London, England
Ho, M., B.S., University of Oxford, England
Houglum, K., M.D., University of California, San Diego
Johnson, R., Ph.D., Hospital for Sick Children, Toronto, Canada
Miller-Davis, S., B.S., Vanderbilt University, Nashville, Tennessee
Perilleux, C., B.S., University of Liege, Belgium
Redhead, C., Ph.D., Columbia University College of Physicians & Surgeons, New York, New York
Rooke, K., B.S., St. Mary's Hospital, London, England
Trejo, J., B.S., University of California, San Diego

SEMINARS

Chandler, V., University of Oregon, Eugene. The B locus of maize: A regulator of specific gene expression and its regulation by paramutation.
Hernandez, N., Cold Spring Harbor Laboratory. Initiation and termination of transcription in RNA polymerase II and III snRNA genes.
Johnson, A., University of California, San Francisco. Yeast homeodomain proteins.
Kingston, R., Harvard Medical School. HSF, Ga1-4, TFIID, and nucleosomes.
McKnight, S., Carnegie Institution of Washington. On the importance of heteromeric interactions in eukaryotic transcription factors.
Myers, R., University of California, San Francisco. Molecular studies of Huntington's disease.
Rio, D., Whitehead Institute. Mechanism of regulation of P-element transposition in *Drosophila*.
Sharp, P., Massachusetts Institute of Technology. Regulation of transcription and splicing.
Treisman, R., Imperial Cancer Research Fund, London. SRF: Relatives and friends.
Winston, F., Harvard Medical School. Analysis of TFIID, histones, and other transcription factors of yeast.
Yamamoto, K., University of California, San Francisco. Factor interactions at a composite GRE.

Yeast Genetics

July 23–August 12

INSTRUCTORS

Michaelis, Susan, Ph.D., Johns Hopkins University, Baltimore, Maryland
Mitchell, Aaron, Ph.D., Columbia University, New York, New York
Rose, Mark, Ph.D., Princeton University, New Jersey

ASSISTANTS

Berkower, Carol, Johns Hopkins University, Baltimore, Maryland
Bowdish, Kathy, Columbia University, New York, New York
Stasenko, Laurie Jo, Princeton University, New Jersey

The major laboratory techniques used in the genetic analysis of yeast were studied, including the isolation and characterization of mutants, tetrad analysis, complementation, and mitotic recombination. Micromanipulation used in tetrad analysis was carried out by all students. Molecular genetic techniques, including yeast transformation, gene replacement, analysis of gene fusions, and electrophoretic separation of chromosomes, were applied to the analysis of yeast DNA. Indirect immunofluorescence experiments were done to identify the nucleus, microtubules, and other cellular components. Lectures on fundamental aspects of yeast genetics were presented along with seminars given by outside speakers on topics of current interest.

PARTICIPANTS

Akiyama, M., Ph.D., Stanford University, California
Breiding, D., Ph.D., New England Medical Center, Boston, Massachusetts
Casadevall, A., Ph.D., Albert Einstein College of Medicine, New York
De Repentigny, L., M.D., University of Montreal, Canada
Guan, K., Ph.D., Purdue University, West Lafayette, Indiana
Kang, H., M.S., University of California, Davis
Kohno, K., Ph.D., Osaka University, Japan
Lesage, P., Ph.D., Institut de Biologie, Paris, France
Lim, A., M.S., Northeastern University, Boston, Massachusetts
McAlear, M., B.S., McGill Cancer Centre, Montreal, Canada
Pande, S., Ph.D., University of Maryland, Baltimore County
Romisch, K., Ph.D., EMBL, Heidelberg, Germany
Schiebel, E., Ph.D., University of California, Los Angeles
Silos, S., B.S., Thomas Jefferson University, Philadelphia, Pennsylvania
Silverman, L., Ph.D., Memorial Sloan-Kettering Center, New York, New York
Zheng, P., M.D., Yale University School of Medicine, New Haven, Connecticut

SEMINARS

Adams, A., University of Arizona. Morphogenesis and the actin cytoskeleton.
Boeke, J., Johns Hopkins University. Transposition in yeast.
Broach, J., Princeton University. Growth control by the yeast adenylate cyclase cascade.
Carlson, M., Columbia University. Transcriptional control of glucose repressible genes.
Fink, G., Whitehead Institute. Diverse topics of broad interest.
Futcher, B., Cold Spring Harbor Laboratory. Coordination of yeast cell growth and division.
Guthrie, C., University of California, San Francisco. How snRNPs snip.
Hieter, P., Johns Hopkins University. Genetics of chromosome segregation in yeast.
Hinnebusch, A., National Institutes of Health. Regulation of initiation factors in *GCN4* transcriptional control.
Hoyt, A., Johns Hopkins University. Mechanisms and regulation of mitosis in *S. cerevisiae*.
Johnson, A., University of California, San Francisco. Regulation of cell-type-specific gene expression in yeast.
Michaelis, S., Johns Hopkins University. Modification and export of the yeast mating pheromone, α factor.
Mitchell, A., Columbia University. Control of entry into meiosis in yeast.
Petes, T., University of North Carolina. Homologous and nonhomologous recombination in yeast.
Rose, M., Princeton University. Microtubule motors and the spindle pole body in yeast mating and mitosis.
Schekman, R., University of California, Berkeley. Protein secretion in yeast.
Winston, F., Harvard Medical School. Analysis of TFIID, histones, and other transcription factors in yeast.

Imaging Structure and Function in the Nervous System

July 23–August 12

INSTRUCTORS

Katz, Larry, Ph.D., Duke University, Durham, North Carolina
Lewis, Richard, Ph.D., Stanford University, California

ASSISTANTS

Callaway, Edward, Duke University, Durham, North Carolina
Dolmetsch, Ricardo, Stanford University, California
Peinado, Alex, Duke University, Durham, North Carolina
Yuste, Rafael, Duke University, Durham, North Carolina

Recent advances in optical and video microscopy, coupled with the development of powerful new fluorescent probes, present unique opportunities for visualizing the structure and function of individual neurons and neuronal assem-

blies. This intesive laboratory/lecture course provided participants with the theoretical and practical tools to utilize these emerging technologies. Course topics included principles of fluorescence and video microscopy; image processing techniques; theory and practice of confocal microscopy; and the use of fluorescent tracers to visualize neuronal subpopulations in slices and in dissociated cell culture. Particular emphasis was placed on the use of calcium-sensitive probes (e.g., fura-2, fluo-3) to monitor cell function in slices and in single cells. Using state-of-the-art equipment, students explored a variety of preparations, including mammalian brain slices, acutely dissociated neurons, and cultured cells.

PARTICIPANTS

Carrive, P., Ph.D., University of Sydney, Australia
Clark, B., B.S., University College London, England
Finch, E., B.A., Cambridge Neuroscience Research Inc., Massachusetts
Henzi, V., B.S., Columbia University, New York, New York
Kania, D., B.S., SUNY at Buffalo
Lim, S., Ph.D., Indiana University Medical Center, Indianapolis
McPherson, D., Ph.D., Marquette University, Milwaukee, Wisconsin
Mooney, R., Ph.D., Stanford University, California
O'Brien, E., M.A., Cornell University Medical College, New York, New York
Reid, C., Ph.D., Rockefeller University, New York, New York
Solomon, J., B.A., Washington University Medical School, St. Louis, Missouri
Wong, R., Ph.D., Stanford University, California

SEMINARS

Augustine, G., Duke University Medical Center. Simultaneous optical recording and voltage clamp measurements.
Bonhoeffer, T., Max Planck Institute, Frankfurt. Roller cultures and optical imaging.
Bookman, R., University of Miami. Excitation-secretion coupling.
Connor, J., Roche Institute of Molecular Biology. Fast calcium imaging and the use of CCD cameras for imaging fura-2.
Fraser, S., California Institute of Technology. Frontiers in imaging: MRI microscopy.
Grinvald, A., Weizmann Institute of Science. Designing optical probes and visualizing intrinsic signals.
Llinas, R., New York University Medical Center. Dendritic dynamics of calcium in mammalian central neurons.
O'Rourke, N., Stanford University. In vivo confocal microscopy.
Purves, D., Duke University Medical Center. Repetitive imaging of neurons in living animals.
Smith, S., Stanford University. Principles of scanning laser confocal microscopy.
Steinbach, P., ETM Systems. Principles of image processors.
Tank, D., Bell Laboratories. Combining imaging with physiology: Modeling Ca dynamics.
Tsien, R., University of California, San Diego. New fluorescent indicators for cell function.

Genetic Approaches to Human Disease Using DNA Markers

August 5–August 12

INSTRUCTORS

Lander, Eric, Ph.D., Whitehead Institute & Massachusetts Institute of Technology, Cambridge
Page, David, M.D., Whitehead Institute & Massachusetts Institute of Technology, Cambridge

This intensive lecture course explored the ways to map the genes underlying human diseases and traits of unknown molecular basis by studying individuals from families and populations with DNA polymorphisms. The goal was to teach the full

range of strategies for mapping genetic diseases and traits by discussing the methods and illustrating their practical application. Specifically, the course discussed important methods in linkage analysis, segregation analysis, cytogenetics, population genetics, epidemiology, and molecular biological techniques for exploring large genomes. Their application was illustrated by a range of examples, spanning the range from simple to complex genetic problems. Among the topics discussed were Alzheimer's disease, Fragile-X syndrome, human cancer, diabetes, hypertension, genetic imprinting, Down's syndrome, rare recessive diseases, and quantitative traits. The course was intended for students with backgrounds in either molecular biology, molecular genetics, or medical genetics, who are interested in undertaking research on the genetic basis of particular diseases, traits, or physiological systems.

PARTICIPANTS

Ebers, G., M.D., University Hospital, London, Canada
Glenn, C., B.S., University of Alabama, Birmingham
Guillem, J., M.D., Lahey Clinic Medical Center, Burlington, Massachusetts
Heinrich, G., Ph.D., Massachusetts Institute of Technology, Cambridge
Kwok, P., Ph.D., Washington University Medical Center, St. Louis, Missouri
O'Flaherty, E., B.S., Alfred Hospital, Victoria, Australia
Peltomaki, P., M.D., University of Helsinki, Finland
Riba-Ramirez, L., M.S., Massachusetts Institute of Technology, Cambridge
Rosenberg, M., Ph.D., USSR Academy of Science, Moscow, Russia
Seiberg, M., Ph.D., Bristol-Myers Squibb Institute, Princeton, New Jersey
Van Dyke, D., M.D., University of Iowa, Iowa City
Vulpe, C., B.S., University of California, San Francisco
Wang, C., Ph.D., Columbia Presbyterian Hospital, New York, New York
Yager, T., Ph.D., California Institute of Technology, Pasadena

SEMINARS

Chakravarti, A., University of Pittsburgh. Down's syndrome and Charcot-Marie-tooth disease.
Fearon, E., Johns Hopkins University School of Medicine. 1. Tumor suppressor genes. 2. Genetic alterations underlying colorectal tumorigenesis.
Gilliam, C., New York State Psychiatric Institute. Spinal muscular atrophy.
Green, E., Washington University Medical School. YAC cloning.
Hobbs, H., University of Texas Southwest Medical School. Dissecting heart disease.
Nicholls, R., University of Florida. Genomic imprinting.
Puck, J., University of Pennsylvania School of Medicine. X-linked immunodeficiency.
Tanzi, R., Massachusetts General Hospital. Mapping Alzheimer's disease.
Warren, S., Emory University. Molecular genetics of Fragile-X syndrome.

Analysis and Genetic Manipulation of Yeast Artificial Chromosomes

October 8–October 21

INSTRUCTORS

Carle, Georges, Ph.D., Universite de Nice, France
Green, Eric, M.D., Ph.D., Washington University, St. Louis, Missouri
Rothstein, Rodney, Ph.D., Columbia University, New York, New York

ASSISTANTS

Schmidt, Laurence, Universite de Nice, France
Sunjevaric, Ivana, Columbia University, New York, New York
Tidwell, Rose, Washington University, St. Louis, Missouri

Cloning in yeast artificial chromosomes (YACs) is rapidly being applied to a wide variety of molecular genetic problems. This course provided basic scientific expertise in current techniques for the analysis and manipulation of YACs. In general, a blend of theoretical and practical information was provided, with the goal to establish a strong foundation for applying YAC cloning to a diversity of scientific problems. Topics included standard yeast genetic techniques (such as the propagation and storage of cells, tetrad dissection, colony hybridization, and DNA transformation), YAC library screening using both PCR- and genetic-based strategies, characterizing YAC inserts by pulsed-field gel electrophoresis and PCR, manipulating YAC clones by recombination-mediated disruption, targeted integration, and YAC-YAC recombination, and techniques for introducing YACs into mammalian cells. Participants learned through hands-on experience, informal discussions, and lectures given by prominent experts designed to complement the experimental activities.

PARTICIPANTS

Best, S., B.S., National Institute for Medical Research, London, England
Boldog, F., Ph.D., University of Nebraska, Omaha
Byrd, P., Ph.D., University of Birmingham, England
Clerc, P., Ph.D., Harvard Medical School, Boston, Massachusetts
Darras, B., M.D., Tufts-New England Medical Center, Boston, Massachusetts
Deloukas, P., B.S., F. Hoffmann La Roche, Basel, Switzerland
Engler, P., Ph.D., University of Chicago, Illinois
Forstner, M., B.S., University of Graz, Austria
Henriksson, J., B.S., Uppsala University, Sweden
Jones, E., Ph.D., Howard Hughes Institute in Dallas, Texas
Lee, Y., Ph.D., Children's Hospital, Boston, Massachusetts
Libert, F., Ph.D., Genmark, Salt Lake City, Utah
Liu, A., Ph.D., VA Medical Center, Los Angeles, California
Palmer, L., B.S., McLaughlin Research Institute, Great Falls, Montana
Polzin, K., Ph.D., Iowa State University, Ames
Tanguay-Stowers, L., B.A., Bio 101, La Jolla, California

SEMINARS

Bentley, D., Guy's Hospital, London. Isolation and analysis of YACs: Application to mapping the human X chromosome.
Burke, D., University of Michigan Medical School. Development and uses of a mouse genomic YAC library.
Garza, D., Stanford University School of Medicine. Application of YACs to the study of model genetic organisms.
Hieter, P., Johns Hopkins University School of Medicine. Modification of YACs using homologous recombination-based techniques.
Huxley, C., Washington University School of Medicine. Transfer of YAC DNA from yeast to mammalian cells.
Olson, M., Washington University School of Medicine. In vitro manipulation of large DNA molecules.
Ward, D., Yale University School of Medicine. Physical mapping of DNA with fluorescent in situ hybridization techniques.

Macromolecular Crystallography

October 10–October 23

INSTRUCTORS

Furey, W., Ph.D., VA Medical Center, Pittsburgh, Pennsylvania
Gilliland, G., Ph.D., Center for Advanced Research in Biotechnology, Rockville, Maryland
McPherson, A., Ph.D., University of California, Riverside
Pflugrath, J., Ph.D., Cold Spring Harbor Laboratory, New York

Crystallography and X-ray diffraction yield a wealth of structural information unobtainable through other methods. This intensive laboratory/computational course focused on the major techniques used to determine the three-dimensional structures of macromolecules. It was designed for scientists with a working knowledge of protein structure and function, but who are new to macromolecular crystallography. Topics covered included protein purification, crystallization, crystal characterization, data collection, data reduction, anomalous dispersion, phase determination, molecular replacement and averaging, electron density interpretation, structure refinement, molecular graphics, and molecular dynamics. Participants learned through extensive hands-on experiments, informal discussions, and lectures on current applications of these and related procedures given by outside speakers.

PARTICIPANTS

Bhandary, K., Ph.D., SUNY at Buffalo, New York
Cueto, M., B.A., Rutgers University, Piscataway, New Jersey
Curry, S., Ph.D., Institute for Animal Health, Surrey, England
Faust, C., Ph.D., Texas Tech University, Lubbock
Gastinel, L., Ph.D., California Institute of Technology, Pasadena
Johansson, A., M.S., University of Copenhagen, Denmark
Lee, P., Ph.D., Brookhaven National Laboratory, Upton, New York
Melhorn, I., Ph.D., University of California, San Francisco
Miller, H., B.S., Wake Forest University, Winston-Salem, North Carolina
Nolte, R., B.S., Boston University, Massachusetts
Phipps, B., Ph.D., NRC Institute for Biological Sciences, Ottawa, Canada
Quirk, S., Ph.D., Johns Hopkins University, Baltimore, Maryland
Rao, J., Ph.D., Texas College of Osteopathic Medicine, Fort Worth
Rech, J., B.A., Vanderbilt University, Nashville, Tennessee
Stoll, V., Ph.D., Max Planck Institute, Heidelberg, Germany
Zhou, G., Ph.D., Oregon State University, Corvallis

SEMINARS

Anderson, J., Cold Spring Harbor Laboratory. Crystallization of proteins with DNA.
Barford, D., Cold Spring Harbor Laboratory. Molecular replacement I and II.
Brunger, A., Yale University. New approaches to crystallographic refinement and molecular replacement.
Burley, S., Rockefeller University. Structure determination of leucine aminopeptidase.
Furey, W., Veterans Administration Medical Center. 1. Isomorphous replacement I and II. 2. Solvent flattening/phase combination.
Gilliland, G., Center for Advanced Research in Biotechnology. 1. Biological macromolecule crystallization database: A tool for the development of crystallization strategies. 2. The three-dimensional structure determination of isozyme 3-3 of a glutathione *S*-transferase from the Mu gene class.
Gronenborn, A., National Institutes of Health. Determination of protein structure by 2D, 3D, and 4D NMR.
Hendrickson, W., Columbia University. Application of multiwavelength anomalous diffraction to direct macromolecular structure determination.
Herzberg, O., University of Maryland. Two crystal structures from the phosphoenopyruvate:sugar phosphotransferase system: A paradigm for protein-protein phosphoryl transfer.
Jones, T.A., University of Uppsala. Map interpretation.
Kuriyan, J., Rockefeller University. Structural studies on disulfide reductases.
McPherson, A., University of California, Riverside. 1. Crystallization of macromolecules I, II, and III. 2. Preliminary crystal characterization. 3. More on Bragg's Law. 4. Precision photography. 5. Difference patterns.
Minor, W., Purdue University. Data collection in protein crystallography: Tricks and traps I and II.
Rose, J., University of Pittsburgh. Crystallographic study of the neurophysin-hormone system.
Sigler, P., Yale University. Crystal structures showing how steroid receptors recognize their DNA targets.
Sweet, R., Brookhaven National Laboratory. 1. Introduction to crystallography. 2. Crystallographic symmetry and unit cells. 3. X-ray sources and optics.
Tronrud, D., Institute of Molecular Biology. Refinement with TNT.
Westbrook, E., Argonne National Laboratory. Structure determination of cholera toxin.

Essential Computational Genomics for Molecular Biologists

October 27–November 5

INSTRUCTORS

Branscomb, Elbert, Ph.D., Lawrence Livermore Laboratory, California
Goodman, Nat, Ph.D., Massachusetts Institute of Technology
Lander, Eric, Ph.D., Massachusetts Institute of Technology
Marr, Tom, Ph.D., Cold Spring Harbor Laboratory, New York
Myers, Gene, Ph.D., University of Arizona

This course was intended primarily for molecular biologists and geneticists who are mounting large-scale projects that require informatics components. No prior experience with the use of computers or mathematics was assumed. Lectures and computer work delved deeply into both the theoretical issues and practical approaches to a number of important computational problems in genomic analysis. The course included an overview of informatics systems analysis and design principles. Topics included such basics as database design and data modeling to more complex analytical methods. Technical issues relating to the analysis of raw data such as image analysis, sequence assembly, physical mapping, genetic-linkage analysis, and data integration were covered. Students were introduced to, and gained hands-on experience with, a variety of software tools used in computer systems design and data acquisition and analysis running on Macintosh computers. It was intended that students gain sufficient knowledge and experience from this course to enable them to initiate active collaborations with computational scientists at their home institutions.

PARTICIPANTS

Bergh, S., M.S., Royal Institute, Stockholm, Sweden
Fernandez-Larsson, R., Ph.D., University of Massachusetts, Worcester
Francke, U., M.D., Stanford University, California
Mahtani, M., B.S., Stanford University, California
Mao, J., Ph.D., Collaborative Research, Inc., Waltham, Massachusetts
Marin, A., M.D., University of Seville, Spain
Rine, J., Ph.D., University of California, Berkeley
Wang, F., M.S., University of Texas, Austin

Molecular Genetics of Fission Yeast

October 24–November 7

INSTRUCTORS

Fantes, Peter, Ph.D., University of Edinburgh, Scotland
Hyams, Jeremy, Ph.D., University College London, England
McLeod, Maureen, Ph.D., State University of New York, Brooklyn

ASSISTANTS

Alfa, Caroline, University College London, England
Devoti, Jamie, State University of New York, Brooklyn
Warbrick, Emma, University of Edinburgh, Scotland

The fission yeast (*Schizosaccharomyces pombe*) is increasingly being used as a model organism for the study of basic aspects of cell biology. This course introduced the student to all aspects of fission yeast biology, but with particular emphasis on genetic manipulation (both classical and with recombinant DNA) and the use of the organism for the study of cell biology. Topics covered included chemical mutagenesis and mutant analysis, transformation and gene replacement techniques, isolation of nuclei, preparation of nuclear DNA, plasmid recovery from yeast into bacteria, cell cycle methods, cytology, and immunocytochemical techniques. In addition to hands-on experience, participants had the opportunity to learn through informal group discussions and a lecture series designed to complement the experimental section.

PARTICIPANTS

Benko, Z., M.S., L. K. University, Debrecen, Hungary
Brazill, D., B.S., University of California, Berkeley
Finkelstein, C., M.S.. Instituto Investigaciones, Buenos Aires, Argentina
Germain, D., Ph.D., National Research Council, Montreal, Canada
Guan, K., Ph.D., Purdue University, West Lafayette, Indiana
He, X., B.S., Baylor College of Medicine, Houston, Texas
Moser, M., B.S., University of Washington, Seattle
O'Connell, M., Ph.D., University of Medicine & Dentistry of New Jersey, Piscataway
Sander, P., B.S., Max Planck Institute, Frankfurt, Germany
Shpakovski, G., Ph.D., National Institute of Mental Health, Bethesda, Maryland
Zou, J., M.S., Johns Hopkins University, Baltimore, Maryland

SEMINARS

Beach, D., Cold Spring Harbor Laboratory. Role of phosphatases in cell cycle regulation.
Chappell, T., Duke University. The Golgi apparatus of *S. pombe*.
Fantes, P., University of Edinburgh. New mitotic control elements.
Hoffman, C., Boston College. Glucose repression of transcription of the *S. pombe fbp1* gene.
Hyams, J., University College London. Cell cycle control in fission yeast.
Klar, A., NCI-Frederick Facility. Fission yeast mating type.
Kohli, J., University of Bern. Recombination in fission yeast.
McLeod, M., SUNY, Brooklyn. Regulation of meiotic initiation.
Niwa, O., Kyoto University. Chromosome stability in fission yeast.
Simanis, V., ISREC, Switzerland. Identification and analysis of a bypass supressor of the *cdc10* gene.
Wigler, M., Cold Spring Harbor Laboratory. *ras* oncogene in fission yeast.
Young, P., Queen's University. Isolation of mutants defective in sodium metabolism.

Seminars

Cold Spring Harbor Seminars were initiated to provide a semiformal avenue for communication between the various research groups at the Laboratory. They are particularly useful for research personnel who have joined the Laboratory during the summer. The seminars also afford a necessary opportunity for the graduate students and postgraduate staff to develop their skills in defending, organizing, and presenting their research. In addition to those listed below, seminars were given by many others involved in research at this laboratory.

1991

January

R. Scott Hawley, Albert Einstein College of Medicine, Bronx, New York. Molecular genetics and cell biology of chromosome segregation in *Drosophila*.

Stan Fields, State University of New York at Stony Brook. Applications of a genetic system to detect protein-protein interactions.

Greg Hannon, Case Western Reserve, Cleveland, Ohio. Trans-splicing of nematode pre-messenger RNA.

Huseyin Mehmet, University College, London, England. Signal transduction in glial cell development.

February

Richard Marais, Imperial Cancer Research Fund, London, England. Posttranslational modification and the control of serum response factor activity.

Carl Pabo, Johns Hopkins University, Baltimore, Maryland. Co-crystal structures of zinc finger/DNA and homeodomain/DNA complexes: New perspectives in protein-DNA recognition.

Joan Massagué, Sloan Kettering Cancer Center, New York, New York. Cell regulation by transforming growth factors.

Brian Holaway, Boehringer Mannheim Corp., Indianapolis, Indiana. Advances in nonradioactive nucleic acid labeling and detection.

Larry Simpson, University of California at Los Angeles. RNA editing in trypanosome mitochondria.

March

Masayori Inouye, Robert Wood Johnson Medical School at Rutgers University, New Brunswick, New Jersey. Retroelements in bacteria.

Ruth Lehmann, Whitehead Institute, Cambridge, Massachusetts. Maternal control of germ line determination and pattern formation in the *Drosophila* embryo.

Chris Hardy, Columbia University, New York, New York. Identification by the two hybrid approach of a gene product that interacts in the RAP1 at yeast silencers and telomeres.

Alan Hinnebusch, National Institutes of Health, Bethesda, Maryland. Regulation of initiation factors in translational control of yeast transcriptional activator GCN4.

April

Erin O'Shea, Whitehead Institute, Cambridge, Massachusetts. Structure/function studies of leucine zipper peptides.

Olivia Peirera-Smith, Baylor College of Medicine, Houston, Texas. Molecular genetic analysis of immortalization of human cells.

Jef Boeke, Johns Hopkins University, Baltimore, Maryland. Retrotransposition: Mechanism and host functions.

Peter Drain, Stanford University, California. Molecular genetic approaches to learning in *Drosophila*.

May

Philippe Soriano, Baylor College of Medicine, Houston, Texas. Genetic manipulation of the early mouse embryo.

June

Nam-Hai Chua, Rockefeller University, New York, New York. Molecular switches for gene expression during plant development.

Jonathan Jones, John Innes Institute, United Kingdom. Transposition of *Ac* in tobacco and *Arabidopsis*.

July

Michel Saure, University Paris-Sud, France. *CDC25* and *SDC25:* Two genes of *S. cerevisiae* that encode exchange factors for small GTP binding of proteins.

Christoph Muller, Albert-Ludwigs-Universitat, Freiburg, Germany. Wild-type and mutant structures of adenylate kinase from *Escherichia coli*.

September

Yi Zhong, University of Iowa, Iowa City. Activity-dependent synaptic plasticity in *Drosophila* memory mutants.

Tom Broker, University of Rochester, New York. Complete productive program of a papillomavirus in culture.

October

Howard Schulman, Stanford University Medical Center, California. Multifunctional Ca^{++}/calmodulin kinase: An enzyme with a memory.

Ming Lei, Cornell University, Ithaca, New York. Cloning and characterization of a rice cell wall protein gene; Evolutionary studies using plant cytochrome *c* genes.

Alex von Gagain, Karolinska Institute, Sweden. Environmentally regulated stability of mRNA in *E. coli*: A model to study the mechanism of mRNA degradation.

Robin Wharton, Columbia University, New York, New York. RNA regulatory elements and the control of posterior segmentation in *Drosophila*.

November

Kerry Bloom, University of North Carolina at Chapel Hill. Molecular structure of a yeast centromere.

Max Gottesman, Columbia University, New York, New York. *v-ras*-induced dedifferentiation in rat thyroid cells.

Gerald Hart, Johns Hopkins University, Baltimore, Maryland. Glycosylation of nuclear proteins: A putative regulatory modification.

Martin Hammer, Berlin. Behavioral and physiological analysis of olfactory learning in bees.

December

Jerry Yin, Massachusetts Institute of Technology. Approaches to cAMP and the establishment of long-term memory in *Drosophila*.

Ken Marcu, State University of New York at Stony Brook. Mechanisms and regulation of lymphoid antigen receptor rearrangement.

Sarah Hake, USDA/Plant Gene Expression Center, Albany, California. Ectopic expression of the maize homeobox gene *Knotted* causes altered leaf development.

Hazel Sive, Whitehead Institute, Cambridge, Massachusetts. Determination of the anteroposterior axis in *Xenopus laevis*.

Undergraduate Research

An important aspect of the summer program at the Laboratory is the participation of college undergraduate students in active research projects under the supervision of full-time laboratory staff members. The program was initiated in 1959. Since that year, 355 students have completed the course, and many have gone on to creative careers in biological science.

The objectives of the program are to provide (1) a greater understanding of the fundamental principles of biology; (2) an increased awareness of major problem areas under investigation; (3) a better understanding of the physical and intellectual tools for modern research and the pertinence of this information to future training; and (4) a personal acquaintance with research workers and centers for study.

The following students, selected from over 173 applicants, took part in the program, which was supported by the Burroughs Wellcome Fund, Baring Brothers, Miles Inc./Bayer AG, Cold Spring Harbor Laboratory Robert H.P. Olney Fund, BioRad/Laboratories, National Science Foundation, Phillips Petroleum Foundation, Inc., Edwin Whitehead Association, and anonymous gifts.

Joseph Beauchamp, SUNY, Cortland
Advisor: **Thomas Peterson**
Sponsor: National Science Foundation
Investigation of mosaic pericarp color in maize.

David Birschbach, University of Wisconsin, Madison
Advisor: **Kim Arndt**
Sponsor: National Science Foundation
Identification of suppressor of transcription (SIT) mutants in *Saccharomyces cerevisiae*.

Lisa Catapano, Dartmouth College
Advisor: **Thomas Marr**
Sponsor: National Science Foundation
Short functional elements in DNA.

Clark Chen, Stanford University
Advisor: **Bruce Stillman/Anindya Dutta**
Sponsor: Burroughs Wellcome Fund
Cloning the human homolog of *Saccharomyces cerevisiae CDC7* gene using cross-species complementation.

Marie-Dominique Galibert, Rene-Descartes, University, Paris
Advisor: **Michael Mathews/Claude Labrie**
Sponsors: Miles, Inc.; Bio-Rad Laboratories; Whitehead Associates
Characterization of DNA sequences mediating the *trans*-activation of PCNA by E1A.

Leena Gandhi, University of Utah
Advisor: **Arne Stenlund**
Sponsor: Burroughs Wellcome Fund
BrdU density-labeling analysis of BPV replication in rodent cell lines.

Per Gesteland, Allegheny College, Pennsylvania
Advisor: **Dafna Bar-Sagi**
Sponsor: Burroughs Wellcome Fund
The elucidation of possible upstream regulators of the *ras* protein in the process of T-cell activation.

Flaviano Giorgini, Purdue University
Advisor: **Jeff Kuret**
Sponsor: Whitehead Associates
Solution of the three-dimensional structure of the catalytic subunit of the cAMP-dependent protein kinase from *Saccharomyces cerevisiae* following a mutagenic approach.

Gilbert L. Henry, University of California, Santa Barbara
Advisor: **Winship Herr**
Sponsor: National Science Foundation
An analysis of the binding specificity of two POU domain proteins: Oct-1 and Pit-1.

Christina M. Hull, University of Utah
Advisor: **Carol Greider**
Sponsor: Anonymous Contributor
Direct assay for telomerase primer binding.

Lei Meng, Barnard College
Advisor: **Bruce Futcher**
Sponsors: Olney Memorial Cancer Fund; Philips Petroleum Foundation, Inc.
Searching of a new *CLN*-like gene by screening a *Saccharomyces cerevisiae* genomic library.

Juan Moreno, University of California, Irvine
Advisor: **Nouria Hernandez**
Sponsor: National Science Foundation
A PCR approach for the identification of the cDNA encoding the largest subunit of human RNA polymerase III.

Adam John Oates, Newcastle-Upon-Tyne University, United Kingdom
Advisor: **Hong Ma**
Sponsor: Baring Brothers
Analysis of the *agamous* gene product in yeast.

Johanna O'Dell, Beloit College
Advisor: **Robert Martienssen**
Sponsor: National Science Foundation
A new PCR technique for cloning suppressible genes in maize.

Frank Papanikolaou, University of Toronto
Advisor: **James Pflugrath/Daniel Marshak**
Sponsor: Anonymous Contributor
Study of the neurite extension factor S100b.

Jennifer L. Saeger, Cedar Crest College
Advisor: **David Helfman**
Sponsor: National Science Foundation
Isolation and identification of a cDNA encoding the polypyrimidine tract binding protein.

Melissa Slawecki, Dickinson College
Advisor: **John Anderson**
Sponsor: Burroughs Wellcome Fund
Crystallization and X-ray diffraction analysis of R.*Pvu*II.

David Stark, Cambridge University, United Kingdom
Advisor: **Nicholas Tonks**
Sponsor: Baring Brothers
Identification of protein tyrosine-phosphatases (PTPases) in PC12 cells.

Kathy Tsapos, Harvard University
Advisor: **B. Robert Franza**
Sponsor: National Science Foundation
Characterization of NFκB/p105 and cRel.

Jin Yang, SUNY, Oneonta
Advisor: **Eric Richards**
Sponsor: Anonymous Contributor
Structure and function of telomere-associated sequences.

Nature Study Program

The Nature Study Program gives elementary and secondary school students the opportunity to acquire a greater knowledge and understanding of their environment. Through a series of specialized field courses, students can engage in such introductory programs as Nature Detectives, Seashore Life, and Pebble Pups, as well as more advanced programs such as Marine Biology and Nature Photography.

During the summer of 1991 a total of 420 students participated in the Nature Study Program. Most classes were held outdoors, when weather permitted, or at the Uplands Farm Preserve, the headquarters of the Long Island Chapter of the Nature Conservancy. The Laboratory has equipped and maintains classrooms/laboratories as well as a darkroom at Uplands Farm. This facility is used as a base for the student's exploration of the local environment. Field classes are held on Laboratory grounds, St. John's Preserve, Fire Island National Seashore, Caumsett State Park, the Cold Spring Harbor Fish Hatchery and Aquarium, as well as other local preserves and sanctuaries.

In addition to the 2-week courses, the 2-day Adventure Education class took students on a 10-mile bike hike to Sagamore Hill and a 12-mile canoe trip on the Nissequogue River.

PROGRAM DIRECTOR

William M. Payoski, M.A., Adjunct Professor,
Nassau Community College

INSTRUCTORS

Tricia Decarmine, B.S., science teacher, St. Anthony's High School
Michael Manfredonia, teaching candidate, Marist College
Kim Hamilton, B.S., Colgate University
Linda Payoski, B.A., science teacher, Uniondale High School
Marjorie Pizza, B.A., science teacher, Glen Cove School District
Donna Stokes, teaching candidate
Edward Tronolone, B.A., M.S., Principal, Locust Valley School District

COURSES

Nature Bugs
Nature Detectives
Advanced Nature Study
Ecology Explorers
Frogs, Flippers, and Fins
Pebble Pups
Bird Study
Fresh Water Life
Seashore Life
Marine Biology
Nature Photography
Adventure Education

FINANCIAL STATEMENT

FINANCIAL STATEMENT

BALANCE SHEET
Year ended December 31, 1991
with comparative amounts for 1990

ASSETS

	Operating Funds			Endowment & Similar Funds	Land, Building, & Equipment Funds	Total All Funds	
	Unrestricted						
	Undesignated	Designated	Restricted			1991	1990
Cash and cash equivalents	$3,117,239	750,000	–	856,904	3,333,907	8,058,050	17,913,193
Marketable securities	780,813	–	–	19,517,935	154,969	20,453,717	11,442,776
Accounts receivable:							
Publications (less allowance for doubtful accounts of $50,000 in both 1991 and 1990)	525,989	–	–	–	–	525,989	729,472
Other	174,269	–	19,296	–	–	193,565	128,830
Grants receivable	–	–	3,047,419	–	–	3,047,419	1,301,854
Accrued interest receivable	–	–	–	506,674	–	506,674	273,426
Publications inventory	1,167,301	–	–	–	–	1,167,301	900,727
Other assets, principally prepaid expenses	95,735	–	–	–	537,910	633,645	836,965
Investment in employee residences	–	–	–	–	1,457,746	1,457,746	1,084,424
Land, buildings, and equipment:							
Land and improvements	–	–	–	–	4,248,012	4,248,012	4,181,050
Buildings	–	–	–	–	46,886,748	46,886,748	24,047,478
Furniture, fixtures, and equipment	–	–	–	–	2,482,465	2,482,465	2,278,950
Laboratory equipment	–	–	–	–	7,063,973	7,063,973	5,044,457
Library books and periodicals	–	–	–	–	365,630	365,630	365,630
	–	–	–	–	61,046,828	61,046,828	35,917,565
Less accumulated depreciation and amortization	–	–	–	–	14,509,155	14,509,155	12,610,734
Land, buildings, and equipment, net	–	–	–	–	46,537,673	46,537,673	23,306,831
Construction in progress	–	–	–	–	3,188,616	3,188,616	20,166,508
Total assets	$5,861,346	750,000	3,066,715	20,881,513	55,210,821	85,770,395	78,085,006

LIABILITIES AND FUND BALANCES

	Operating Funds			Endowment & Similar Funds	Land, Building, & Equipment Funds	Total All Funds	
	Unrestricted						
	Undesignated	Designated	Restricted			1991	1990
Liabilities:							
Accounts payable and accrued expenses	$2,633,248	–	511,692	–	410,288	3,555,228	3,192,036
Notes payable	–	–	–	–	910,127	910,127	964,869
Bonds payable	–	–	–	–	20,000,000	20,000,000	20,000,000
Deferred revenue	1,290,707	–	2,555,023	–	–	3,845,730	2,818,458
Total liabilities	3,923,955	–	3,066,715	–	21,320,415	28,311,085	26,975,363
Fund balances:							
Unrestricted–undesignated	1,937,391	–	–	–	–	1,937,391	1,652,547
Unrestricted–designated	–	750,000	–	–	–	750,000	650,000
Endowment and similar funds	–	–	–	20,881,513	–	20,881,513	18,039,437
Land, buildings, and equipment:							
Expended	–	–	–	–	31,352,674	31,352,674	28,222,713
Unexpended–Donor restricted	–	–	–	–	1,079,986	1,079,986	1,460,522
Unexpended–Board authorized	–	–	–	–	1,457,746	1,457,746	1,084,424
Total fund balances	1,937,391	750,000	–	20,881,513	33,890,406	57,459,310	51,109,643
Total liabilities and fund balances	$5,861,346	750,000	3,066,715	20,881,513	55,210,821	85,770,395	78,085,006

STATEMENT OF SUPPORT, REVENUE AND EXPENSES, AND CHANGES IN FUND BALANCES
Year ended December 31, 1991
with comparative amounts for 1990

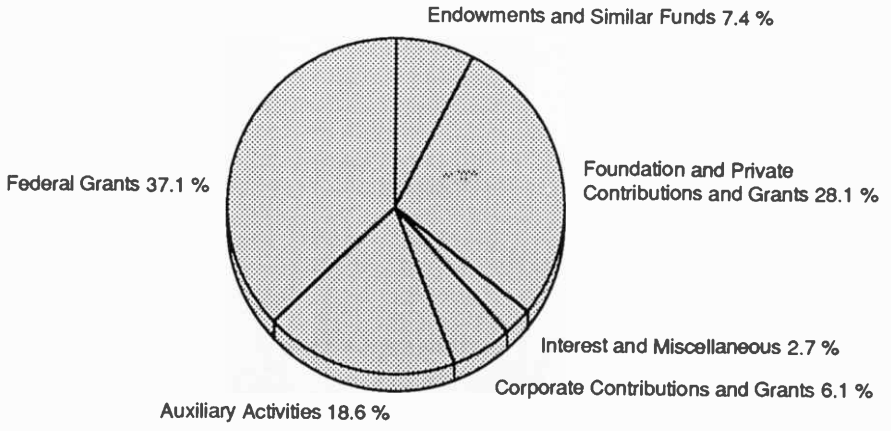
	Operating Funds						
	Unrestricted			Endowment & Similar Funds	Land, Building, & Equipment Funds	Total All Funds	
	Undesignated	Designated	Restricted			1991	1990
Support and revenue:							
Public support	$1,898,003	–	6,540,534	3,584,438	1,093,533	13,116,508	7,557,930
Government grant awards	–	–	7,982,835	–	–	7,982,835	10,250,777
Indirect cost allowances	7,280,352	–	–	–	–	7,280,352	6,633,895
	9,178,355	–	14,523,369	3,584,438	1,093,533	28,379,695	24,442,602
Other revenue:							
Program fees	1,329,875	–	–	–	–	1,329,875	1,123,793
Rental income	278,840	–	–	–	–	278,840	248,333
Publications	3,078,938	–	–	–	–	3,078,938	4,222,694
Dining services	1,439,224	–	–	–	–	1,439,224	1,154,033
Rooms and apartments	1,239,585	–	–	–	–	1,239,585	795,995
Distribution from Robertson Funds	375,000	–	975,000	–	–	1,350,000	1,150,000
Investment income	164,371	–	–	1,333,374	475,836	1,973,581	2,853,915
Royalty & licensing	318,029	–	–	–	–	318,029	84,677
Miscellaneous	92,042	–	–	–	–	92,042	84,887
Total other revenue	8,315,904	–	975,000	1,333,374	475,836	11,100,114	11,718,327
Total support and revenue	17,494,259	–	15,498,369	4,917,812	1,569,369	39,479,809	36,160,929
Expenses:							
Program services:							
Research	–	–	13,026,253	–	–	13,026,253	11,348,636
Summer programs	849,168	–	2,145,416	–	–	2,994,584	3,052,125
Publications	3,488,100	–	–	–	–	3,488,100	3,708,062
Banbury Center conferences	204,801	–	242,243	–	–	447,044	499,942
DNA Education Center programs	6,643	–	373,161	–	–	379,804	323,219
Total program services	4,548,712	–	15,787,073	–	–	20,335,785	18,931,984
Supporting services:							
Direct research support	903,032	–	–	–	–	903,032	609,502
Library	433,496	–	–	–	–	433,496	388,221
Operation and maintenance of plant	4,221,594	–	–	–	–	4,221,594	4,070,385
General and administrative	3,005,496	–	–	75,791	–	3,081,287	2,877,460
Dining services	1,322,422	–	–	–	–	1,322,422	1,296,009
Interest	–	–	–	–	934,105	934,105	1,413,344
Total supporting services	9,886,040	–	–	75,791	934,105	10,895,936	10,654,921

Depreciation	–	–	–	–	1,898,421	1,898,421	1,485,084
Total expenses	14,434,752	–	15,787,073	75,791	2,832,526	33,130,142	31,071,989
Excess (deficiency) of support and revenue over expenses before designation	$3,059,507	–	(288,704)	4,842,021	(1,263,157)	6,349,667	5,088,940
Designation:							
Funds designated for research program	(100,000)	100,000	–	–	–	–	–
Excess (deficiency) of support and revenue over expenses and designation	2,959,507	100,000	(288,704)	4,842,021	(1,263,157)	6,349,667	5,088,940
Other changes in fund balances:							
Capital expenditures	(2,564,962)	–	(1,925,942)	–	4,490,904	–	–
Transfer to restricted funds	–	–	2,214,646	(2,214,646)	–	–	–
Transfer to endowment funds	(109,701)	–	–	214,701	(105,000)	–	–
Net increase in fund balances	284,844	100,000	–	2,842,076	3,122,747	6,349,667	5,088,940
Fund balances at beginning of year	1,652,547	650,000	–	18,039,437	30,767,659	51,109,643	46,020,703
Fund balances at end of year	$1,937,391	750,000	–	20,881,513	33,890,406	57,459,310	51,109,643

Copies of our complete, audited financial statements, certified by the independent auditing firm of KPMG, Peat, Marwick & Co., are available upon request from the Comptroller, Cold Spring Harbor Laboratory.

SOURCES OF REVENUE

YEAR ENDED DECEMBER 31, 1991



COMPARATIVE OPERATING HISTORY
1987-1991
(Dollars in Thousands)

	1987	*1988*	*1989*	*1990*	*1991*
Income:					
Main Lab:					
Grants & contracts	10,409	10,799	13,062	13,535	15,172
Indirect cost reimbursement	4,779	5,707	6,412	6,558	7,170
Other	2,727	3,205	4,034	3,976	5,056
CSH Press	1,556	1,641	4,450	4,223	3,079
Banbury Center	982	976	1,012	1,120	1,090
DNA Learning Center	349	660	622	585	744
Total income	20,802	22,988	29,592	29,997	32,311
Expenses:					
Main Lab:					
Grants & contracts	10,409	10,799	13,062	13,535	15,172
Operation & maintenance of plant	2,791	3,010	3,412	3,759	3,904
General & administrative	1,975	2,102	2,377	2,414	2,468
Other	2,633	3,049	3,165	2,973	3,375
CSH Press	1,311	1,719	3,934	3,708	3,488
Banbury Center	947	910	1,038	1,125	1,063
DNA Learning Center	260	590	635	615	752
Total expenses	20,326	22,179	27,623	28,129	30,222
Excess before depreciation and designation of funds	476	809	1,969	1,868	2,089
Depreciation	(1,127)	(1,286)	(1,399)	(1,485)	(1,898)
Designation of funds (1)	–	–	(400)	(250)	(100)
Net operating excess (deficit)	$ (651)	(477)	170	133	91

The above amounts are presented on a combined basis for all funds for which Cold Spring Harbor Laboratory prepares operating budgets.

(1) Funds designated to underwrite future direct and indirect expenses of the neuroscience and other research programs.

FINANCIAL SUPPORT OF THE LABORATORY

SOURCES OF SUPPORT

Cold Spring Harbor Laboratory is a nonprofit research and educational institution chartered by the University of the State of New York. Less than half of its annual support is derived from Federal grants and contracts. Contributions from the private sector are tax exempt under the provisions of Section 501(c)(3) of the Internal Revenue Code. In addition, the Laboratory has been designated a "public charity" and, therefore, may receive funds resulting from the termination of "private foundations."

Over the years, the Laboratory has earned a reputation for innovative research and high-level science education. By training young scientists in the latest experimental techniques, it has helped spur the development of many research fields, including tumor virology, cancer genes, gene regulation, movable genetic elements, yeast genetics, and molecular neurobiology. This continual development of new research programs and training courses requires substantial support from private sources.

Because its endowment is small and because government support is highly competitive and the uses of research grants are restricted, the Laboratory depends on **annual** contributions from the private sector (foundations, corporations, and individuals) for its central institutional needs.

As of December 31, 1991, the Second Century Campaign had raised $45,965,194, exceeding its goal of $44,000,000. These are **capital** funds and will be used for construction of new facilities and for staff and student endowment.

METHODS OF CONTRIBUTING TO COLD SPRING HARBOR LABORATORY

Gifts of money can be made directly to Cold Spring Harbor Laboratory.

Securities You can generally deduct the full amount of the gift on your income tax return, and, of course, you need pay no capital gains tax on the stock's appreciation. We recommend any of the following methods:

(1) Have your broker sell the stock and remit the proceeds to Cold Spring Harbor Laboratory.

(2) Deliver the stock certificates to your broker with instructions to him to open an account for Cold Spring Harbor Laboratory and hold the securities in that account pending instructions from Cold Spring Harbor Laboratory.

(3) Send the *unendorsed* stock certificates directly to the Laboratory: Comptroller, Cold Spring Harbor Laboratory, Box 100, Cold Spring Harbor, New York 11724. In a separate envelope send an *executed* stock power.

Pooled Income Funds Combine gifts from a number of donors in a pool for attractive investment and tax purposes.

Appreciated real estate or personal property Sizable tax benefits can result from such donations; the Laboratory can use some in its program and can sell others.

Life insurance and charitable remainder trusts can be structured to suit the donor's specific desires as to extent, timing, and tax needs.

Bequests Most wills probably need to be updated. Designating Cold Spring Harbor Laboratory as beneficiary ensures that a bequest will be utilized as specified for continuing good.

Conversion of private foundation to "public" status on termination This may be done by creating a separate fund within Cold Spring Harbor Laboratory whereby the assets of the private foundation are accounted for as a separate fund and used for the purposes specified by the donor. Alternatively, the private foundation can be established as a "supporting organization of Cold Spring Harbor Laboratory."

For additional information, please contact the Director of Development, Cold Spring Harbor Laboratory, Box 100, Cold Spring Harbor, NY 11724, or call 516-367-8840.

GRANTS

January 1, 1991–December 31, 1991

COLD SPRING HARBOR LABORATORY

Grantor	*Program/Principal Investigator*	*Duration of Grant*	*Total Award*
FEDERAL GRANTS			
NATIONAL INSTITUTES OF HEALTH			
Program Projects	Cancer Research Center, Dr. Mathews	1/87 - 12/91	$16,787,416
	Cancer Center Support, Dr. Roberts	8/87 - 7/95	8,823,360
	PEBRA-HIV Grant, Dr. Herr	9/88 - 8/91	3,672,816
	Oncogene Program Project, Dr. Wigler	3/88 - 2/93	4,869,923
Research Support	Dr. Anderson	4/90 - 3/93	176,000
	Dr. Arndt	4/88 - 3/93	1,288,964
	Dr. Arndt	1/91 - 12/94	773,411 *
	Dr. Bar-Sagi	7/91 - 6/96	1,280,538 *
	Dr. Beach	12/84 - 1/93	1,481,482
	Dr. Beach	9/86 - 8/94	3,032,957
	Dr. Beach	7/88 - 6/93	1,488,740
	Dr. Beach	8/90 - 7/93	1,287,507
	Dr. Davis	9/91 - 8/95	847,368 *
	Dr. Franza	9/85 - 11/94	1,497,483
	Dr. Franza	10/91 - 8/94	487,949 *
	Dr. Frendewey	4/87 - 3/92	775,814
	Dr. Futcher	4/88 - 3/93	1,115,434
	Dr. Futcher	1/91 - 12/94	604,467 *
	Dr. Garrels	1/85 - 12/92	5,016,584
	Dr. Gilman	9/87 - 8/92	608,291
	Dr. Greider	12/89 - 11/94	1,130,019
	Dr. Greider	8/91 - 7/96	1,465,240 *
	Dr. Helfman	9/85 - 3/94	2,109,264
	Dr. Hernandez	7/87 - 6/92	1,293,754
	Dr. Hernandez	9/91 - 8/96	1,141,822 *
	Dr. Krainer	7/89 - 6/94	1,298,402
	Dr. Kuret	7/89 - 6/94	1,312,808
	Dr. Kuret	5/91 - 4/95	760,241 *
	Dr. Marr	9/89 - 7/91	156,455
	Dr. Marr	8/91 - 7/94	1,122,933 *
	Dr. Moran	3/88 - 6/91	473,959
	Dr. Moran	8/91 - 6/96	1,299,986 *
	Dr. Moran	8/91 - 5/95	1,122,557 *
	Dr. Peterson	4/88 - 3/93	704,475
	Dr. Pflugrath	4/88 - 3/91	573,503
	Dr. Richards	7/90 - 6/93	424,932
	Dr. Roberts	7/88 - 6/93	1,598,876
	Dr. Roberts	9/88 - 8/94	1,180,152
	Dr. Roberts	4/91 - 3/95	891,380 *
	Dr. Spector	4/90 - 3/95	1,441,475
	Dr. Stillman	7/83 - 5/96	2,672,015
	Dr. Stillman	7/91 - 6/95	763,393 *
	Dr. Tonks	8/91 - 5/96	1,631,345 *
	Dr. Tully	9/91 - 1/93	126,301 *
	Dr. Wigler	7/85 - 6/92	8,426,929
	Dr. Wigler	8/90 - 7/93	640,923

* New Grants Awarded in 1991

Grantor	Program/Principal Investigator	Duration of Grant	Total Award
Equipment Support	Dr. Roberts	8/90 – 7/91	37,583
	Dr. Roberts	6/91 – 5/92	37,327 *
	Dr. Roberts	4/91 – 3/92	54,922 *
	Dr. Spector	4/90 – 3/91	180,000
Fellowships	Dr. Ballester	2/89 – 1/92	63,996
	Dr. Brill	8/89 – 7/92	46,667
	Dr. Connolly	1/90 – 1/93	69,000
	Dr. Del Vecchio	12/91 – 11/93	22,700 *
	Dr. Grueneberg	10/90 – 9/93	69,000
	Dr. Jones	10/91 – 9/94	85,800 *
	Dr. Kessler	8/88 – 7/91	63,996
	Dr. Otto	9/90 – 8/93	100,500
	Dr. Regulski	8/91 – 6/92	49,658 *
	Dr. Stern	11/88 – 11/91	63,996
	Dr. Szymanski	11/90 – 11/92	49,000
Training Support	Institutional, Dr. Grodzicker	7/78 – 4/94	2,364,146
Course Support	Advanced Bacterial Genetics, Dr. Grodzicker	5/80 – 4/93	559,540
	Cancer Research Center Workshops, Dr. Grodzicker	1/83 – 3/92	1,010,057
	Neurobiology Short Term Training, Dr. Hockfield	5/82 – 4/96	1,300,588
	CSH Analysis Large DNA Molecules	1991 – 1996	475,538 *
	Essential Computational Genomics for Molecular Biologists	1991 – 1996	132,619 *
Meeting Support	Genome Mapping and Sequencing Conference	1990 – 1993	78,040
	56th Symposium: The Cell Cycle	1991	10,000 *
	Ribosome Synthesis Conference	1991	2,000 *
	Regulation of Eukaryotic mRNA Transcription	1991	2,000
	Molecular Neurobiology of *Drosophila*	1991	12,000 *
	RNA Processing	1988 – 1991	9.500
NATIONAL SCIENCE FOUNDATION			
Research Support	Dr. Anderson	9/90 – 8/93	315,000
	Dr. Herr	6/88 – 5/91	240,000
	Dr. Ma	5/91 – 4/93	399,000 *
	Dr. Ma	8/91 – 7/94	360,000 *
	Dr. Marr	7/90 – 6/91	49,290
	Dr. Martienssen	7/89 – 6/92	305,000
	Dr. Peterson	11/91 – 4/93	106,753 *
	Dr. Roberts	8/87 – 7/92	63,106
	Dr. Roberts	2/90 – 2/93	210,000
	Dr. Sundaresan	5/87 – 4/91	360,000
Training Support	Undergraduate Research Program, Dr. Herr	6/91 – 5/94	150,000 *
Course Support	Plant Molecular Biology, Dr. Grodzicker	8/86 – 8/93	160,929
Meeting Support	RNA Processing Conference	4/88 – 3/91	30,000
	RNA Processing Meeting	4/91 – 3/94	30,000 *
	Eukaryotic DNA Replication Conference	5/91 – 4/92	12,000 *
	56th Symposium: The Cell Cycle	5/91 – 4/92	13,000 *
	Regulation of Eukaryotic mRNA Transcription	7/91 – 6/92	10,000 *
	Ribosome Synthesis Conference	7/91 – 6/92	10,000 *
	Molecular Biology of Signal Transduction in Plants	8/91 – 1/92	4,000 *
	Molecular Neurobiology of *Drosophila*	7/91 – 6/92	10,700 *

* New Grants Awarded in 1991

Grantor	Program/Principal Investigator	Duration of Grant	Total Award
DEPARTMENT OF ENERGY			
Research Support	Dr. Marr	7/91 -2/94	762,000 *
	Dr. Martienssen	8/91 -8/93	144,000 *
Meeting Suport	56th Symposium: The Cell Cycle	1991	15,000 *
	Molecular Biology of Signal Transduction	1991	5,000 *
OFFICE OF NAVAL RESEARCH			
Course Support	Computational Eye Movement Workshop	7/88 -6/91	87,125
U.S. DEPARTMENT OF AGRICULTURE			
	Dr. Sundaresan	8/91 -8/94	221,000 *
	Dr. Martienssen	9/91 -8/93	128,000 *
NONFEDERAL GRANTS			
Research Support			
Aaron Diamond Foundation	Dr. Anderson	12/88 -11/91	200,000
American Cancer Society	Dr. Bar-Sagi	7/89 -6/91	194,000
	Dr. Spector	7/87 -6/91	363,000
	Dr. Spector	7/91 -6/92	50,000 *
	Dr. Wigler	4/91 -3/92	10,000 *
	Dr. Wigler, Professorship	1986 -2012	1,333,333
	Dr. Gilman	7/91 -5/94	300,000 *
	Dr. Gilman, Institutional Award	7/90 -6/92	100,000
	Dr. Helfman	7/91 -6/96	175,000 *
American Heart Association	Dr. Krainer	11/86 -10/92	827,925
Amersham International plc	Dr. Laspia	12/91 -11/92	60,000 *
American Foundation for AIDS Research	Dr. Mathews	10/91 -9/92	60,000 *
Argonne National Laboratories	Dr. Pflugrath	2/91 -8/93	89,473 *
Sara Chait Foundation	Dr. Marshak	12/91 -11/96	125,000 *
Council for Tobacco Research	Dr. Helfman	7/91 -6/92	80,000 *
Donaldson Charitable Trust	Dr. Enikolopov	12/91 -5/92	100,000 *
Hechler Private Investments	Dr. Enikolopov	12/91 -5/92	26,328 *
Howard Hughes Medical Institute	Neurobiology Support	1987 -1993	1,000,000
Istituto Farmaco-terapico Italiano (IFI)	Dr. Marshak	5/91 -4/92	22,221 *
Lucille P. Markey Charitable Trust	Neurobiology Support	7/90 -6/96	2,000,000
	Neuroscience (Building Fund)		2,000,000
Mathers Charitable Foundation	Dr. Davis	8/91 -7/94	1,028,000 *
Mellam Family Foundation	Dr. Franza	12/88 -11/91	150,000
	Dr. Franza	12/91 -11/95	200,000 *

* New Grants Awarded in 1991

Grantor	Program/Principal Investigator	Duration of Grant	Total Award
Richard Meltzer Fund	Dr. Stillman	3/90 -2/92	50,000
Fund for Cancer Research (World Business Council)			
Massachusetts Institute of of Technology (Subcontract)	Dr. Marr	11/91 -7/92	49,006 *
Muscular Dystrophy Association	Dr. Helfman	1/91 -12/93	126,100 *
National Down's Syndrome Society	Dr. Marshak	7/90 -6/92	50,000
Oxnard Foundation	Dr. Gilman	2/91 -1/94	150,000 *
Pall Corporation	Swiss Project	8/91 -12/91	50,000 *
Pew Memorial Trust	Dr. Greider	7/90 -6/94	200,000
	Dr. Tonks	7/91 -6/95	200,000 *
Pioneer Hi-Bred International Inc.	Cooperative Research	8/85 -9/91	2,500,000
	Dr. Peterson	8/91 -7/94	150,000 *
Rita Allen Foundation	Dr. Hernandez	10/89 -9/94	150,000
Samuel Freeman Charitable Trust	Freeman Laboratory of Cancer Cell Biology	7/89 -6/94	1,000,000
Equipment Support			
Fannie E. Rippel	Neurobiology Equipment	10/89 -7/91	250,000
Florence Gould Foundation	Neuroscience Equipment	7/91 -6/94	300,000 *
Fellowships			
American Cancer Society	Dr. Nefsky	7/90 -6/93	69,000
	Dr. Caligiuri	1/91 -12/93	69,000 *
	Dr. Dutta	1/91 -12/92	47,000 *
American Foundation for AIDS Research	Dr. Laspia	7/89 -6/92	112,200
	Dr. Phares	10/91 -9/92	5,000 *
	Dr. Scheppler	7/91 -6/94	112,000 *
Bristol-Myers Company	Fellowship Support	6/86 -12/91	500,000
Damon Runyon-Walter Winchell Cancer Fund	Dr. Melendy	1/89 -12/91	69,000
	Dr. Walworth	9/90 -8/93	84,000
	Dr. Wilson	1/91 -12/93	69,000 *
	Dr. Ruppert	8/91 -7/94	84,000 *
Ford Foundation	Dr. Peña	9/91 -5/92	30,000 *
Garfield Foundation	Dr. Skowronski	12/91 -5/92	100,000 *
Government of Canada	Dr. Tyers	9/90 -8/91	1,099
	Dr. Labrie	10/90 -9/91	1,300
	Dr. Demetrick	11/91 -10/92	2,923 *
Joyce Green Family Foundation	Dr. Sun	12/91 -2/92	5,000 *
Helen Hay Whitney Foundation	Dr. Bell	7/91 -6/94	75,000 *
Human Frontier	Dr. Clarke	7/91 -6/93	76,000 *
Science Program	Dr. Mayeda	7/91 -6/93	77,000 *
Irvington Institute for Medical Research	Dr. Giordano	7/90 -6/93	78,000
Ladies Auxiliary to the VFW	Dr. Yaciuk	7/90 -6/91	20,000
The Leukemia Society of America	Dr. Das	7/89 -6/92	76,140
	Dr. Riabowol	7/90 -6/91	29,160
	Dr. Bischoff	7/91 -6/94	76,140 *
LIBA	Four Fellowships a Year	1987 -1992	750,000
Life Science Research Foundation	Dr. D. Roberts	9/89 -8/93	105,000
McKnight Endowment	Dr. Davis	11/91 -12/91	5,167 *
John Merck Fund	Dr. Tully	9/91 -5/94	180,000 *

* New Grants Awarded in 1991

Grantor	Program/Principal Investigator	Duration of Grant	Total/Award
Muscular Dystrophy Association	Dr. Tsukahara	7/91-6/93	76,000*
Andrew Seligson Memorial Fellowships	Two Postdoctoral Fellows (Cancer Research Support)	9/90-12/91	70,000
Weizmann Institute of Science	Dr. Gerst	9/89-8/91	57,000
Training Support			
Anonymous	Summer Undergraduate Program	1991	18,750*
Baring Brothers & Co., Ltd.	Summer Undergraduate Program	1990-1992	75,233
Bio-Rad Laboratories	Summer Undergraduate Program	1991	1,000*
Burroughs Wellcome Foundation	Summer Undergraduate Program	1991	21,000*
Grass Foundation	Neurobiology Scholarship Foundation	1980-1991	181,670
Phillips Petro Co.	Summer Undergraduate Program	1991	2,500*
Merck Sharp & Dohme Research Laboratories	Summer Undergraduate Program	12/88-11/90	30,000
Miles Inc.	Summer Undergraduate Program	1991	5,250*
Robert H.P. Olney Memorial Fund	Undergraduate Research Program	1991	4,000*
Whitehead Associates	Summer Undergraduate Program	1991	10,000*
Course Support			
Howard Hughes Medical Institute	Neurobiology Courses	1987-1990	1,000,000
	Postgraduate Courses	1991-1995	1,000,000*
Esther A. and Joseph Klingenstein Fund, Inc.	Advanced Neurobiology Courses	3/91-2/94	180,000*
Meeting Support			
Abbott Laboratories	Synthesis of Ribosomes	1991	250*
Accurate Chemical & Scientific Corp.	Synthesis of Ribosomes	1991	250*
Affinity Bioreagents, Inc.	Stress Proteins and Heat Shock	1991	500*
Anheuser-Busch Companies	Yeast Cell Biology	1991	500*
Berlex Biosciences, Inc.	Stress Proteins and Heat Shock	1991	1,500*
Biogen, Inc.	Stress Proteins and Heat Shock	1991	1,000*
Bio-101 Inc.	Synthesis of Ribosomes	1991	250*
Bristol-Myers Squibb Company	Synthesis of Ribosomes	1991	250*
Connaught Laboratories, Inc.	Synthesis of Ribosomes	1991	250*
Connaught Laboratories, Inc.	Modern Approaches to New Vaccines Including Prevention of AIDS	1991	2,000*
Council for Tobacco Research-USA, Inc.	Role of Isoform Diversity in Cytoskeletal Functions (Meeting Support)	7/90-6/91	1,000
E.I. du Pont de Nemours & Co.	Synthesis of Ribosomes	1991	250*
Fidia Research Foundation	Molecular Neurobiology of *Aplysia*	1991	5,000*
Fondazione Sigma-Tau, Foundation	Molecular Neurobiology of *Aplysia*	1991	5,000*
GIBCO BRL Research Products Division	Stress Proteins and Heat Shock	1991	500*
Kureha Chemical Industry Co., Ltd.	Stress Proteins and Heat Shock	1991	3,600*
John Labatt Limited	Yeast Cell Biology	1991	100*

* New Grants Awarded in 1991

Grantor	Program/Principal Investigator	Duration of Grant	Total Award
Merck Sharp & Dohme Research Laboratories	Isoform Diversity in Cytoskeletal Functions	1991	250*
Merck Sharp & Dohme Research Laboratories	Synthesis of Ribosomes	1991	500*
Miller Brewing Company	Yeast Cell Biology	1991	1,000*
Miscellaneous Support	Isoform Diversity in Cytoskeletal Functions	1991	422*
Muscular Dystrophy Association	Isoform Diversity in Cytoskeletal Functions	1991	7,500*
Pioneer Hi-Breed International, Inc.	Signal Transduction in Plants	1991	500*
Rockefeller Foundation	Modern Approach to New Vaccines (Publication Support)	9/90–8/91	20,000
Alfred P. Sloan Foundation	Evolution: From Molecules to Culture	3/90–2/28/91	30,000
StressGen Biotechnologies Corp.	Stress Proteins and Heat Shock	1991	2,000*
Supelco, Inc.	Synthesis of Ribosomes	1991	250*
Takara Shuzo Co., Ltd.	Stress Proteins and Heat Shock	1991	3,700*
U.S. Biochemical Corporation	Sythesis of Ribosomes	1991	500*
Worthington Bio-chemical Corp.	Synthesis of Ribosomes	1991	250*
Carl Zeiss, Inc.	Isoform Diversity in Cytoskeletal Functions	7/90–6/91	1,000

* New Grants Awarded in 1991

BANBURY CENTER

Grantor	Program/Principal Investigator	Duration of Grant	Total Award
FEDERAL GRANTS			
NATIONAL INSTITUTES OF HEALTH			
	Workshop on the Genome of *E. coli* (NLM)	1991	6,000 *
	Workshop on the Genome of *E. coli* (NCHGR)	1991	23,000 *
	Molecular Genetics and Cell Biology of Marfan Syndrome Meeting (NIAMSD)	1991	10,000 *
NATIONAL SCIENCE FOUNDATION			
	Issues in Training Computational and Mathematical Biologists: A Workshop	1991	19,650 *
	Workshop on the Genome of *E. Coli*	1991	6,000 *
DEPARTMENT OF ENERGY			
	Human Genetics and Genome Analysis: Practical Workshop for Public Policy Makers and Opinion Leaders	3/91 -3/93	128,059 *
	Workshop on DNA Sequence Acquisition and Interpretation	9/91 -9/92	6,000 *
NONFEDERAL SUPPORT			
Meeting Support			
Alfred P. Sloan Foundation	Journalists and Congressional Workshops	1990 - 1992	150,000
Allen & Hanburys	Molecular Immunobiology of Lyme Disease	1991	10,000 *
Amgen, Inc.	Intellectual Property and Biotechnology	1991	5,000 *
Baring Brothers & Co.	Baring Brothers Conference	1991	88,324 *
E.I. du Pont de Nemours & Co.	Intellectual Property and Biotechnology	1991	5,000 *
Eli Lilly and Company	Intellectual Property and Biotechnology	1991	5,000 *
Genentech, Inc.	Intellectual Property and Biotechnology	1991	5,000 *
Glaxo Group Research Limited	Seven-transmembrane Segment Proteins	1991	4,000 *
Hoffmann-La Roche Inc	Intellectual Property and Technology	1991	5,000 *
Home Infusion Pharmaceutical Services	Molecular Immunobiology of Lyme Disease	1991	5,000 *
Merck Sharp & Dohme Research Laboratories	Seven-transmembrane Segment Proteins	1991	3,000 *
MetPath Inc.	Molecular Immunobiology of Lyme Disease	1991	10,000 *
National Multiple Sclerosis Society	Molecular Immunobiology of Lyme Disease	1991	2,000 *
NovoNordisk	Seven-transmembrane Segment Proteins	1991	1,000 *
Repligen Corporation	Control of HIV Gene Expression	1991	5,000 *
The William Stamps Farish Fund	Meetings on Complex Genetic Diseases	1991 - 1993	150,000
ZymoGenetics	Seven-transmembrane Segment Proteins	1991	1,000 *

* New Grants Awarded in 1991

DNA LEARNING CENTER

Grantor	Program/Principal Investigator	Duration of Grant	Total Award
FEDERAL GRANTS			
NATIONAL SCIENCE FOUNDATION			
	High School Faculty Enhancement	1990 – 1993	474,036
	Middle School Faculty Enhancement	1990 – 1993	252,614
	College Faculty Enhancement	1991 – 1993	264,467 *
DEPARTMENT OF EDUCATION			
	College Faculty Enhancement	1991 – 1993	177,925 *
DEPARTMENT OF ENERGY			
	Opinion Leader Workshops (joint grant with Banbury Center)	1991 – 1993	128,059 *
NONFEDERAL GRANTS			
Brinkmann Instruments, Inc.	Special Programs	1991	2,000 *
CMP Foundation	Special Programs	1991	1,000 *
Corporate Advisory Board	Special Programs	1991	14,500 *
CSHL Centennial Fund	"Partners in the Future"	10/91 – 6/92	27,000 *
Harweb Foundation	Special Programs	1991	2,000 *
William Randolph Hearst Foundation	Middle School Program	1991 – 1992	35,000 *
Howard Hughes Medical Institute Montgomery County, Maryland	High School Faculty Enhancement	1990 – 1993	46,500
The Esther A. and Joseph Klingenstein Fund, Inc.	Core Support	1989 – 1991	75,000
Josiah Macy, Jr. Foundation	Core Support	1990 – 1991	98,905
Richard Lounsberry Foundation	Core Support	1991	35,000 *
Natural Heritage Trust, New York State	Exhibit Support	1991	66,300 *
Stone Foundation	Capital Support	1991 – 1994	250,000 *
Nancy Van Vranken	Core Support	1991	500 *
Edwin S. Webster Foundation	Capital Support	1991	15,000 *
The Weezie Foundation	Exhibit Support	1991 – 1992	100,000 *
John R. Young	Special Programs	1991	5,000 *
Hillsborough County, Tampa, Florida	Vector Workshop	1991	2,410 *
Independent School Districts, Houston	Vector Workshop	1991	2,400 *
Mississippi School for Math and Science	Vector Workshop	1991	7,300 *
Northside Independent School District, San Antonio	Vector Workshop	1991	2,410 *
Project Share, Killingworth, Connecticut	Vector Workshop	1991	1,900 *
SUNY at Stony Brook	Middle School Program	1991	10,000 *
Commack Union Free School District	Curriculum Study	1991	500
East Williston Union Free School District	Curriculum Study	1991	500
Garden City Union Free School District	Curriculum Study	1991	1,500
Great Neck Public Schools	Curriculum Study	1991	500
Half Hollow Hills Central	Curriculum Study	1991	500

* New Grants Awarded in 1991

Grantor	*Program/Principal Investigator*	*Duration of Grant*	*Total Award*
Harborfields Central School District	Curriculum Study	1991	500
Herricks Union Free School District	Curriculum Study	1991	500
Huntington Union Free School District	Curriculum Study	1991	500
Island Trees Union Free School District	Curriculum Study	1991	500
Jericho Union Free School District	Curriculum Study	1991	500
Kings Park Central School District	Curriculum Study	1991	1,500
Lawrence Union Free School District	Curriculum Study	1991	500
Lindenhurst Union Free School District	Curriculum Study	1991	500
Locust Valley Central School District	Curriculum Study	1991	500
Manhasset Union Free School District	Curriculum Study	1991	500
Massapequa Union Free School District	Curriculum Study	1991	2,000 *
Northport-East Northport Union Free School District	Curriculum Study	1991	500
North Shore Central School District	Curriculum Study	1991	500
Oyster Bay-East Norwich Central School District	Curriculum Study	1991	500
Plainview-Old Bethpage Central School District	Curriculum Study	1991	500
Plainedge Union Free School District	Curriculum Study	1991	500
Portledge School	Curriculum Study	1991	500
Port Washington Union Free School District	Curriculum Study	1991	500
Roslyn Public Schools	Curriculum Study	1991	500
Sachem Central School District at Holbrook	Curriculum Study	1991	500
South Huntington Union Free School District	Curriculum Study	1991	500
Syosset Central School District	Curriculum Study	1991	500

* New Grants Awarded in 1991

Long Island Biological Association (LIBA)

The Long Island Biological Association is the oldest supporting organization for Cold Spring Harbor Laboratory. Over the years a most unique and productive partnership has developed wherein LIBA has become our "Friends of the Laboratory" and accounts for the largest amount of unrestricted annual giving for the Laboratory. (The detailed report of their activities appears later in this Annual Report.)

Summary of Annual Contributions

Unrestricted Annual Contributions

CSHL Associates (1/1/91–12/31/91)	$265,245	
LIBA Members (1/1/91–12/31/91)	75,728	
General	5,722	
Memorials	8,055	
		$354,750

Restricted Annual Contributions

Miscellaneous	2,500	
		$ 2,500

Total Annual Contributions

$357,250

CORPORATE SPONSOR PROGRAM

Restricted

The response of the corporate world to invitations to renew or become new members of the Cold Spring Harbor Laboratory Corporate Sponsor Program in 1991 can only be described as extraordinary. In a period that experienced increasing economic recession and correspondingly increasing financial constraints on companies, membership in the Program increased from 28 to 32 in 1991! The companies that we are proud to acknowledge as friends of Cold Spring Harbor Laboratory are listed below. It was particularly pleasing that we now have three Japanese companies with us. In 1991, Sumitomo Pharmaceuticals Co., Ltd. and Toyobo Co., Ltd. joined Kyowa Hakko Kogyo Co., Ltd. in becoming members of the Program.

This level of funding enabled us to go ahead with confidence in putting together a truly outstanding meetings program at the Laboratory and at the Banbury Center. Details of these meetings are described elsewhere in this Annual Report. Corporate Sponsor attendance was strong at all meetings, but especially at the 56th Cold Spring Harbor Laboratory Symposium on Quantitative Biology on *The Cell Cycle*, the meeting on *Genome Mapping and Sequencing*, and the Cancer Cells meeting on *Regulation of Eukaryotic mRNA Transcription*. At the Banbury Center, no fewer than 15 Corporate Sponsors came to the meeting on *Adhesion Molecules and Their Receptors*, a record number!

It is, of course, the remarkable quality of the Laboratory's meetings programs that persuades so many of the world's leading biotechnology companies to join with us in promoting biological research. In acknowledgment of our members' contributions, we waive all on-site fees for eight representatives at our meetings. Three of these meetings may be at the Banbury Center, where attendance is otherwise only by invitation of the organizers. Furthermore, Corporate Sponsors receive gratis copies of all publications of the Cold Spring Harbor Laboratory Press, including the internationally acclaimed *Genes & Development* journal and the new, rapidly rising journal *PCR*. These benefits add up to a package that accounts for a substantial part of the membership fee.

Quite apart from the direct benefits that our sponsors receive, they are also acknowledged in all relevant publications, abstract books, and other materials published by Cold Spring Harbor Laboratory Press. In this way, the several thousand scientists coming here each year learn which are the companies that look beyond their own immediate interests to promote research throughout the world by their support of meetings at Cold Spring Harbor Laboratory.

Jan A. Witkowski

Alafi Capital Company
American Cyanamid Company
AMGen Inc.
Applied Biosystems, Inc.
Becton Dickinson and Company
Boehringer Mannheim Corporation
Bristol-Myers Squibb Company
Ciba-Geigy Corporation/Ciba-Geigy Limited
Diagnostic Products Corporation
The Du Pont Merck Pharmaceutical Company
Eastman Kodak Company
Genentech, Inc.
Hoffmann-La Roche, Inc.
Johnson & Johnson
Kyowa Hakko Kogyo Co., Ltd.
Life Technologies, Inc.
Eli Lilly and Company
Millipore Corporation
Monsanto Company
New England BioLabs, Inc.
Oncogene Science, Inc.
Pall Corporation
Perkin-Elmer Cetus Instruments
Pfizer Inc.
Pharmacia Inc.
Sandoz Research Institute
Schering-Plough Corporation
SmithKline Beecham Pharmaceuticals
Sumitomo Pharmaceuticals Co., Ltd.
Toyobo Co., Ltd.
The Upjohn Company
The Wellcome Research Laboratories, Burroughs Wellcome Co.
Wyeth-Ayerst Research

SECOND CENTURY CAMPAIGN

January 1, 1986–December 31, 1991

Unrestricted Contributions $4,976,620

Anonymous
Anonymous
Anonymous
Mr. Amyas Ames
Mr. S. Reed Anthony
Mr. and Mrs. Benjamin H. Balkind
Mr. Edmund Bartlett
Mrs. Patricia Bell-Thomson
Mr. Rodney B. Berens
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Mrs. Jean Cattier
Chemical Bank
Dr. Eleanor Chernick
Dr. Bayard Clarkson
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Mr. Theodore N. Danforth
Mr. and Mrs. Norris Darrell, Jr.
Ms. Katya Davey
Mrs. Rosamond Arthur Dean
Mr. and Mrs. Raymond deClairville
Mr. Charles Delafield
Mrs. Jamie Tilghman Deming
Mr. and Mrs. Donald Deming
Mrs. Carolyn C. Dilworth
Mr. and Mrs. Joseph P. Downer
Dr. and Mrs. Lester Dubnick
Mr. and Mrs. James Eisenman
Mr. Martin Elias
Mr. William Everdell
Mr. and Mrs. Harold L. Fates
Mr. and Mrs. Jack B. Friedman
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Dr. Barry Michael Gaines
Mr. and Mrs. Clarence Galston
Mr. and Mrs. Robert B. Gardner, Jr.
Dr. and Mrs. H. Bentley Glass
BF Goodrich Company
Mr. Maurice Green
Mr. and Mrs. Edward Greenberg
Mr. C. Groel, Jr.
Mr. and Mrs. Gordon S. Hargraves
Mr. and Mrs. Henry U. Harris, Jr.
Mr. and Mrs. Sinclair Hatch
Dr. and Mrs. Alfred D. Hershey
Mrs. Julie Hunnewell
Mr. and Mrs. Robert Dean Jay
Mr. and Mrs. Morris I. Karpen
Mr. and Mrs. Albert Key
Dr. and Mrs. Alan M. Kisner
Mr. and Mrs. Townsend J. Knight
Dr. Barbara B. Knowles
Mr. and Mrs. Abraham Krasnoff
Mr. and Mrs. Mort Kunstler
Mr. and Mrs. Edward M. Lamont
Mrs. John H. Leib
Mr. and Mrs. George N. Lindsay, Jr.
Mr. Angus C. Littlejohn
Mr. and Mrs. John Livingston
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Ms. Martha Makanna
Lucille P. Markey Charitable Trust
Marks Family Foundation
Mr. and Mrs. Robert R. Maxfield
Mr. and Mrs. Edward McCann II
Mr. and Mrs. Warren J. McEntee
Miss Diana McIlvaine
Mr. and Mrs. Robert M. McLane
Mr. and Mrs. Robert G. Merrill
Mr. and Mrs. Clarence Michalis
Mr. William H. Miller
Mr. and Mrs. William R. Miller
Mr. O.B. Miller
Milliken Foundation
Leo Model Foundation
Mr. and Mrs. Grinnell Morris
Dr. and Mrs. Larry Nathanson
Mr. and Mrs. Adams H. Nickerson
Mr. and Mrs. Charles Noyes
Mr. and Mrs. George O'Neill
Mr. and Mrs. William H. Osborn, Jr.
Dr. and Mrs. David B. Pall
Mr. Edward L. Palmer
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Mr. and Mrs. Martin Pollak
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Mrs. H. Irving Pratt
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William and Maude Pritchard Charitable Trust
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Mrs. Edward M. Shepard
Mr. Richard Slade
Mr. and Mrs. H. Turner Slocum
Mr. and Mrs. Peter Solbert
Dr. George F. Sprague, Jr.
Seth Sprague Educational and Charitable Trust
The Stebbins Fund
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Drs. Carol and Sidney Strickland
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Mr. and Mrs. Seth A. Thayer
Mrs. Robert Tilney, Jr.
Mr. and Mrs. George Toumanoff
Dr. and Mrs. Robert N. Van Son
Mr. and Mrs. Philip R. von Stade
Mr. and Mrs. Colton Wagner
Mrs. Bayard Walker
Dr. Jonathan Warner
Dr. and Mrs. James D. Watson
Mr. and Mrs. Bradford G. Weekes III
John L. Weinberg Foundation
Mr. and Mrs. Taggart Whipple
Mr. and Mrs. A. Morris Williams
Mrs. Henry A. Wilmerding
Mrs. John C. Wilmerding
Mr. and Mrs. Robert Winthrop
Mr. and Mrs. Sigi Ziering

Restricted Contributions — $39,450,474

Endowment — $5,764,470

Anonymous Professorship in Molecular Neuroscience
Anonymous Senior Staff Fellowship for Cancer Research
Doubleday Professorship for Advance Cancer Research
Russell and Janet Doubleday Fund
Garfield Undergraduate Internship
George Garfield
Oliver and Lorraine Grace Director's Chair
Libby Undergraduate Internship
Holly Brooks
Margaret S. Pomeroy Cancer Research Chair
Charles Robertson Neuroscience Chair
Frederica von Stade Undergraduate Internship
Posy White Fund
Elinor White Montgomery
Mrs. Edward H. Auchincloss
Mrs. Jean Cattier
Mr. and Mrs. David C. Clark
Mr. and Mrs. Paul Sheeline
Mrs. Alexandra White Smith
Mrs. George G. Walker

Program — $2,000,000

Howard Hughes Medical Institute

Facilities Development — $31,686,004

Cancer Biology

Michel David-Weill Foundation
Lita Annenberg Hazen

Fannie E. Rippel Foundation
Starr Foundation
Margo Geer Walker

Neuroscience Center

The Achelis Foundation
Anonymous
George F. Baker Trust
Arnold and Mabel Beckman Foundation
The Bodman Foundation
Booth Ferris Foundation
Mary Livingston Griggs and Mary Griggs Burke Foundation
Mr. and Mrs. Arthur M. Crocker
The Ira W. DeCamp Foundation
Mrs. Duncan Elder
Dolan Family Foundation
Samuel Freeman Charitable Trust
Florence J. Gould Foundation
Lita Annenberg Hazen
Howard Hughes Medical Institute
W.M. Keck Foundation
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The Kresge Foundation
Litchfield Charitable Trust
Lucille P. Markey Charitable Trust
James S. McDonnell Foundation
Nichols Foundation
Pew Charitable Trusts
Fannie E. Rippel Foundation
Mr. and Mrs. Gilbert Tilles

Infrastructure

Boehringer Mannheim
Bristol-Myers Squibb
Burroughs Wellcome
CibaGeigy
E.I. DuPont
Lita Annenberg Hazen
Hoffman-LaRoche
Merck and Company
Pall Corporation
Rorer Group
Schering Plough
SmithKline Beecham
Upjohn Corporation
Westvaco Corporation

Structural Biology

Oliver and Lorraine Grace
Lucille P. Markey Charitable Trust

Page Laboratory of Plant Genetics

Anonymous
Charles E. Culpeper Foundation
Ira W. DeCamp Foundation
Long Island Biological Association
Morgan Guaranty Trust Company of New York
National Institute of Health
National Science Foundation
North Country Garden Club
Dr. and Mrs. David B. Pall
Pioneer Hi-Bred International
William and Maude Pritchard Charitable Trust

Guest Facilities

Cabins
- Alumni Cabin Contributors
- Dr. Herbert Boyer
- The Hyde-Watson Foundation
- Dr. Thomas Maniatis
- New England Biological Laboratories
- Dr. Mark Ptashne

Visiting Scientist Guest House
- Russell and Janet Doubleday Fund

DNA Learning Center
- Banbury Fund #1
- Banbury Fund #2
- The Brinkmann Foundation
- Mr. and Mrs. Henry U. Harris, Jr.
- New York State
- Stone Foundation, Inc.
- Weezie Foundation

Total Second Century Campaign Contributions	**$44,415,094**
Net Interest Earned 12/31/91	**$1,538,100**
Final Total	**$45,953,194**

SECOND CENTURY CAMPAIGN COMMITTEES

David L. Luke III, *Chairman*

Steering Committee

Oliver R. Grace, *Co-chairman**
George W. Cutting, Jr., *Co-chairman*
Mrs. Sinclair Hatch, *Co-chairman*
Townsend J. Knight, *Co-chairman*
Taggart Whipple, *Co-chairman*

Leadership Gifts Committee

Oliver R. Grace, *Chairman**
G. Morgan Browne
Bayard D. Clarkson, M.D.
David L. Luke III
James D. Watson, Ph.D.
Taggart Whipple

Major Gifts Committee

Mrs. Sinclair Hatch, *Chairman*
Amyas Ames
Mrs. Harry A. Brooks
Robert L. Cummings
William Everdell
Mrs. Henry U. Harris, Jr.
Abraham Krasnoff
Laurie Landeau, V.M.D.
Dr. Cynthia MacKay
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H. Turner Slocum
Colton Wagner

Special Gifts Committee

George W. Cutting, Jr., *Co-chairman*
William H. Miller, *Co-chairman*
Mrs. Philip R. von Stade, *Co-chairman*
Mrs. Edward Greenberg, *Co-chairman*
Edmund Bartlett
David C. Clark
H. Gray Colgrove*
Arthur M. Crocker
Lawrence L. Davis
Mrs. Donald L. Deming
Joseph P. Downer
Charles J. Hubbard
Gordon Lamb
Edward Pulling*
John R. Reese
J. Wright Rumbough, Jr.
Mrs. James F. Stebbins

Corporate Committee

David L. Luke III, *Chairman*
G. Morgan Browne
Peter B. Cannell
Robert E. Cawthorn
William R. Miller
David B. Pall, Ph.D.
Douglas E. Rogers
James D. Watson, Ph.D.
Henry Wendt
Sigi Ziering, Ph.D.

Foundation Gifts Committee

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Mrs. C. Payson Coleman
Robert Ebert, Ph.D.
James W. Fordyce
John Klingenstein
Clarence F. Michalis
William E. Murray

Planned Gifts Committee

Townsend J. Knight, *Chairman*
Maureen Augusciak
John Cleary
William J. Kramer
William Parsons, Sr.
Colton Wagner

*deceased 1992


LONG ISLAND
BIOLOGICAL
ASSOCIATION

Officers

George W. Cutting, President
Mrs. Henry U. Harris, Jr., Vice-President
Mrs. E. Maxwell Geddes, Secretary
Byam K. Stevens, Jr., Treasurer
G. Morgan Browne, Asst. Secretary/Treasurer

Directors

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Mrs. Harry A. Brooks
John P. Cleary
George W. Cutting
Mrs. Donald L. Deming
Joseph P. Downer
John R. Drexel IV
Mrs. Duncan Elder
Mrs. E. Maxwell Geddes, Jr.
Mrs. Edward Greenberg
Mrs. Henry U. Harris, Jr.
Mrs. Sinclair Hatch
Alan M. Kisner, M.D.
Gordon E. Lamb
Mrs. James L. Large
Edward F. McCann II
Mrs. Walter C. Meier
Mrs. Vincent Nesi
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James L. Spingarn
Byam K. Stevens, Jr.
Mrs. Philip R. von Stade
James D. Watson, Ph.D.

CHAIRMAN'S REPORT

With the "number crunching" in Washington in recent years, it seemed a particularly appropriate time to redefine the mission of the Long Island Biological Association (LIBA) today and make whatever changes would best serve the interest of the Cold Spring Harbor Laboratory in the future. In February 1991, the Board of Directors determined that it should gather for an extensive study of LIBA and its relationship to the Laboratory. Jane Pierson of Cavanaugh, Hagan, and Pierson in Washington, D.C. was engaged as "Facilitator," and the Arden Homestead was reserved for the second weekend in November. The process of preparing ourselves was in itself an interesting effort, and this effort produced a "mission statement" for LIBA today:

- raising unrestricted annual support for research and education at the Laboratory
- informing the public about the science and education programs of the Laboratory and their importance to us all
- assisting the scientific staff with personal and family needs.

The "retreat" itself was attended by 19 of the 27 Directors. Most importantly, the mission was affirmed, and the Directors resolved to implement this mission through a more organized effort using the expertise and interests of its members and their contacts in ever broadening areas.

As this report goes to press, the membership of LIBA will be asked to approve some changes in the By-Laws of the corporation and a change of its name to Cold Spring Harbor Laboratory Association. Although we have become very accustomed to "LIBA" over the years, it seemed to the Directors that in today's world, particularly with our attempts to draw support from more distant areas, the confusion of LIBA's identity and its relationship to the Laboratory had become more and more apparent.

The role that the Cold Spring Harbor Laboratory plays in the search for answers to many afflictions through basic research will require an ever-increasing level of unrestricted support from individuals. Therefore, all effort has been taken to make the association of supporters as effective as possible.

As for 1991 operations, I am pleased to report that in LIBA's fiscal year, which ended September 30, 1991, significant progress was made in all three areas. Contributions and membership have reached new all time highs. These results come from a number of fund-raising activities involving many members of the Laboratory staff and volunteers.

ANNUAL GIVING

Year ended September 30,	1991	1990
Contribution	$384,571	$284,214
Associates (Donors of $1,000 or more)	156	139
Total number of contributors	829	671
LIBA endowment funds	$115,686	$87,216

Included within the Annual Giving totals are Memorial Contributions which were given in memory of:

Agnes Alesi
Carmela Barbagallo
Caroline Bomeisler
Mamie Botwick
Leonie Brigham
Rudy Caputo, Sr.
Margaret Carroll
Merrill Christiansen
Garner Cline
Beverly Davis
William M. Duryea
Harry Dwoskin
Nick Giammalvo
Arthur Greenberg
Herbert Hallaran
Maxine Harrison
Philip K. Hills
Frank Hohner
Thomas Holland
Robert Humphrey
James M. Kay
Robert Kalka
Lilly Kurfess
Louis Maniscalco
Mimi Matthaei
Helen Morris
Eva Nagy
James L. O'Conner
Daniel Olian
Jimmy Parsons
Edward Pulling
Madeline Ridder
Salvatore Santoro
Anthony Snow
Joseph Squillaro
Elizabeth Schneider
Clara Wisnewski

Group and individual tours of the facilities increased as well as the total attendance at LIBA lectures, and new efforts have been taken to assist the families of our scientists with some particular success in finding affordable housing in the area. All this has been made possible through the efforts of the Laboratory's small, but most effective, development staff, LIBA Directors, and the special efforts of many members.

Winter '91

The Annual LIBA Meeting was held on Sunday, January 13th in the Grace Auditorium with more than 250 LIBA members, guests, and members of the North Country Garden Club in attendance. After a call to order, retiring Directors Eleanor Campbell, Jack Evans, John Reese, and Susie Trotman were thanked for

Members of the Long Island Biological Association (LIBA)

their loyal service. In addition, Taggart Whipple was named Ambassador of the Year for his continuing efforts to bring the Laboratory's importance and its needs to the attention of the surrounding community and to his friends and associates nationwide. Larry Davis, Chairman of the Nominating Committee, which included Eleanor Campbell, John Cleary, and Phyllis Weekes, presented a slate of new Directors. Holly Brooks, Nick Drexel, Laurie Nesi, and Jim Spingarn were approved by acclamation.

Judith Chapman, Chairperson of the North Country Garden Club, and her committee arranged for Richard W. Underwood to address us. Mr. Underwood is a pioneer in space photography, worked for NASA for 30 years, and authored numerous technical papers in photogrammetry and aerospace technology. He presented an interesting talk on "The World's Environment: The View from Space." Startling were the pictures of pollution in our harbors and rivers and the uncontrolled advance of the Sahara Desert.

Spring '91

Through the invitation of Dr. Alan Kisner, a LIBA Director, a "Saturday Afternoon at the Lab" was arranged for doctors at Huntington Hospital and their families. More than 50 accepted and were given tours through the facilities including discussions with several scientists. The afternoon ended with the guests attending an Associate lecture by Drs. Nouria Hernandez and Winship Herr on their AIDS research. Nouria and Winship and many other scientists provide LIBA with a most valuable link in supporting our effort to explain scientific research to the community in terms the public understands. This is an important part of LIBA's mission, and we sincerely thank them all for giving us their most valuable time.

As the Laboratory's Banbury Center had arranged a special conference on Lyme disease, the LIBA Board of Directors felt that it would be a service to the community to present a panel discussion on the disease for the public. Some of the world's leading authorities on Lyme disease presented the latest information on research, diagnosis, and treatment. The announcement was sent to LIBA membership, as well as to Long Island newspapers and veterinary clinics. The attendees included many residents who are suffering from Lyme disease and who had never visited the Laboratory. As a number of new contributors joined the LIBA rolls, this event served as another Laboratory outreach.

On April 28, James L. Spingarn and his wife Jane gave a cocktail reception for 35 of their friends at the Laboratory following a tour of the campus. This introduction has resulted in numerous new members and Associates.

In the past several years, the Dorcas Cummings Memorial Lecture has been given during the Cold Spring Harbor Laboratory Symposium on Quantitative Biology. The topic in 1991 was "The Cell Cycle," and LIBA invited Dr. Michael Bishop, a 1989 Nobel laureate, to address its membership. Following in the footsteps of Dr. Francis Crick the year before, Dr. Bishop's talk titled "Misguided cells: The Genesis of Human Cancer," which was augmented by excellent slides and films, was delivered with great humor. The more than 500 LIBA members, guests, and visiting scientists received a "primer" on how cells divide and how cancers are formed when control of cell division is lost. Dr. Bishop was one of the earliest scientists to recognize the impact of oncogenes. Following the talk, the traditional "Symposium dinner parties" were given for the visiting scientists and many members of the Laboratory's staff. This year's hosts and hostesses were

Mr. and Mrs. Harry A. Brooks
Mr. and Mrs. Morgan Browne
Mr. and Mrs. John P. Cleary
Mr. and Mrs. W. Tucker Dean
Mr. and Mrs. James J. Eisenman
Mr. and Mrs. Henry U. Harris
Mrs. Sinclair Hatch
Mr. and Mrs. David L. Luke III
Mr. and Mrs. Vincent R. Nesi
Mr. and Mrs. David B. Pall
Mrs. Francis T.P. Plimpton
Mr. and Mrs. Thomas A. Saunders III
Mr. and Mrs. James F. Stebbins
Mr. and Mrs. Byam K. Stevens, Jr.
Mr. and Mrs. John C. Stevenson
Mr. and Mrs. Martin B. Travis, Jr.
Mr. and Mrs. Robert P. Walton
Mr. and Mrs. Taggart Whipple

Summer '91

On June 16, Dr. and Mrs. Watson gave a dinner party for Dr. John Cairns and his wife Elfie, who were returning to their native England after many years in this country. Dr. Cairns was Director of the Laboratory from 1960 to 1968 and a distinguished researcher in Cambridge, Massachusetts since that time. Attending this wonderful event were the present LIBA Directors as well as many former Directors who had close personal ties with the Cairns family over the years.

Fall '91

Through the great generosity of Mrs. Eric Ridder, who loaned 32 of her miniature rooms for display in the Plimpton Seminar Room on September 21, a benefit reception for the Laboratory was held giving the attendees an opportunity to view the exquisite creations which varied from colonial to the most elaborate rococo. Many of the rooms included whimsical animals which added to their charm. The success of this event was ensured by the tireless effort of Mrs. Sinclair Hatch and her committee.

The following day, the 5th annual Associates cocktail reception was held to give the Cold Spring Harbor Laboratory Associates (contributors of $1000 or more) a chance to see Mrs. Ridder's wonderful works of art. The Associates, whose number now stands at 156, provide the Laboratory with the cornerstone in its Annual Giving campaign. It is with great sadness that we note the sudden death of Mrs. Ridder on November 2, 1991, so soon after her wonderful show.

Probably the most important contribution that LIBA has made to date in its mission to inform the community about elements of biology was a lecture on "Genetics of Breast Cancer" given by Dr. Mary-Claire King and Dr. Marc Lippman on November 11th. They were attending a conference at the Banbury Center and agreed to talk to our membership about some of the recent discoveries. As Dr. King is an epidemiologist and Dr. Lippman a surgeon, the exchanges between them and the audience were most informative. We hope to be able to present a similar program again in early fall 1992.

These specific LIBA events were augmented throughout the year by the special efforts of LIBA Directors and other members who introduced some of our scientists to new funding sources, and new members of the community to the Laboratory.

It is only through the combined energetic response from so many LIBA members that the results shown in this report were achieved. Clearly, this partnership has made the Cold Spring Harbor Laboratory a unique, independent, research institution.

George W. Cutting, Jr., Chairman

Members of Long Island Biological Association

COLD SPRING HARBOR LABORATORY ASSOCIATES*

Anonymous
Mr. Warren S. Adams II, Esq.
Mr. Amyas Ames
Mr. & Mrs. David L. Banker
Mr. & Mrs. Vincent C. Banker
Mrs. William N. Bannard
Mr. & Mrs. Sherburn M. Becker
Mr. & Mrs. Rodney B. Berens
Ms. Jane S. Block
Mr. & Mrs. Allen L. Boorstein
Dr. David Botstein & Dr. Renee Fitts
Mrs. William T. Brigham
Mr. & Mrs. Lester E. Brion, Jr.
Mrs. Harry Brooks
Drs. John & Elaine Broome
Mr. & Mrs. G. Morgan Browne
Ms. Mary Griggs Burke
Mr. & Mrs. John P. Campbell
Mr. & Mrs. Peter B. Cannell
Mr. & Mrs. Vincent Carosella
Centerbrook Architects
Mr. John T. Chandler
Mr. & Mrs. Thomas H. Choate
Mr. & Mrs. David C. Clark
Dr. & Mrs. Bayard D. Clarkson
Mrs. Robert L. Clarkson, Jr.
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