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DIRECTOR’S REPORT

I have now been closely associated with Cold Spring Harbor Laboratory for more than 45 years. During this almost half-century interval, the Lab has both stayed the same and changed itself radically. What initially made me so admire, if not love, the Lab was the beauty of its harbor and buildings, then populated by individuals such as Barbara McClintock and Alfred Hershey, who had extraordinarily high intellectual objectives and standards. There was then, as still today, no snobbery or hypocritical obeisance to age or authority. Instead, what mattered was the truth or falsity of the facts and ideas of genetics. As a 20-year-old summer student, I felt both liked and needed for wanting passionately to unravel the essence of the gene, the master unit of heredity that we now know to be composed of DNA.

This preoccupation with genetics, which for so long made Cold Spring Harbor Laboratory so unique, goes back to the turn of the century. It was then that Mendel’s laws, effectively lost for 35 years, were rediscovered independently by the European plant breeders Correns, de Vries, and Tschemark. In 1904, Cold Spring Harbor became one of the first sites to take on the challenge of the new genetics when money from Andrew Carnegie’s steel fortune was used to create a Station for Experimental Evolution here. It was headed by the Harvard-trained zoologist Charles B. Davenport, who was already in charge of a summer teaching program at The Biological Laboratory. This neighboring institution had been started in 1890 by the Brooklyn Institute of Arts and Sciences and supported by the local Jones family, one of whose houses adjacent to the Lab was the largest on Long Island until its destruction by fire in 1861. Initially, the main preoccupation of the Station, later to be renamed the Department of Genetics of the Carnegie Institution of Washington, was to extend the validity of Mendel’s laws to a large variety of plants and animals including our own human species.

To carry out these studies, which soon made Cold Spring Harbor a world-class leader in genetics, several new buildings in Italianate style were constructed using designs from leading architects of the time. Initially, these new structures must have seemed discordant with the more traditional New England style buildings along the western shore of the inner harbor, where the whaling industry of the Jones family had been located from the 1830s until the start of the Civil War. Particularly stark and ungraceful in early photographs was Blackford Hall. This residence and dining hall was built in 1907 from reinforced concrete at the suggestion of the Lab’s early patron William John Matheson, a founder of Allied Chemical, whose massive, brick, Tudor style home, “Fort Hill,” graced the entrance from Long Island Sound into Cold Spring Harbor. But by the time I first arrived here some 40 years later, the various buildings worked well together nestled amidst the trees that had grown up between them. I remember well one particular early June day at sunrise when, already exposed to the magnificent buildings of the old world Cambridge, I thought Blackford to be one of the world’s most beautiful buildings.

But these buildings from the past were not equal to the next phase of genetics, whose objective was to understand the gene at the chemical level. The
director at that time, Milislav Demerec, rose to the challenge by selling off Lab-owned land along both sides of Stewart Lane to build, in 1953, the box-shaped, concrete laboratory that today bears his name. Seen at night from across the harbor, its long regular rows of windows conveyed a commercial feeling that jarred with the sylvan feeling of the inner harbor then dominated by the Moorings Restaurant, whose red leather interior long graced Long Island’s fabled Gold Coast. Also strange must have seemed the new Scandinavian style auditorium that later was to be named after Vannevar Bush, who then led the Carnegie Institution of Washington. This 250-seat edifice was sorely needed to maintain the viability of the annual June Symposium when attendance was routinely creeping beyond 100 and thus impossible to accommodate in the Fireplace Room of Blackford. No one, however, then protested its appearance, since it gave to the Symposium a home appropriate for its ever-growing reputation as the world’s most influential meeting in biology.

These 1950s additions soon blended with those of the past, with motorists driving along route 25A always much less conscious of the Lab than of the elegant 1836 Jones family church, St. John’s, and the adjacent Fish Hatchery, whose 1881 founding by John D. Jones and E.G. Blackford just preceded that of The Biological Laboratory in 1890. Even when I became the director in 1968, the Lab’s existence was unknown to many nearby residents who, if asked, would most likely have said that the buildings stretching northward along Bungtown Road were connected to the Fish Hatchery. Equally at variance with reality were the perceptions of those biologists who only knew the Lab indirectly through its yearly Symposium volumes and from the growing reputation of its summer courses in which the experimental techniques of the new DNA-based molecular biology were taught. It was presumed that such prestigious activities very likely had to emerge from up-to-date laboratories and auxiliary buildings. But all insiders knew that except for two relatively new buildings dating from the 1950s, virtually all of the other buildings on the 95-acre waterfront campus bordered on the derelict. If such decay was not attended to soon, high-level science at Cold Spring Harbor would soon disappear.

The thought that Cold Spring Harbor Laboratory might vanish obviously worried its many friends, and it was to prevent that from happening that I became director in 1968. Key to that decision was the reassurance from Harvard University that I would remain a salaried professor there as long as I continued to give my lectures and oversee those graduate students doing their Ph.D. thesis work under my supervision. So I could worry about the future of the Lab without fear that my salary might vanish along with the Lab. My decision to take on the task of saving science at Cold Spring Harbor was not, however, an entirely selfless act. As the director here, I would have the ability to start a major effort focused on using tumor viruses to work out the genetic basis of cancer, an intellectual objective that increasingly dominated my thoughts after 1966. By then we knew the main features of how RNA is involved in protein synthesis, my main intellectual goal since finding the double-helical structure of DNA in 1953. From the start, I saw that the task of understanding cancer would require large team efforts involving many senior scientists with highly different backgrounds and so beyond my resources as a professor at Harvard. There my research space linked me to a research group of at most ten students and postdocs. In contrast, there would be no limitation of available laboratory space at Cold Spring Harbor provided I could obtain the funds to upgrade the unoccupied but dilapidated structures.

Ordinarily, incoming directors of research institutions inherit significant numbers of scientists appointed by previous directors and whose continued
presence inhibits the new director from moving swiftly in new intellectual directions. But the poor physical condition of most of our laboratories coupled with the lack of any long-term fiscal stability had led many previous staff members to move to more stable academic positions. At least initially, we had all the space we needed as long as I could find the funds to renovate preexisting laboratories into acceptable facilities for molecular work on cancer-causing viruses.

Fortunately, this proved to be an easy task, since in 1968 there was far more federal money available for research on cancer than could be used effectively by the scientists then working on this disease. At that time, cancer was thought to be deadly not only for its unfortunate victims, but also for the careers of those scientists who invariably failed to understand its underlying diabolically complex molecular mechanisms. Most first-class biochemically oriented scientists in the prime of their careers thus stayed away from work on cancer.

But the public did not know this, and each year Congress could be counted on to vote even more money for cancer research, and so the money was there. Soon after I became director, the National Cancer Institute was asked for funds both to renovate James Laboratory, parts of which remained virtually unchanged since its construction in 1929 as a concrete shell for biophysics research, and to bring to the Lab some top-level younger scientists already trained to work on tumor viruses. Key to our getting the grant was Joe Sambrook's decision to move here from the still almost new Salk Institute. At the Salk, Joe was one of the younger stars working in Dulbecco's DNA tumor virus group. With his arrival, we instantly had the potential to soon become one of the world's best cancer research laboratories. But I worried about the disparity between the facilities here and those in the magnificent Louis Kahn-designed Salk laboratories and soon was out seeking funds for an annex to James that would allow the scientists to have offices separate from where they worked with viruses as well as a library seminar room where experiments could be discussed over coffee. Then only $200,000 was needed to build the imaginative wooden structure that had been designed by the talented New York architect Harold Edelman. But even this relatively small sum greatly exceeded any funds at our disposal, and so it was to our great good fortune that retired Pfizer executive John Davenport decided to make the major gift that allowed the Annex to be completed early in 1971. By design, the unpainted cedar boards of the Annex blended nicely into the hill behind it, and the grand Norway spruce in front of it made it virtually invisible from across the harbor, except at night when its large windows reflected the fact that science here goes on until very late in the evening.

The James Annex was not the first new building of my directorship. Upon accepting the directorship, I and Liz, whom I had just married, decided to live in Osterhout, then an older building dating from just before 1800. Feeling flush with the royalties from my just published, best-selling book The Double Helix, I gave the Lab the funds to totally renovate Osterhout and expand it to the designs of Harold Edelman. When construction started in the summer of 1968, however, the builder quickly told us that Osterhout had decayed beyond saving, but he offered to build a completely new structure for the price agreed upon for the renovation. Everyone liked the final result, which still had early New England features, and no one complained that we had given up part of our whaling-era identity.

Happily, we were able to save the 1830-era Wawepex, despite its advanced decay which had required us to abandon its use. Using monies from Manny Delbrück, who with her husband Max had been a summer visitor here for 25 years, Wawepex was converted from a primitive laboratory to a year-round residence capable of housing 16 students during the summer teaching intervals. By then,
the crisis atmosphere that had enveloped Cold Spring Harbor for more than a
decade had passed, and our scientists could worry about their experiments with
tumor viruses rather than about whether they would have jobs over the foresee-
able future. The Lab thus looked very alive when we learned in the summer of
1972 that our neighbor Charles S. Robertson, whose large estate in Lloyd Harbor
bordered on Cold Spring Harbor, was seeking an institution to make use of the
land and buildings on Banbury Lane that he no longer wished to keep after the
unexpected death that spring of his beloved Marie.

A visit was soon arranged for him to tour the Lab and to have lunch at Oster-
hout, whose large picture windows on the harbor side revealed the summer
beauty of Cold Spring Harbor at its best. Within days, we knew we had won his
support to a degree that we had never anticipated. Not only would he deed to us
his wonderful Lloyd Harbor property, but even more important, he also planned
to give us an endowment of 8 million dollars to support research at the
Laboratory. Gifts like this generally go only to distinguished universities whose
elegant buildings convey the fact that they will live forever. The fact that both the
James Annex, in which by then my office was situated, and Osterhout were ar-
chitecturally elegant had to have been a factor in convincing Charles Robertson
to choose us as beneficiaries of his great philanthropy. The message was clear:
Whenever possible, we should design attractive buildings appropriate for the fu-
ture of an elite institution.

The means were thus available to expand further the research done at the
Lab by additional renovations to preexisting spaces. At the same time, ever-
increasing community support for the Lab, marshaled by Edward Pulling's firm
leadership of the Long Island Biological Association (LIBA), allowed us to sys-
tematically renovate the other buildings on the Lab grounds, in particular to con-
vert many from summer-only to year-round use. In this way, Blackford Hall ac-
quired the heating system that lets this dining hall function year-round. It was also
LIBA's help that let us buy the circa 1885 Takami House (now Olney) that was
surrounded by Lab grounds and that by then included the field to the north which
had once been owned by Charles Davenport. Several years later, thanks again to
LIBA support, a new Williams House modeled after the original unsalvageable
1830 "tenement" gave us five new apartments for visiting scientists and course in-
structors.

My decision to give up our house near Harvard and move full-time to Cold
Spring Harbor led to the 1973–1974 renovation of Airlsle, the director's residence
since 1943. This 1806 former Jones family farmhouse had been purchased in
1943 from Mrs. de Forest following the death of her noted philanthropic and capi-
talist husband and the subsequent breakup of their large estate at the end of
Bungtown Road. Their specimen-tree-covered property adjacent to the Lab
stretched up over Ridge Road, extending northward to virtually contact the estate
of their close friend Louis Comfort Tiffany. Following the acquisition of Airlsle, the
Lab did not have the monies to renovate it properly, and its north-facing rooms
remained largely unheatable during the severe cold of the winter. But by 1973 we
had the resources to carry out a clever renovation scheme proposed by the al-
ready celebrated architect Charles W. Moore, who then headed the School of
Design at Yale. Moore's plan left the 1806–1850 exterior virtually unchanged but
radically changed the inside space, in particular by creating a three-storied
entrance hall that went to the top of the house. When so renovated, Airlsle was a
major architectural triumph, photos of which later graced architectural books and
magazines.

I had to feel highly privileged living in such an exciting yet so livable house,
whose renovation costs totaled almost $200,000 for its 6600 square feet of space. My intent, however, was to raise all of our housing to such standards, and we later spent roughly the same sum to renovate the by then totally derelict Victorian house located at the Bungtown Road entrance to the Lab. Now called Davenport House, it was built in 1884, and since the 1930s has continually served as a large residence for postdoctoral fellows and graduate students. Most important, my wife Liz, who oversaw the renovation project, had the house repainted in its original six Victorian colors which now give continuous pleasure to the thousands of motorists who drive past it on route 25A each day.

With the renovation of Davenport, virtually all of the previously derelict buildings had been restored to functional use. Our gamble that we could do first-class research on tumor viruses using preexisting space more than paid off, with Cold Spring Harbor already acknowledged by the mid 1970s as one of the world's leaders in this area. Admittedly, a 1000-square-foot west annex to James had to be built in 1973 to provide special rooms for the growth of viruses, but that we could so quickly leap-frog scientifically ahead of the Salk shows that the heart of success depends much more on the esprit and competence of specific scientists than on the grandeur of the facilities in which they work. Certainly the 1970s represented one of the best decades in the Lab's history, with last year's Nobel Prize in Medicine or Physiology awarded to Richard Roberts for research done here between his arrival in 1972 and the historic discovery of RNA splicing in 1977.

The challenge of the 1980s was completely different with the world of molecular biology exploding because of two new important methodological developments of the 1970s—that of recombinant DNA (1973) and that of monoclonal antibodies (1975). Effective utilization of recombinant DNA procedures was held up for several years because of misplaced concerns about potential biohazards, but no such inhibitions held up work on monoclonal antibodies. This technique had already been brought here in 1978 when David Lane arrived from London to use monoclonal antibodies to study the T antigen, the SV40 tumor virus protein at the heart of the cancer-causing potential of this virus. The making of such antibodies required the facilities for a mouse colony, and the tiny *mouse house* that we had placed in the former sheep shed next to McClintock Laboratory was inadequate for the tasks ahead. Using funds from the Kresge Foundation and the National Cancer Institute, a modern, barn-like, animal facility, the Harris Building, was finished in 1982 on the site where a woolen-mill-era building had existed since the early nineteenth century.

At the same time, to create the additional laboratories needed to properly use monoclonal antibodies for cancer research, a major addition to James Laboratory was finished in 1985. Its planned existence allowed us in 1983 to recruit the American-born and -educated Ed Harlow from London, where his Ph.D. thesis research had involved monoclonal antibodies. In retrospect, the building of this turreted wooden-clad north annex, now known as Sambrook Laboratory, was a crucial step in the Lab's development since it let Ed make one of the great discoveries of all tumor virus research. To our amazed delight, in 1987 he showed that the key adenovirus E1A protein binds to a normal cellular protein that functions to inhibit cell growth—a tumor suppressor protein. Without the opportunity the Sambrook Laboratory gave to Harlow to form his own lab group, the discovery that capped some 30 years of worldwide research on DNA tumor viruses would most likely have occurred elsewhere. Instead, our leading position in cancer research was retained.

There also arose the need to build two successive additions to the 1926
waterfront "Long Island Colonial" style building just off Bungtown Road that I first knew as Davenport Laboratory. Robertson Research Funds had permitted it to be upgraded into a heated year-round facility in 1973 so that it could function as the home of our tiny but intellectually powerful Yeast Genetics group. The aim of this group was to determine a molecular genetic mechanism for how yeast cells can suddenly switch from one sex to the other. Using the new procedures of recombinant DNA, Jim Hicks, Amar Klar, and Jeff Strathern hoped to establish the validity of their "cassette model," which time has shown to be a revolutionary step forward in understanding how genetic information is moved from one chromosomal site to another. But they had to do so in the context of routinely losing their labs when the summer courses were taught. To solve this dilemma, a modest addition, designed to look from across the harbor like a separate whaling-era house, was made to the south in 1981. The same architectural trick was used six years later when a slightly larger north addition was added that let us seriously move back into plant genetics to exploit Barbara McClintock's jumping genes idea, which by then had widespread implications for both pure science and commercial plant breeding.

During the same period, we made two additions to the by then 30-year-old Demerec Laboratory to let it function more effectively as a center for the further development of recombinant DNA technology, particularly in the area of protein and nucleic acid chemistry. Of these, the first addition, made in 1982, was the largest, being built using monies from Exxon for facilities needed for our joint program to introduce advanced recombinant DNA technologies into our respective research programs. Before we signed this major agreement with Exxon, which was to provide us with 7.5 million dollars over five years, we had reason to worry that Cold Spring Harbor would become an increasingly marginal player in science as recombinant DNA techniques more and more dominated biological research. Happily, Exxon's help came just in the nick of time to let us compete successfully with major institutions doing biomedical research, virtually all of which were adding large new facilities for DNA-based research. Now, in retrospect, the 1980s were every bit as successful scientifically as the 1970s, with the computerized citation indices, which track the key scientific articles that are quoted by other scientists, now revealing us as the most influential research institution in the world doing biomedical research over this two-decade interval.

A major contributing factor to this success has been our advanced courses and meetings which set out the important challenges and dilemmas of biology. During the spring, summer, and fall sessions, we function, in effect, as the international center for DNA-based research. By the middle 1980s, however, these teaching and conference activities were coming under increasing strain as they continued to grow in size and number. Not only was Bush Auditorium much too small for meetings attended increasingly by more than 300 scientists, but also our dining and on-site housing could no longer handle a meeting schedule that now stretched into summer months when the rain could no longer be counted on to feel warm. Closed-circuit TVs, long food lines, dining tents, and massive busing to not always near motels could no longer be the norm if we were to have a future for disseminating important biological facts and techniques.

The first challenge we faced was to build a bigger home for our world-famous Symposium, which we did in the shape of the strikingly elegant, brick-faced, Oliver and Lorraine Grace Auditorium. This 360-seat almost-square structure embodies both modern and traditional features, with its dormer-windowed south-facing facade warmly compatible with neighboring multicolored Davenport House. Upon its completion in 1986, we possessed for the first time a building
more appropriate for our increasingly year-round, as opposed to summer-camp-like, existence. That our new auditorium enhanced, not diminished, our always cherished sense of history led to a complete change in our thoughts as to how we should further develop our "middle campus" land on the hill to the west of Blackford Hall. In the mid 1950s, part of this hill had been flattened for the Page Motel and its companion parking space to provide additional housing for summer visitors. Three decades later, we began to think about replacing this by then very dilapidated relic with a much larger Adirondack style year-round lodge. It was to be adjacent to a modest new laboratory for neurobiology, also designed to look like a component of a summer camp but also suitable for winter use.

That we needed to expand in the area of neurobiology had been obvious ever since the Sloan Foundation had given us the funds in 1970 to initiate a program of advanced courses on nerve cells and how they underlie perception, memory, and thinking. Understanding the brain was very likely to be the ultimate frontier for biology, an undertaking soon to be populated by many of the brightest and most courageous minds in science. Increasingly obvious to virtually all perceptive biologists was the fact that the brain would be to the twenty-first century what the gene was to the twentieth century—the place where the real action lies. Moreover, especially after the onset of the recombinant DNA revolution, genetic methodologies were likely to prove indispensable to understanding how the brain develops and functions. Much useful synergism would develop by promoting neurobiology at institutions with strong genetic heritages. So in the late 1970s, the recently created Marie H. Robertson Fund for Neurobiology helped us attract several young neurobiologists to use our summer neurobiology teaching labs for their year-round research, retreating during the summer course season to the former mouse house now converted to a tiny laboratory. The research they did was first-class, but, for lack of appropriate research space, all too soon they were recruited away to institutions where they could properly exploit their new advances.

In 1985, when Sue Hockfield, our last remaining talented young neurobiologist, had left us for Yale, we decided to seek the funds for a modest neurobiology laboratory in which both advanced teaching and research would occur. Our planning, however, necessarily only became focused when we knew that we had a realistic chance of raising the required multimillion-dollar sums. That moment came in the summer of 1987 when the Howard Hughes Medical Institute made a gift of 7 million dollars toward the building, its equipment, and staff support. By then the success of Grace Auditorium led us to decide on an "academic" as opposed to "rustic" design. So we asked our architects, Centerbrook of Essex, Connecticut, the descendant of Charles W. Moore's original firm, to design a building complex in which the neurobiology laboratory is separated by a courtyard from a 60-room residence for visitors to our meetings and courses. Particularly appealing in their resulting design was an imaginative bell tower that also functioned as smoke stacks.

No building anywhere near the size of the 1991-completed 65,000-square-foot Neuroscience Center had ever existed on our Bungtown Road site, and naturally concerns were voiced as to whether its existence would fundamentally change the way we do science at Cold Spring Harbor. But these worries vanished as soon as some of us had moved into Beckman Laboratory and the summer courses had been successfully run in its labs. Much more sophisticated experiments could be done in the new teaching laboratories than in the older facilities in James and Jones Laboratories, totally justifying the decision of the Howard Hughes Medical Institute to fund the new teaching laboratories. Although
we deliberately retained a hillside layer of deciduous trees that would make the Neuroscience Center effectively invisible from across the harbor in the summer, there was no way to keep it invisible during the winter, and its massive size upset some neighbors who would have preferred us to remain perpetually old-fashioned. But the planting of appropriate conifers eventually will give the Neuroscience Center the appearance of a lower building and, with time, I'm sure that its clever architectural details will begin to be appreciated in their own right as more and more of our neighbors accept the fact that we should look like the major academic institution that we in fact are.

Less attention will also be drawn to the Neuroscience Center because of alterations more recently made elsewhere on our grounds. To do away with the need for tents to feed attendees at the Symposium and other similar size meetings, a major addition to Blackford Hall, largely to its east, was finished in 1992. It effectively doubled its size, creating in particular the elegant new Clarkson Dining Room on the ground floor. Although its appearance from Bungtown Road has hardly changed, Blackford looks different from the harborside, with the new staircase to lower floors topped by our second bell tower.

Also presenting a larger facade to the western shore is the newly renovated McClintock Laboratory, which had a third, penthouse-like floor added to it to make it over into a very effective facility for cancer research. Ever since I became the director, I saw the eventual need to give McClintock proper offices and seminar space through adding a tastefully designed upper floor. Without such an addition, McClintock, perhaps the most historic of all our buildings through the Nobel prize winning research done within it by Barbara McClintock and Alfred Hershey, was increasingly too small for the complexities of modern science. Happily, the upper floor further enhances the Italianate elegance of the original 1914 design while providing breathtaking views of the inner harbor.

Although we take much deserved pleasure from the way we propelled Cold Spring Harbor Laboratory into a world-leading role in science while simultaneously preserving the flavors of its historic past, our rapid growth of the past decade worries some of our nearby neighbors. They say we have already grown too much and want to take legal steps that will restrict how we grow in the future. That such fears exist should not surprise us, since rapid change is invariably unsettling to those comfortable with the present and who would like their environment to present only the challenges that they had faced in preceding years. Moreover, we share the concerns of those who wish to preserve forever the great intrinsic beauty of Cold Spring Harbor. It greatly enhances the value of the houses of those fortunate, like me and my family, to live in our vicinity as well as the quality of the commuting lives of those whose work necessitates driving daily along route 25A. No one, in fact, has a greater vested interest in the beauty and ambiance of our site than the Lab and its residents.

Had we not been located on such an idyllic site, I'm sure that the Laboratory would have gone under during the financial crises of the 1960s. Moreover, the chief reason why we have functioned so well as a meeting and teaching center is the almost rural feeling that still characterizes our buildings along Bungtown Road, as well as the inner harbor itself. The desire to keep this atmosphere is what led us in the still fiscally tricky early 1970s to buy the Whaler's Cove Marina that lies across from us on the eastern shore of the inner harbor. If we had not so acted, its size would have soon doubled to 100 slips, its owner having just won a lengthy legal battle for the expansion. Such a move would have radically changed our inner harbor, paving the way for the awful congestion that already characterized nearby Huntington Harbor. We saw our purchase of the property to
be totally for the public good, but we did displease a tiny minority who felt that we should close down the marina instead of letting it continue to operate at its current size. But we did not want to upset those who had their boats there, since they did not seriously disturb us or even their closer neighbors. In addition, the rent paid to us by the marina, then and still now, each year covers the stipends of several young postdoctoral fellows. Finally, the small, quiet marina preserves the bucolic ambience of Cold Spring Harbor that we love.

More recently, we have taken the equally important step of successfully working to see that the main Laboratory grounds and those of our neighboring Fish Hatchery were in January 1993 nominated to the State and National Registers of Historic Places. Toward this recognition, my wife Liz, assisted by Nathaniel Comfort and Susan Cooper, diligently documented our achievements as one of the major founding sites for American science. By being on the register, we will have to demonstrate that future major changes to our campus do not have a negative impact on its historic flavor. This designation, however, in no way means that we cannot make additions to clearly historic structures like those just done to McClintock Laboratory and Blackford Hall. Nor does this designation inhibit us from building the new laboratories that we will need to continue to be a major research and educational establishment. Such changes, however, must be done in ways that respect Cold Spring Harbor’s unique environment for science and its teaching. Since this is the way we have proceeded over the past 25 years, I do not see our freedom to act imaginatively being seriously restricted. During all this time, we have always instructed our architects to design structures compatible with their preexisting neighbors. This does not mean, however, that invariably their resulting designs will please everyone, since often many years, if not decades, must pass before a new building finds general acceptance. We must thus always take care that we do not fall into the trap of perpetuating architectural styles long past the time when they are the best solution for a given era. Imagine Yale or Princeton slavishly burdened by the need to build perpetually in Gothic Revival styles.

Our freedom to continue to build imaginatively upon the past is aided by the fact that we still utilize only a small fraction of our 95-acre site in Laurel Hollow, with nearly all our science and conference activities done on land at the south end of our property. Moreover, within this region, which comprises less than a quarter of our land, there remains much room on our middle-campus slopes to expand these activities further when the need arises. So we should never have reason to change the basic rural character of the land along the northern reaches of Bungtown Road. Future visitors to our meetings and courses should perpetually have the pleasure of walking along Bungtown Road, which has the feeling of a narrow country lane. Of course, there will be subsequent changes along it, but we hope that they will further add to its charm, for example, by the construction near our tennis courts of a small, barn-like athletic building containing a squash court and a modest exercise room. When we find the funds, we plan to replace the split-level former residence of the Kurahara family with a hillside, barn-like building for our Buildings and Grounds Department, which is now crowded into inadequate facilities along the waterfront below Blackford Hall.

Even within our more built-up region at the head of the harbor, we should have the ability to expand our research and educational activities without significantly increasing the number of individuals working on our main campus. This can be done by moving certain programs to nearby off-campus sites. We, in fact, began this process in 1976 when we were given the 45-acre Lloyd Harbor estate of Charles S. Robertson to use as a site for small conferences where some 30–40
individuals come together for several days. From the start, a scenic easement was attached to the land preventing us from ever building upon the fields that still convey the character of past days when it was farmed. So the estate-like ambiance that now makes our Banbury Conference Center so successful should be forever preserved and help Lloyd Harbor keep some of the atmosphere of 50 years ago, when it was largely covered by the magnificent properties of many of New York's best-known families.

We likewise were able to restart a serious program in plant genetics by utilizing land and farm buildings, purchased from the Nature Conservancy, on the former 90-acre estate of Mrs. George Nichols, less than a half mile away from us to the east on Lawrence Hill Road. By sharing the maintenance of its buildings, we helped the Nature Conservancy keep intact what was once one of Long Island's finest estates maintained for agricultural purposes.

We have helped the village of Cold Spring Harbor preserve the handsome Georgian style school building located on Main Street at the foot of Goosehill Road. By purchasing it from our local school district for use as our DNA Learning Center, we prevented its transformation into commercial office space that would have destroyed its academic flavor. It was built in 1925 using funds provided by Mrs. Walter Jennings, whose Standard Oil fortune husband had built in 1906 the magnificent Burrwood mansion at the top of Snake Hill Road. Sadly, Burrwood itself has just been demolished, having served as a residence for the blind long since the end of World War II following the deaths of its original owners. For years I had dreamed of finding the funds to renovate Burrwood as a residence for our increasing number of graduate students who now number some 50. But a multimillion-dollar sum would have been necessary to restore it properly, and I never seriously shared this hope with others, knowing it to be unrealistic.

But now that Burrwood is gone, our community has lost an important expression of its fabled past. We should work to see whether further opportunities exist whereby we could save remaining historic properties through utilizing them for our current needs. In particular, we now desperately need more residences for our younger scientists, few of whom have the resources to live within our well-to-do local neighborhoods. Although we possess hilltop land off Moore's Hill Road in Laurel Hollow suitable for such residences, we would prefer now to keep that land vacant, both to maintain a buffer space between us and our nearby neighbors and to retain the future possibility of devoting some of this land to scientific uses. Thus, I believe, it would be in the best interests of both ourselves and our neighboring villages if we can acquire additional off-ground sites whose historic flavor we can maintain by our occupancy.

I am thus optimistic that the Cold Spring Harbor Laboratory will continue to serve well both its own scientists and those working outside it while at the same time further enhancing the quality of life for those citizens who live nearby. We must always remember that only by continuing to do science at its best will we maintain the financial strength needed to allow us to design and maintain buildings that our neighbors can forever take delight in. So we must necessarily always look more to the future than to its past. What we look like must reflect the way we need to be to do the science that the world wants.

I will always remember our sage neighbor Amyas Ames remarking that if it were not for the Lab, Cold Spring Harbor would be no different from the Greenwich that lies just across Long Island Sound. We will not let it go that way.
HIGHLIGHTS OF THE YEAR

A Nobel for Splicing: The Tumor Virus Program Bears Fruit

Beginning in 1969, Joe Sambrook and I began assembling a tumor virus group at Cold Spring Harbor. After the successes of the 1960s in understanding the genetic code and the way genetic information was translated into proteins, a next logical step was to apply this new knowledge to understanding cancer. Tumor viruses seemed to be an ideal model system. These tiny packages of proteins and genes somehow contained the necessary information to generate cancer. In addition, the National Cancer Institute was soon to embark on its "War on Cancer" and so funding for cancer research was readily available. So I began to recruit the best scientists I could find to tease out the functions of the handful of genes in DNA viruses such as simian virus 40 (SV40) and the somewhat larger adenovirus 2, which Ulf Pettersson brought from Sweden to Cold Spring Harbor in 1971. That year, a young postdoc named Phillip Sharp came from Caltech to work with Joe Sambrook, and the next year Richard Roberts, an expert DNA chemist from England, came to set up his own research group in Demerec Laboratory. Soon, both of them turned their attention to the wide-open adenovirus field, which was relatively unpopulated at the time. As with the discovery of a new continent, the first job to be done is the cartography. In genetics, this means mapping out the locations of the genes. So Roberts and Sharp collaborated on an early paper on adenovirus gene mapping.

Sharp accepted a job offer from the Massachusetts Institute of Technology in 1974, where he took his postdocs and set up shop to continue working on adenovirus. Roberts stayed at Cold Spring Harbor and pursued among other projects his own adenovirus research. Soon his laboratory began identifying novel restriction enzymes, proteins discovered in the 1960s that are used by host cells to defend themselves against invading viruses by cutting up their DNA. Each one of these enzymes recognizes a specific DNA sequence and cuts the DNA at each occurrence of that sequence. Roberts realized that many such enzymes were likely to exist and quickly developed techniques for seeking them out and purifying them. Here he saw, perhaps more than anyone else, their immense potential as tools for molecular biologists. Not only could these enzymes break up a long DNA sequence into manageable pieces, but they would cut a genome the same way every time. Moreover, if you knew what sequence a given enzyme cut, you therefore knew the sequence at each cut it made in novel DNA.

Soon Roberts effectively set up a restriction enzyme clearing-house in his laboratory, with bottles and flasks and a line out the door of scientists from all over the country wanting to borrow a little EcoRI or BamHI. This project was his bread and butter, allowing him to pursue riskier projects with adenovirus.

With his talented postdoc Richard Gelinas, who had come from Harvard, Roberts began in 1975 to try to identify the sequences of adenovirus promoters, the DNA sequences used to turn on gene transcription. No one had yet sequenced a promoter from an organism higher than a bacterium, and Roberts reasoned that a virus which infected a higher cell would likely have promoters similar to that of its host. Furthermore, they reasoned that each of the 20 or so viral genes would have its own promoter. To find them, Roberts and Gelinas devel-
oped a way to isolate just the left-hand (5' ) end of the RNA messages for some 20 different adenovirus proteins. But when they did the experiment, they only got one sequence, an 11-nucleotide-long string. This seemed certainly to be an artifact, since they expected some 20 different sequences, one for each protein. When they repeated the experiment, however, they got the same result. Two conclusions were possible: Either the same 11-base sequence was repeated throughout the adenovirus genome at the beginning of every gene or it only occurred once in the genome and was somehow tacked onto each RNA message during transcription. The latter possibility was unheard of, but it turned out to be correct.

Dr. Richard Roberts (left) receiving 1994 Nobel Prize for Physiology or Medicine from King Carl XVI Gustaf of Sweden
It was easy enough to check whether a given sequence occurred with unusual frequency in the adenovirus genome, and when they looked, they found that it did not. At the same time, another postdoc in Roberts' laboratory, Sayeeda Zain, unexpectedly found another piece of the puzzle. She had been looking at recombination between SV40 and adenovirus. A recombination event happened to occur at a spot just in front of one of the adenovirus genes that Gelinas and Roberts had been studying. A natural assumption was that Zain would therefore encounter a DNA sequence complementary to the now-notorious 11-base sequence in the RNA, but she did not. The DNA complement to that 11-base sequence therefore had to lie somewhere "upstream" in the genome. The gene, in other words, had to be in at least two parts, which were somehow joined during transcription to make a single messenger RNA.

At this point, late in 1976, Roberts and Gelinas began to suspect the existence of what we call today split genes. A key paper they coauthored in January 1977 suggested that the DNA complement to the 11-base message fragment might not lie adjacent to the DNA for the rest of the RNA message. Roberts and Gelinas then began to design and carry out experiments to show that this 11-base message fragment was not a contaminant or other artifact. One Saturday morning in the early spring of 1977, Roberts conceived the key experiment during one of his regular meetings with Gelinas. It would use electron microscopy to show that a DNA fragment from upstream in the adenovirus genome would bind to the end of the RNA message from the downstream gene. Neither Roberts nor Gelinas knew electron microscopy, but fortunately right downstairs in Demerec Laboratory were Louise Chow and Tom Broker, who knew the specific techniques needed to do the experiment. Roberts and Gelinas explained to Broker and Chow what they wanted to do. Chow said she thought it sounded possible and agreed to do the experiment. Within 3 days, they had the result, and it looked almost exactly as Roberts had drawn it on the blackboard the previous Saturday.

Chow, however, had a good microscopist's eye for detail, and she explained to Roberts and Gelinas that her pictures showed that the RNA was a composite of more than two distinct regions of DNA. The adenovirus gene was instead made up of four separate regions that were spliced together to make a single RNA message.

Over the next few months, many people contributed to fleshing out the final picture of split genes. Dan Klessig, from Ray Gesteland's laboratory, had been applying the techniques Gelinas had been using to specific RNA messages, and he showed much more detail in terms of how splicing occurred. He also articulated the probable mechanism by which the splicing of RNA message fragments from different regions of the genome could occur. Other scientists at Cold Spring Harbor had results that in fact could only be adequately explained by splicing. John Hassell and Ashley Dunn, from Joe Sambrook's laboratory, and Jim Lewis, Carl Anderson, and John Atkins, from Ray Gesteland's laboratory, reinterpreted their results in terms of RNA splicing, making a very strong case indeed. Roberts coordinated a group of four papers that were shipped off to the journal Cell and were published back-to-back in the October 1977 issue.

At the same time up at MIT, Phil Sharp and Sue Berget had made the same discovery. A slightly different approach to the same electron microscopy experiment had led them directly to the conclusion that the adenovirus messenger RNA molecules were coded by four discrete regions of the DNA. Their results were published in the August 1977 issue of Proceedings of the National Academy of Sciences.
If some scientific discoveries are like time bombs, lingering in the literature for years until they become widely accepted or their generality to all life becomes clear, this was a land mine. As soon as Roberts and Sharp stepped on it, it blew up the entire field of eukaryotic gene structure. At the Cold Spring Harbor Symposium in June, 1977—still 3 months before the first publication—the papers by Sharp and Tom Broker reporting on the discovery of splicing were followed by another on splicing in adenovirus and two more on splicing in SV40. Soon everyone discovered that RNA splicing explained results they had been getting for months. It was almost instantly accepted.

This year in October, 16 years after the discovery, RNA splicing was recognized by the Nobel Committee, and Roberts and Sharp shared the Nobel Prize in Physiology or Medicine. The week after the announcement from Stockholm, we held a party for Rich that was attended by many of the Laboratory staff who wanted to celebrate this most glorious of all prizes awarded for scientific achievement. Our pleasure in the occasion was tempered by the omission of those other Laboratory scientists who contributed to the discovery. The Nobel Committee’s “Rule of Three” has often precluded acknowledgement of all who shared in the burden of research, most notably in this case the vital contributions of Louise Chow and Richard Gelinas. Last year, Rich concluded 20 years at Cold Spring Harbor and moved to the Boston area to head research at New England Biolabs, the premier source for restriction enzymes. He continues his ties with the Laboratory, however, not least through his exciting collaboration on DNA methylases with staff scientist Xiaodong Cheng.

The phenomenal growth of the splicing field, now with its own subdisciplines, such as the exciting field of alternative splicing, is proof of the value of basic research and yet another reminder that most real scientific innovations come from "pure" investigations, undertaken for their own intrinsic interest. Splicing has clinical interest as well—dozens of diseases, including β-thalassemia, Tay Sachs, and some forms of cystic fibrosis and muscular dystrophy, have been shown to result from defects in RNA splicing.

A Good Year for Cancer Research, Crystallography, Plant Genetics

In 1981, in Demerec Laboratory, Mike Wigler was one of the first scientists to discover a human oncogene, called ras. The Ras protein, made by the ras gene, plays a key role in telling a cell when to grow and divide. When genetically damaged, the ras gene’s respective protein product can become stuck in the "on" position, sending the cell into uncontrolled growth. This discovery began what has become a worldwide effort to understand exactly how the Ras protein functions, how it is activated, and what it in turn activates to initiate cell growth. This year saw large advances in our understanding of the Ras pathway, and several laboratories at Cold Spring Harbor contributed significant pieces to this large jigsaw puzzle. We now have a nearly complete molecular pathway, from the extracellular signal all the way down to the DNA.

The Ras pathway begins at the cell membrane, with a receptor protein such as the epidermal growth factor (EGF) receptor. The EGF receptor receives a molecule of growth factor, which circulates in the blood, to trigger the Ras pathway and at least one other pathway called SIF. The SIF pathway has fewer steps to the DNA than the Ras pathway, and it activates a different set of genes. In James Laboratory, Mike Gilman’s group showed that the EGF receptor’s SIF pathway activity is independent of its Ras pathway activity. By mixing cell mem-
branes, cytoplasm, and EGF, they reconstituted the activity of the EGF receptor in a beaker and showed that they could recover SIF activity.

In the Ras pathway, the EGF receptor becomes activated when EGF binds to it. It can then bind a complex of two proteins, GRB2 and Sos. Dafna Bar-Sagi, working in Demerec Lab, showed that GRB2 binds the EGF receptor, and when Ras is in the activated state binds Sos. Mike Wigler's laboratory furthermore showed that activated Ras in turn binds a protein called RAF. The RAF protein is the first in a bucket brigade of protein kinases, each one activating the next by phosphorylating it, that results in the activation of a protein such as AP-1, which binds DNA to turn on genes. Also essential to controlling cell growth is deactivating the proteins in this pathway. Nick Tonks' laboratory isolated a protein that deactivates MAP kinase, the last step in the kinase bucket brigade. The protein, called a tyrosine phosphatase, is one of the first such proteins to be purified and understood functionally in the cell.

In our Keck Laboratory of Structural Biology, David Barford has just worked out the three-dimensional structure of the first tyrosine phosphatase. Equally exciting has been the achievement of Xiaodong Cheng, who produced a high-resolution three-dimensional map of Hhal methylase. This project, part of a collaboration between Xiaodong and Rich Roberts, promises to elucidate the function of an enigmatic kind of enzyme. Methylases attach a small chemical group called a methyl group (CH₃) to DNA. Some researchers hypothesize that methylation blocks other proteins from binding to the DNA. Thus, if a promoter or an enhancer region were heavily methylated, the gene might be "silenced." Methylation is at the center of a hot debate over how gene activity is controlled in the cell.

DNA-binding proteins have specific "domains" that recognize a particular DNA sequence. Some bind to a gene's promoter, which directly turns on the gene, whereas others bind to an enhancer, which, as its name implies, enhances the activity of the promoter, providing another level of regulatory control. Winship Herr's group, in James Laboratory, helped determine the structure of the DNA-binding domain for a protein that binds to an SV40 enhancer. The structure of this DNA sequence, called the POU-specific domain, is strikingly similar to that of the DNA-binding domain of the repressor of bacteriophage λ, a bacterial virus. Together with other results, this revealed that human dwarfism can occur by defects similar to those that affect λ replication—an amazing similarity between such distantly related organisms.

Laboratory scientist David Beach is one of the world leaders in the cell cycle field. This year, his group in McClintock Laboratory identified two new genes and their proteins, which are key players in regulating cell growth. One, called p21, interacts with the nearly ubiquitous tumor suppressor protein p53. In response to DNA damage, p53 activates p21, which then interacts with a variety of other proteins to halt cell growth. The p21 protein functions by blocking the activity of cyclin-dependent kinases, which are proteins that have a key regulatory role in the cell cycle. If p21 is damaged, this check on growth could be removed, leading to tumor formation. The other gene, which they called p16, inhibits a major cycle protein complex called cyclin D/Cdk4. This protein complex inhibits yet another protein called Rb, which inhibits cell growth. Through this triple negative, the p16 protein holds cell growth in check. Early in 1994, scientists in Utah found a gene that was damaged in a large proportion of cancers, including cancers of the lung, brain, bladder, breast, blood, and skin. The discovery that this gene was the same as p16 marks the most direct link to date of cancer to the genes that control the cell cycle.
Formation of the Dana Consortium on Manic Depressive Diseases

An exciting new collaborative research project was begun this year to identify the genetic underpinnings of manic depressive illness (MDI). The project is funded by the Charles A. Dana Foundation and involves research teams at Cold Spring Harbor, Johns Hopkins, and Stanford. The team at Johns Hopkins is selecting very carefully families with manic depressive illness for genetic analysis, and this analysis is being carried out at both Johns Hopkins and Stanford. The Laboratory’s contribution is to develop an integrated database that will have at its core programs for linkage analysis but will as well incorporate information on all aspects of the diagnoses and laboratory analyses of these families. This database is being designed in Tom Marr’s Computational Biology group, using a program developed by Marr called Genome Topographer. An especially exciting feature of this approach is that the database will be useful for genetic research on other disorders. We are also promoting research on manic depressive illness through meetings being held at Banbury Center and through courses intended to train psychiatrists in basic molecular genetics.

Double Helix Turns 40

The year 1993 marked the 40th anniversary of the discovery of the double helix. The Laboratory celebrated with a special 3-day meeting entitled DNA and Biotechnology, which spanned both the history and the future of DNA science. Among the audience were leading scientists from the now almost 40 pharmaceutical, diagnostic, and biotechnology companies that are the corporate sponsors of our meetings programs. On the opening night, Francis Crick gave a thoroughly engaging public evening lecture to a packed Grace Auditorium, after which I presented him with a miniature golden helix to celebrate the occasion. The next day featured talks by many of the pioneers in DNA science, including...
Mahlon Hoagland, Rich Roberts, Wally Gilbert, Stanley Cohen, and Mark Ptashne. Kary Mullis' talk on his discovery of the polymerase chain reaction, for which he won the Nobel Prize in Chemistry this year, was a colorful and entertaining highlight of that afternoon. Then after a banquet, I talked about how I have tried to adapt to the various stages of my life as a student, a scientist, a professor, a writer, an administrator, and a bureaucrat. As befits the central role played by DNA, talks on future developments in biotechnology covered a wide range of topics. We can now control gene expression (Peter Dervan), design proteins (Jim Wells), and study how the brain works (Sol Snyder). New strains of plants can be made with special characteristics (Robert Fraley) and human proteins can be produced in both animals and plants (Alan Colman). Genetic disorders can be diagnosed (Tom Caskey) and, most spectacularly, gene therapy is being used to help those afflicted (French Anderson). The whole meeting was a testament to the extraordinary power of molecular genetics and to the work of thousands of scientists over the past 40 years.

Symposium LVIII

This year's Symposium was on DNA and Chromosomes, a further tribute to the 40th anniversary of the double helix. Forty years after the 1953 Symposium on Viruses where I first presented our model for the structure of DNA, Bruce Alberts of the University of California, San Francisco, and Bruce Stillman put together a first-class program featuring sessions on some of the most interesting new research areas, such as silencing, imprinting, and chromosome domains, as well as productive mainstream areas such as transcription and regulation. Eric Lander, of the Whitehead Institute and MIT, gave the annual Dorcas Cummings public lecture, which occurs midway through each Symposium. Lander is one of the world's leading genome scientists, and his talk was entitled "Mapping Genes and Genomes." Lander is adept at accurately simplifying complex issues, and he outlined with elegance the arguments for mapping and sequencing the human genome. A highlight of the event was a gift from Laboratory employees and many of my friends and colleagues of a 15-foot bronze sculpture of a double helix, crafted by local artist Charles Reina. 'Bronze Helix' has become the focal point of the Grace Auditorium lobby and is a fitting tribute to four decades of excellent science as well as the world's prettiest molecule.

A Rich Program at Banbury Center

Banbury Center continues to hold exciting meetings, ranging from "hard" science to social issues affecting the way modern research is pursued. An outstanding meeting in 1993, and one typical of the Banbury style, was that on Apoptotic Cell Death: Functions and Mechanisms. This is a topic that has changed beyond recognition in recent years, and cell death is now seen to be a normal and essential process in the lives of cells, including cancer cells. For this meeting, we brought together scientists working on cell death in diverse biological systems, who find now that their research has converged on common molecular pathways. All of us hope that the new knowledge coming from the Human Genome Project and other research can be put to use to help people. For disorders like Duchenne muscular dystrophy, we want methods to treat boys afflicted by this disorder, and Banbury Center was the site of a meeting to examine a novel strategy to do this. Molecular studies suggest that it may be possible to turn on a gene for a protein called utrophin, which might substitute for the protein...
dystrophin that is missing or damaged in boys with Duchenne muscular dystrophy. We must now see whether this can be achieved in practice.

With each passing year, federal funding for research becomes more difficult to obtain, and so we must turn increasingly to private sources. But close associations between academic institutions and the corporate world are fraught with potential conflict of interest problems. In a very interesting and important meeting at Banbury Center held in May, the benefits and problems were reviewed and assessed by scientists, institutions, and politicians. No definitive conclusions were reached, but at least the complexities of the situation were appreciated.

**Baring Brothers Executive Meeting**

For the eighth year of our October meetings for senior executives in the worlds of pharmaceuticals and biotechnology, we turned to the topic of Human Molecular Genetics, a field that we drew attention to in our first executive conference held in 1986. The advances that have been made since then have been quite astounding. The major successes up to 1986 were locating the gene for Huntington's disease and cloning the gene for Duchenne muscular dystrophy. But in 1993, our speakers were able to discuss the trinucleotide repeat genes, including the gene for Huntington's disease, the hunting of genes involved in breast cancer and other complex disorders, approaches to developing new genetic diagnostic techniques, progress in genome research, and, far in advance of our expectations, gene therapy for inherited diseases and cancer. The recent meetings in this series have been supported by Baring Brothers of London together with Dillon Read of New York. I would like to pay a special tribute to Robin Broadley of Baring Brothers, London, who has been such an enthusiast for bringing together the worlds of commerce and biology.

**Robertson Research Fund Marks 20th Anniversary**

This was a year of milestones. In addition to the 40th anniversary of the double helix, we celebrated the 20th anniversary of the Robertson Research Fund. The more than generous gift in May 1973 from Charles Robertson and his family of $8 million and their family estate instantly secured the Laboratory's future as a continuing leader in molecular biology research. Until then, the Laboratory did not have a meaningful endowment. Today, thanks to prudent, conservative management, the original Robertson sum has grown to over $48 million. The Robertson estate, originally endowed with its own $1.5 million endowment that now has risen to $7.8 million, has become our Banbury Conference Center, home to an eclectic and engaging series of meetings and workshops on biology and social topics. In early June, we feted the Robertsons with a special evening featuring talks by some of our scientists and a brief but moving acknowledgment from Bill Robertson, a leather-bound booklet outlining the history and achievements of the Fund, and a wonderful dinner in Blackford Hall. A bronze plaque on the wall in Grace Auditorium lobby now commemorates the Robertson Fund.

**Major Gifts**

The Laboratory received a number of major gifts in 1993 from corporations, foundations, and private individuals. Major gifts are an important part of our ability to support young scientists. Many of these were gifts to the Cancer Research Fund,
which supports all aspects of cancer research at Cold Spring Harbor. Nearly two thirds of the research done at Cold Spring Harbor has implications for understanding cancer. The Cancer Research Fund now stands at $4.1 million, thanks to a number of substantial recent gifts. Among these are gifts of $100,000 from the Booth Ferris Foundation, $94,000 from Sherburn Becker, $25,000 from the David Koch Foundation, $150,000 from William and Irene Miller, $55,000 from Dr. David Pall, $50,000 from the Perkin Fund, and $10,000 each from the Daphne Seyboldt Culpeper Foundation and Oncor, Inc. The Fannie E. Rippel Foundation has made a challenge grant of $250,000 to raise the $750,000 needed to cover the $1 million equipment budget for the Cancer Research Fund. We are particularly grateful to George and Mary Lindsay for the $250,000 unrestricted gift.

The Infrastructure Fund was concluded this year with great success. It raised a total of $4,124,000 that will be used to build and improve facilities and support research. The Fund is being named in honor of William Miller, in recognition of his leadership. Research Support Funds will continue to target the pharmaceutical industry, but the focus will now shift to program support. Henry Wendt and Maxwell Cowan were the first contributors to this fund, with pledges of $150,000 and $25,000, respectively, for neuroscience fellowships.

This year, we also launched the Genetic Diseases Fund, which will be used to support research into the biology of hereditary illness with initial emphasis on muscular dystrophy. Inaugurating this fund were gifts from John Cleary and his family, Owen Smith, the Daphne Seyboldt Culpeper Foundation, and myself. The Fort Hill Foundation provided $500,000 toward the William J. Matheson Chair, and the Banbury Fund contributed $50,000 for the Charles S. Robertson Chair.

We received Planned Gifts this year from Townsend and Elise Knight, Robert Gardner, the Taggart Whipple estate, Charles Henry Leach II, and Ed Palmer. The Annual Fund, made up of contributions from the nearly 900 members of the Cold Spring Harbor Laboratory Association, continues to be strong and vigorous. The 100 new members of the Laboratory helped bring the Annual Fund total to $526,241.

**McClintock Remodeled, Reinhabited**

Renovations to the 1912 McClintock Laboratory were completed this year, and David Beach and Dick McCombie moved in. The main externally visible change is the addition of a mostly glass third floor, topped with an already-patinaed copper roof, set atop the flat roof of the original structure. The third floor houses offices and the William and Eleanor Everdell Seminar Room. Interestingly, a third floor was first considered for the building in 1937, but it was thought to be architecturally impossible. Indeed, even today it would have been cheaper and easier to raze the historic structure and build a new, larger facility. But the old Animal House is a Laboratory landmark; both Barbara McClintock and Al Hershey did their Nobel-winning research there. So we brought in the ever-talented Bill Grover and Centerbrook Architects to remodel the interior, design a tasteful third-floor addition, and plan out the structural strengthening that would be needed to turn the 80-year-old building into a modern cancer and gene-sequencing laboratory. The old Animal House is now very much a yeast house. Upstairs, David Beach uses the yeast *Schizosaccharomyces pombe* to probe the genes and proteins that control the cell division cycle. Downstairs, Dick McCombie is mounting the “Pombe Genome Project,” a large-scale effort to sequence all
of the yeast DNA. The two laboratories are complementary and further collaborations take place with Tom Marr’s computer laboratory across the road in Hershey building.

On the second floor, the two main laboratories for cancer research now have plaques acknowledging the great generosity of the late Charles Leech II and the Farish Fund. The ground floor is the gene laboratory made possible by a gift from Lita Annenberg Hazen, who long has been one of the Laboratory’s most devoted supporters. So it was with great pleasure that we have attached her noted name to our Gene Sequencing Laboratory. Early in October, we formally dedicated the new McClintock Laboratory in the presence of the major donors who made possible this latest facility for modern science at Cold Spring Harbor.

A Great Architect Passes

Charles Moore, founder of Centerbrook Architects and one of the great architects of the 20th century, died of a heart attack on December 15 at his Austin, Texas, home. Born in 1925 in Benton Harbor, Michigan, Moore took a Bachelor of Architecture degree at the University of Utah in 1947 and a Ph.D. at Princeton in 1957. He accepted an associate professorship at the University of California, Berkeley, in 1959. Three years later, he became chairman of the Berkeley architecture department and formed a partnership with three colleagues, Dorilyn Lyndon, William Turnbull, and Richard Whitaker (MLTW). Moore first achieved national recognition with the development of the popular Sea Ranch Condominiums on California’s North Coast. Over the next 8 years, MLTW designed many prominent structures in California, including several at the new University of California campuses at Santa Barbara and Santa Cruz. In 1965, Moore took the chair of the architecture department at Yale. He began a solo practice in 1970, after the MLTW partners separated, and in 1975, he formed Moore Grover Harper in Essex, Connecticut—the forerunner of Centerbrook Architects.
It was during June 1972 that the Laboratory first became acquainted with Moore. On a trip to California, my wife and I drove north of San Francisco where, after spending a night at the Sea Ranch Lodge, we toured the extraordinary collection of new homes that surrounded it, including Moore’s now famous Condominium. Upon returning East, we realized that Charles Moore was also active on Long Island, being responsible for the design of the Whitman Village Apartments in Huntington Station. So we asked him to visit the Lab with the thought that he would help renovate Airlie, the director’s house into which my wife and I were soon to move. Soon he and his talented collaborator, Bill Grover, gave an elegant design to the interior of Airlie. Two years later, they also gave us super plans for remodeling the Robertson family carriage house to create the Banbury Meeting Center. In 1981, when his Palladian-styled Sammis Hall residence at the Banbury Center was dedicated, Moore was again on the West Coast, a professor of architecture at the University of California, Los Angeles. In 1985, he moved again, this time to Austin, Texas, where he took a professorship and opened a new practice with Arthur Andersson. Throughout his long career, Moore showed style, imagination, respect for tradition, and dissidence from current fashions in architecture. He won the most prestigious awards in his field, including the AIA/ACSA Topaz Medallion for architectural education and the AIA Gold Medal. Beyond that, he was simply one of the great minds in architecture. We are proud to have his stamp on our institution.

DNA Learning Center Renovates, Goes Multimedia

The DNA Learning Center underwent an extensive set of renovations, designed by Centerbrook of Essex, Connecticut, that left it with a new exhibit space, sophisticated computer lab, and, most spectacularly, a state-of-the-art auditorium, complete with a multimedia show about Long Island.

Sponsored by Cablevision Systems Corp., the Stone Foundation, Inc., Edwin S. Webster Foundation, and the Weezie Foundation, the renovations included more than $600,000 worth of work and affected 90% of the building’s 7600 square feet. A new computer lab is linked to the “wet” lab next door, enabling classes to do experiments and then go into the next room to work computer simulations that explain the biology. A new exhibit space features an elaborate mural of the inside of a cell, painted by art students at Cooper Union and New York Tech.

The highlight of the new Learning Center is the auditorium. Located in what was once the Cold Spring Harbor elementary school’s gymnasium, the 104-seat hall now features a bank of 5-foot by 7-foot video screens, 36-inch monitors, and 12 sets of speakers. The hall can accommodate nearly any type of presentation, from “chalk talk” to slides to computer-generated movies. The showpiece of the hall, though, is “Long Island Discovery,” a 28-minute multimedia presentation sponsored by Cablevision Systems Corporation and developed for the Learning Center. The movie uses the multiple screens and surround-sound capabilities of the auditorium to give a rich picture of the history and diversity of Long Island. The DNA Learning Center staff encourage local teachers to use the show as a teaching tool and to draw classes to the other features of the Learning Center.

Nick Tonks Wins Colworth Medal

Nick Tonks received a high honor when he was awarded the Colworth Medal this year. This prestigious award is given to the “top British biochemist under the age
of 35." Nick's exciting work on tyrosine phosphatases, a vital but poorly understood class of regulatory enzymes, makes him a leader in today's cohort of hot young molecular biologists.

David Beach Honored with Lilly Award

Senior Staff Scientist David Beach was honored with the Eli Lilly and Company Research Award, given by the American Society of Microbiology. The award, given for work "of unusual merit for an individual on the threshold of his or her career," is among the most prestigious in microbiology. Previous winners include Joshua Lederberg (1953), Matthew Meselson (1964), and David Baltimore (1971). Established in 1936, the Award was first given to Harry Eagle, a Laboratory trustee from 1962 to 1991 and chairman of the board of trustees from 1974 to 1979.

Bruce Stillman, Fellow of Royal Society

In March of this year, Bruce Stillman was elected a Fellow to the Royal Society, one of the most prized honors for scientists of British or Commonwealth citizenship. Bruce, an Australian citizen, joins an elite group of scientists stretching back to the sixteenth century. Bruce will be the second director to have been elected to the Royal Society, the first being my predecessor John Cairns who was elected to the Society in 1974 soon after his return to England.

A Boom Year for the Press

The Press continued an ambitious publishing program by adding 15 new books and 2 new videotapes, bringing its total titles in print to more than 120, doubling the frequency of both its current journals, and announcing plans for the launch of a third journal in 1994. Sales increased by 16% to over $4.3 million, and the balance after expenses, including significant one-time charges, rose by 25%.

New titles were added in all categories of the catalog. Sales of laboratory manuals continued to flourish, particularly the perennial bestsellers Molecular Cloning, Antibodies, and the recent success A Short Course in Bacterial Genetics. There were new manuals on molecular probes of the nervous system and fission yeast biology. The Symposium volume on The Cell Surface, published in the 60th anniversary year of the Symposium's founding, was a worthy and colorful addition to this prestigious series. The second volume of the authoritative monograph Transcriptional Regulation was published, and the entire book was almost immediately reprinted in paperback in response to the strong demand for an edition affordable by graduate students. A paperback edition of the widely appreciated memoir of Barbara McClintock, The Dynamic Genome, was released, featuring the elegant cover that won a national design award.

Two new books made a dramatic impact both visually and scientifically. The RNA World revisited a prebiotic era in which evolution may have depended on replicating RNA, not DNA, and reviewed current ideas about the structure and function of this ancient molecule. A notable feature of this book is the frontispiece—an RNA hologram. Even more arresting is the lavish artwork in The Development of Drosophila melanogaster, a monograph on the molecular analysis of fruit fly embryology. This book set includes 114 color plates and a separate stunning 50-page atlas created entirely with computer graphics.
It was a year of expansion for our journals. *Genes & Development* was again listed as the most cited journal in genetics and in developmental biology and, as in previous years, had a steady increase in circulation. Because of rising demand to publish in the journal, the decision was taken to produce issues semi-monthly in 1994. The success of *PCR Methods and Applications*, launched in 1991, necessitated an acceleration from quarterly to bimonthly publication with the start of Volume 3 in August. The growing tide of interest in the molecular analysis of mental function prompted the decision to launch a new journal, *Learning & Memory*, in 1994. A distinguished group of editors and advisors was readily assembled, attracted by the idea of a journal devoted exclusively to papers on this broad and fascinating topic.

**Undergraduate Research Program**

This year’s cohort of promising undergraduates included 19 students from nine states and four countries. Like their predecessors of the last 24 years, these students spent 10 weeks during the summer in the laboratories of Cold Spring Harbor scientists. They learned the fundamental techniques of molecular biology, carried out semi-independent research projects, and, most important, were immersed in the culture of science. The program is highly competitive; this year, we received 176 applications. Thirteen of the successful applicants were women. Research projects undertaken by this year’s students included RNA splicing, plant molecular biology, genetics of the yeast cell cycle, DNA sequencing, and *Drosophila* learning and memory. This highly successful program would be impossible without the sponsorship of the National Science Foundation, Burroughs-Wellcome, the Robert P. Olney Fund, Hanson Industries, Bio-Rad Laboratories, the Phillips Petroleum Foundation, Libby, Zeneca, and the Cold Spring Harbor Fund.

**Partners for the Future**

This was the fourth year for our Partners for the Future program. Born in 1990 out of the Laboratory’s centennial celebration, Partners for the Future extends the Undergraduate Research Program to the level of high school seniors. Local businesses support five talented Long Island students in a 6-month after-school research effort, undertaken with the guidance of a Laboratory scientist “Mentor.” These students spend at least 10 hours per week from October through March, learning new techniques and concepts as well as something about what the day-to-day life of a scientist is like. This year’s Partners, followed by their school and Mentor, were: Edwin Yoo, Half Hollow Hills High School West (Dr. James Pflugrath); Olivia Cheng, Plainview-Old Bethpage JFK High School (Dr. Venkatesan Sundaresan); Seema Gupta, Syosset High School (Dr. Jerry Yin); Jason Kass, Oceanside High School (Dr. Daniel Marshak); Joann Romano, Great Neck South High School (Drs. Tim Tully and Mike Regulski).

**Long-term Service**

Seventeen Laboratory staffers celebrated big anniversaries of employment. Celebrating 15 years with the Lab were Katya Davey, Banbury Hostess; Gus Dulis, Mechanical Technician; Jim Garrels, Senior Staff Scientist; Patti Maroney, Payroll Administrator; Chris McEvoy, Assistant Grounds Foreman; Charlie
Employees celebrate 15- and 20-year anniversaries at the Lab: (Top, left to right) Jim Garrels, Chris McEvoy, Carol Caldarelli, Dorothy Youngs, James D. Watson. (Middle) Patti Maroney, Gus Dulis, Bea Toliver, Mike Wigler, Charlie Schneider. (Bottom) Laura Hyman, Nancy Ford, Katya Davey, Elizabeth Watson

Schneider, Estimator/Draftsman; Bea Toliver, Banbury Administrative Assistant; Mike Wigler, Senior Staff Scientist; Jeanne Wiggins, Laboratory Technician; and Dorothy Youngs, Head Housekeeper. Celebrating 20 years of service were Carol Caldarelli, Housekeeper; Nancy Ford, Managing Editor, CSHL Press; Laura Hyman, Administrative/ Business Manager, Library; and Hans Trede, Grounds Foreman.

Changes in Scientific Staff

We said good-bye to Drosophila neurobiologist and Senior Staff Scientist Ronald Davis, who returned to Baylor College of Medicine in Houston, where he was offered an endowed chair. Several of his laboratory group accompanied him, including Staff Associate Jim Cherry. Senior Staff Investigator Elizabeth Moran left to take a joint appointment at Temple University’s School of Medicine in Philadelphia, as Associate Professor of Biochemistry and Director of the Fels Institute for Cancer Research and Molecular Biology’s program in molecular genetics. Betty got her start at Cold Spring Harbor as a postdoc in Mike Mathews’s laboratory in 1983, where she began working on oncogenes in adenovirus. Senior Staff Investigator Tom Peterson will pursue his maize transposon work in the heart of corn country, Iowa State University, in Ames, where he has an endowed chair in Plant Genetics. Tom came to Cold Spring Harbor in 1987, from CSIRO, Division of Plant Industry, in Canberra, Australia.

Staff Investigator Roymarie Ballester has left Mike Wigler’s laboratory for a faculty position at the University of California, Santa Barbara. Michael Laspia, a Staff Investigator in Mike Mathews’ laboratory, has taken a position at Dartmouth Medical School, in Lebanon, New Hampshire. Scott Patterson, a Staff Investigator in the 2D gel facility, has taken a research position at AMgen, Inc., in Thousand Oaks, California. Gil Morris, a Staff Investigator under Mike Mathews, has taken a job at Tulane Medical Center in New Orleans.

Graeme Bolger, a Staff Associate in the Wigler laboratory and a postdoc here from 1985 to 1990, has moved to the University of Utah Medical Center in Salt Lake City. Susan Lobo, Staff Associate with Nouria Hernandez, has moved to the University of Alabama in Birmingham as an assistant professor.
Montage presented to J.D. Watson by staff members. Photos represent long-time employees who worked at Cold Spring Harbor Laboratory between 1968, when Watson became Director, and 1993, his 25th anniversary.

Several visiting scientists have completed their work here and returned to their home institutions. These include: Jacques Camonis, from Mike Wigler's laboratory, who returned to Centre Hospitalier Lariboire, Paris; Javier Diaz-Nido, who came from the Universidad Autonoma de Madrid to Dan Marshak's laboratory; Igor Galkavtsev, from the Medical Genetics Center in Moscow, who spent time in David Beach's laboratory; Nicholas Muzyczka, who took sabbatical leave from SUNY Stony Brook to work in Bruce Stillman's laboratory; Kikuo Sen, a member of Shinshu University, Faculty of Agriculture, in Nagano, Japan, who worked in the Wigler laboratory; Huan Ran Tan, visiting from the Beijing Medical University, Department of Pharmacology, to work with Bob Franza; Robert West, Jr., from SUNY Health Science Center in Syracuse, who worked with Ron Davis; and Daisuke Yamamoto, from Mitsubishi Kasei Institute of Life Sciences, in Tokyo, who also worked with Ron Davis.

New Staff Members

A large number of visiting scientists came to the Laboratory this year. Peter Barker, from the German Cancer Research Center in Heidelberg, is working with Mike Wigler. Brian Cox, from Oxford University, is working with Bruce Futcher. William Eckberg, from Howard University in Washington, D.C., is working in Nick Tonks' laboratory. Alexandre Melnikov, from the State Control Institute of Standardization of Medical and Biological Preparations in Moscow, is working with Dick McCombie. Carol Prives is on sabbatical from Columbia University and working in Bruce Stillman's laboratory. John Scott is also on sabbatical from the University of Hawaii at Hilo to work in Bruce Stillman’s laboratory. Tokio Tani, from Kyushu University in Fukuoka, Japan, is working in David Spector’s laboratory. Rui-Ming Xu, from SUNY Stony Brook, is working with Jeff Kuret.
Promotions

Senior Staff Investigators Mike Gilman and Nouria Hernandez were promoted to Senior Staff Investigator in July. Mike came to Cold Spring Harbor in 1986, following a fruitful postdoc at the Whitehead Institute with ras co-discoverer Bob Weinberg. His work now focuses on signal transduction, the set of chemical reactions that relay growth messages from outside the cell into the nucleus. Nouria also arrived in 1986, from Alan Weiner’s laboratory at Yale. Her field is gene transcription. She focuses her efforts on two systems: small nuclear RNAs (molecules that function within the nucleus to carry out RNA splicing) and transcription of genes of the AIDS virus, HIV.

Two scientists were promoted to Senior Staff Investigator this year. Grigori Enikolopov, who came in 1988 as a visiting scientist from the Institute of Molecular Biology at Moscow’s Academy of Science, has found several surprising functions of the increasingly remarkable gas nitric oxide, including the ability to initiate gene transcription. Jacek Skowronski, promoted from Staff Investigator, came to Cold Spring Harbor in 1986 from the National Institutes of Health, in Bethesda, Maryland. Jacek studies the effects and function of the HIV gene nef in mice.

Postdoc Harriet Feilotter was promoted to Staff Investigator in November. As liaison for the Laboratory’s collaborative effort into the genetic basis of manic depressive illness, Harriet will coordinate efforts among scientists at Cold Spring Harbor, Johns Hopkins, and Stanford.

Postdocs Eric Chang, from Mike Wigler’s laboratory, Erich Grotewold, in the plant genetics group, Shobha Gunnery from Mike Mathews’ laboratory, Misha Jung in the structural biology group, as well as Stevan Marcus, Akila Mayeda, and Linda Van Aelst from Mike Wigler’s laboratory were promoted to Staff Associate. Visiting scientist Natalia Peunova was also appointed to Staff Associate.

Postdoctoral Fellows

A large number of postdoctoral fellows moved on to other positions this year. Cyrille Alexandre, a postdoc with Mike Gilman, has moved to the ICRF in London. Also from Gilman’s laboratory, Ricardo Attar has moved to Princeton, New Jersey, for a position with Bristol-Meyers Squibb. Prasanna Athma, from Tom Peterson’s laboratory, has left for New York Medical College, in Westchester County. Kanagasabapathi Balandran, a postdoc with John Anderson, has moved to the Fels Cancer Institute at Temple University, in Philadelphia. James Bischoff has left David Beach’s laboratory for a job with Onyx Pharmaceuticals, in Richmond, California. Christian Brandes, a postdoc with Tim Tully, has moved to Brandeis University, in Waltham, Massachusetts. Paul Clarke, a postdoc with Mike Mathews, has returned to the UK, to the Institute of Cancer Research Royal Marsden Hospital, Sutton, Surrey. Simon Green, also from Mathews’ laboratory, has gone to Ribogene Inc., in Hayward, California. Mubasher Dar, from Betty Moran’s laboratory, has left for Georgetown University. Brigitte Dauwalder, Kyung-An Han, Victoria Meller, and Efthimios Skoulakis have accompanied Ron Davis to the Baylor College of Medicine, Houston. Salah Ud Din, who worked under Jim Garrels and Bruce Stillman, has gone to pursue other activities. Ahmed Ebrahim, from Betty Moran’s laboratory, is now at home in Bayville. Ian Fitch, who worked with Bruce Futcher, has moved to the University of California, San Francisco, while Michael Tyers, also from Futcher’s laboratory, has taken a faculty position at the University of Toronto. Kyuhyung Han, a postdoc with Ron Davis, has gone...
to Hallym University, South Korea. Chris Hardy left Bruce Stillman's laboratory for a position at Washington University in St. Louis, and John Ruppert went on to a position at Johns Hopkins University School of Medicine, in Baltimore. Luis Jiménez-García, from David Spector's laboratory, has gone to the National Autonomous University of Mexico, in Mexico City. Betty Leichus, a postdoc with Jeff Kuret, has moved to the University of Iowa, in Iowa City. Maarten Linskens, from Bruce Futcher's laboratory, and Karen Prowse, from Carol Greider's laboratory, have taken research positions at Geron Corporation, in Menlo Park, California. Karen Lundgren and Nancy Walworth, who worked with David Beach, have both moved on to other jobs: Karen took a position at Glaxo, in Research Triangle Park, North Carolina, while Nancy went to the Netherlands Cancer Center, Amsterdam. Alan Nighorn finished his postdoc with Ron Davis and took a job at the University of Arizona, Tucson. Arne Östman, who worked with Nick Tonks, went to the Ludwig Institute for Cancer Research in Uppsala, Sweden. Gian-Luigi Russo, a postdoc with Dan Marshak, has moved across the ocean to the University of Naples, while Anthony Rossomando, also from Marshak's laboratory, has moved across the Sound to Miles Pharmaceuticals, in West Haven, Connecticut. Robert Swanson, from Mike Wigler's laboratory, has taken a position at Pharmacopeia, Inc., Monmouth Junction, New Jersey. Ales Vancura has left Jeff Kuret's laboratory for St. John's University, in Jamaica, New York, while Pi-Chao Wang has moved to the National Taiwan University, in Taipei. Heidi Wang left Betty Moran's laboratory for a research job with Bristol-Myers Squibb Pharmaceutical Institute, in Wallingford, Connecticut. Peter Yaciuk also finished up his work in Betty's laboratory and moved to an assistant professorship in microbiology and immunology at St. Louis University Medical Center. Both Yue Xiong, from David Beach's laboratory, and Qing Yang, from Nick Tonks' laboratory, took positions at the University of North Carolina, Chapel Hill.

Graduate Students

Several graduate students have moved on. Jill Crittenden and Kwok Hang Wu have followed Ron Davis to Baylor. Fred Bunz, from Bruce Stillman's laboratory, has left to complete his M.D. at SUNY Stony Brook. Lea Harrington finished up her degree under Carol Greider and took a postdoctoral fellowship at the University of Toronto. Jiann-Shiun Lai, from Winship Herr's laboratory, has begun a postdoctoral fellowship with David Baltimore at The Rockefeller University. Robert Nash finished his degree under Bruce Futcher and headed for California, for a postdoc at the University of California, San Francisco. Michael Sheldon left Nouria Hernandez's laboratory for a postdoc at Rutgers University. Wei Guo finished her degree with David Helfman and continues as a postdoctoral fellow. John Connolly, a visiting graduate student with Tim Tully, came from Dr. C.P. Kyriacou's laboratory at the University of Leicester in England. He has since returned to England, but may return as a postdoctoral fellow.

A Rock-solid Board of Trustees

After the major turnover in our board of trustees in 1992, it was a relief to have few changes this year. We did bid a sad farewell to two members, however. Retiring were George Cutting, whose board membership started in 1986 when he became president of LIBA, and David Pall, the renowned Long Island scientist and corporation founder, who has served since 1987. Both have long been close
Hanna Gray

personal friends and I will miss much the invaluable advice and help they have
given to our board. Charles Dolan, Shirley Tilghman, and Owen Smith were re-
elected to the board this year following their first three-year term. The officers of
the board remain David Luke, Chairman; Mary Lindsay, Vice Chairman; John
Reese, Treasurer; Wendy Hatch, Secretary; and Townsend Knight, Assistant Sec-
retary.

On Friday night before the annual trustees meeting, the Laboratory Trustees
held a special lecture in honor of my 25 years as Director. Trustees and guests
were treated to a wonderful talk by my friend Hanna Gray, the University of Chi-
icago’s President Emeritus. After the talk, David Luke and Mary Lindsay present-
ed Liz and me with a beautiful handpainted tray-table emblazoned with a
landscape of the Laboratory.

George Cutting Turns Over Presidency of the Cold Spring Harbor
Association to Mary Lindsay

For the past 7 years, we were extraordinarily fortunate in having George Cutting
as President of the Long Island Biological Association and its successor organi-
zation, the Cold Spring Harbor Laboratory Association. During his leadership, he
played an essential role in the success of the Second Century Campaign, as well
as in turning the Association’s annual fund-raising objective toward providing re-
search support for the younger scientists of the Laboratory. In every way,
George’s presidency was an unqualified success, with especially important his
decision to create the donor category of Associate to designate those donors
who annually contribute $1000 or more to the Association. Such Associates now
provide more than 75% of the Association support, allowing it in this year’s an-
nual drive to raise more than $530,000. It was thus to our great regret that
George decided that the time had come to retire, his term ending at the Associa-
vital role he has played in ensuring the Laboratory’s healthy scientific future.

Succeeding George as President is Mary Lindsay, also a long-time neighbor of
the Laboratory with her home in Laurel Hollow nearby on Ridge Road. I now have
known Mary and her husband, George, since the summer of 1967 and look for-
ward to even greater Lindsay roles in providing vital leadership skills to the
Laboratory.

I Make the Transition from Director to President

This past year marked my final year as Director, my appointment occurring in
February of 1968. My tenure of slightly more than 25 years has brought me many
moments of happiness as I watched the Laboratory become an even more im-
portant instrument for science than it was when I arrived from Harvard, newly
married, and just turned 40 years of age. Leading our research meetings and
teaching programs has constantly renewed my mind both through the excitement
of important new ideas and facts and through intellectual interactions with so
many talented individuals, many of whom have become lifelong friends. Equally
wonderful has been the opportunity to live on the Laboratory grounds, which
surely must be one of the most attractive sites for conducting science anywhere
in the world.

I am thus very grateful to our Board of Trustees not only for creating the new
position of President for me, but also for acquiring the adjacent parcel of land that is enabling the Laboratory to build a new President’s House which we hope will be completed by the early fall of 1994. The costs of the house will be covered by me as a way of expressing my faith in the continued importance of Cold Spring Harbor for the promotion of biomedical research and education. Upon the occupancy of the President’s House by my wife Liz and me, the Director’s House, Airlie, will be further renewed to suit its future occupancy by our new Director Bruce Stillman and his family. We shall be leaving Airlie after almost exactly 20 years of contented dwelling. Before we moved in, a year had been spent totally renovating this 1806 Jones family farmhouse according to the inspired plans given to us by Charles W. Moore and his talented colleague, William Grover. Airlie remains an extraordinarily fine home to occupy, and I anticipate that Bruce and his family also will take great pleasure from its beautiful lawns and specimen trees.

April 15, 1994

James D. Watson
DEPARTMENTAL REPORTS
1993 was a remarkable year to be at Cold Spring Harbor Laboratory with a ring-
side view of the stunning progress driving the fields of molecular biology and
genetics. There were breakthrough discoveries at the Laboratory, some receiving
much attention in the national media, and most everything new and interesting
from other places was being discussed at our meetings, taught in our courses, or
published by the Cold Spring Harbor Laboratory Press. Against this background
of extraordinary science, it should not be surprising that other aspects of
Laboratory operations were very satisfactory as well.

Financial results were good with revenues at a new high of $39 million. For
the fifth consecutive year, there was a surplus from operations after provision for
depreciation and reserves for future contingencies. During the last 5 years, opera-
tions have provided nearly $12 million of cash flow for investment in new and
improved facilities and programs. Final 1993 results were more than $1 million
better than projected at the beginning of the year due to higher than expected
meetings attendance, a strong level of government grants, higher royalty and in-
terests receipts, and careful cost control by administrative departments. Environ-
mental Health and Safety, under the effective leadership of Art Brings, saved
some $240,000 through innovative programs for managing and disposing of
radioactive and hazardous waste.

The Cold Spring Harbor Laboratory Press, Banbury Center, and the DNA
Learning Center, which operate as individual cost centers, each improved their
financial results over the previous year. The Press, with its successful journal pro-
gram and increased new book publications, has become reliably self-sufficient,
which is important for the Laboratory at this time when Federal grants for science
are difficult to obtain. Both Banbury and the Learning Center conduct innovative
and consistently first-rate programs with broad national significance. They are
successful in attracting the funding they require and operate at a modest
surplus
before depreciation.

The Laboratory's endowment, consisting of the Robertson and Cold Spring
Harbor funds, ended the year at a new high level of approximately $92 million.
The investments include a balanced mix of domestic and foreign equities, fixed
income securities, and short-term liquid instruments. The endowment represents
the Laboratory's primary internal source of funding for science and also provides
important support for $30 million of outstanding tax-exempt civic and research fa-
cility development bonds. Over the years, the drawdown from endowment has
been maintained at a conservative level, averaging about 3.5% per year. This al-
lows for substantial reinvestment of income to protect against inflation and to pro-
vide for future programs. The endowment enjoys excellent investment manage-
ment from Miller, Anderson, and Sherrerd and from the U.S. Trust Company. In
1993, the Robertson and Cold Spring Harbor funds had a total return of about
13.5% compared with a benchmark index of 10.5%. The Cold Spring Harbor
Fund also benefited from new gifts of $2.9 million, which included $500,000 from
the Fort Hill Foundation to establish the William J. Matheson Chair and an addi-
tion of $50,000 from the Banbury Fund for the Charles S. Robertson Chair.

The Trustees of the Laboratory have set as a goal to increase the endowment
to a level of $100 million, net of outstanding debt, by the year 2000. At their No-
vember annual meeting, the Trustees established a new program entitled The
Next 100 Years, which seeks new funds for the endowment in the form of
planned giving. Such gifts, which include various forms of trusts, bequests, and outright donations of property, are regarded by most institutions as the largest potential source for future endowment funds.

Much discussion has occurred in recent years concerning the rates of indirect cost recovery that institutions receive on government research grants and use to pay the cost of overhead incurred in providing support and facilities for science. There has been pressure from Congress to correct a few much publicized cases of abuse and for a broad reduction in such rates. We are pleased that in 1993, the government accepted the Laboratory's past procedures without adjustment and negotiated with us a new 5-year predetermined rate incorporating a reduction of about 5%. This removes considerable uncertainty and greatly facilitates future planning. Bill Keen, our Controller, deserves much credit for his very competent past and present handling of this important subject.

Particularly noteworthy during 1993 were the ongoing efforts by John Maroney, Director of Commercial Relations, to match up the discoveries of Laboratory scientists with pharmaceutical and biotech companies interested in providing research support in return for various rights to existing intellectual property and also with venture capitalists looking to fund new start-ups. The Laboratory's Trustees in 1992 established the Science Fund as a permanent part of the endowment into which are placed all of the Laboratory's royalties and equity shares from such relationships, to be used for the exclusive support of science. Last year, royalty income exceeded $1 million for the first time, and already the Science Fund includes equity holdings in new biotech names such as ICOS, Geron, Mitotix, Pharmacopeia, and Oncor. Soon Amplicon and PathoGenesis will be added to this list.

1993 marked a watershed year for the Laboratory’s Meetings Department with the retirement of Barbara Ward in October. Barbara began her career at Cold Spring Harbor some 25 years ago as secretary to Edward Pulling, who then personified the Long Island Biological Association, now the Cold Spring Harbor Laboratory Association. In recent years, as Meetings and Courses Manager, Barbara has led the very competent Meetings staff in caring for the needs of our nearly 6000 visiting scientists per year. We shall miss her a great deal. Fortunately, we were successful in recruiting Dr. David Stewart from a biotech and academic science background at Cambridge, England; he joined the Laboratory as Director of Meetings in September. Arriving at a time of record meetings attendance, David moved quickly to modernize the department’s computer system to better handle the increased registration and established a small computer center for visiting scientists.

For the future, David is thinking about adding new meetings, perhaps in the normally dormant months of late fall, winter, and early spring. These, for example, might involve academic retreats or meetings sponsored by members of the pharmaceutical or biotech industry to discuss applied uses of basic research. The only caveat would be that such meetings be scientifically interesting and conform to the tradition here of excellence. In this manner, more year-round use can be made of our facilities.

In the interest of improving the overall quality of food service at Blackford Hall, we recruited in November a new chef, Ron Padden, who has extensive restaurant experience. Laboratory employees benefited first at winter meals, and this spring, our meetings visitors confirmed the improvement.

An important change during the year was consolidation of the Development and Public Affairs Departments into a new Department of Public Affairs and De-
velopment with Susan Cooper as Director. There is much overlap between the functions of these areas, and already there is clear improvement in effectiveness and efficiency. The change enables Gordon Hargraves, appointed to the new position of Director of Planned Giving, to devote his efforts to the success of the very important "The Next 100 Years" effort.

The effective functioning of the Buildings and Grounds Department, under the leadership of Jack Richards, has always been of critical importance to the Laboratory. In 1992, Jack delegated the responsibility for maintenance of grounds and the renovation of laboratories, offices, and other facilities to Peter Stahl, and Peter and his staff have responded with skill and dedication. This has enabled Jack to devote the major portion of his time to new construction, and once more, we are in awe of what has been accomplished. The 2-year project to completely gut and rebuild the more than 80-year-old McClintock Laboratory as a state-of-the-art cancer research laboratory was completed on time and on budget of approximately $4.6 million. With the major assistance of subcontractor Bill Baldwin, the entire structure was strengthened and equipped with new mechanical systems, a third floor was added, and the classically detailed brick and stucco exterior was restored to its former beauty.

In the spring of the year, the Laboratory purchased the previously leased DNA Learning Center building in Cold Spring Harbor Village from the Cold Spring Harbor Central School District. Immediately thereafter began a thorough renovation of the building, including a new front entrance, handicap accessibility features, and a new sprinkler system. A computer laboratory was added, and thanks to generous support from Cablevision, Inc., a new multimedia-equipped 100-seat auditorium was built that may well be the finest installation of its type on Long Island.

The year also witnessed the start of construction of a new President's House, which has a spectacular view of Cold Spring Harbor and far-off Connecticut from the northernmost corner of the campus. Paid for entirely through Jim Watson's generosity to the Laboratory, the new structure will be home to Jim and Liz for many years and also house the Watson archives and other important papers dealing with the history of molecular biology and genetics.

Less interesting, but very important, was the completion of all the necessary engineering and approvals for the start of construction at year end of a new sewage force main and pumping station that this spring replaced our nearly 25-year-old sewage treatment plant. For an investment of $1.6 million, the Laboratory is now connected to Nassau County's sewage line in Syosset and then to the Cedar Creek treatment plant on the South Shore. The need to discharge treated effluent into the inner harbor has been ended forever. The assistance of the Trustees of the Village of Laurel Hollow in approving and supporting this project is much appreciated, and we thank the residents of Stewarts Lane and Laurel Hollow Road for their patience during the winter construction.

Every year, other key administrators such as Barbara Wang, Assistant Controller; Susan Schulz, Grants Manager; Sandy Chmelev, Purchasing Manager; and Cheryl Sinclair, Human Resources Manager play key roles in running our complex and larger institution. Roberta Salant deals with all the requirements of our very active and interested Board of Trustees and is of immeasurable help to John Maroney and myself. The administrative departments and their staff do much to provide the facilities and support that make the Laboratory a leader in its field, and they should all feel great satisfaction in what they have accomplished.

Looking to the future, we are concerned by a small but important cloud on an
otherwise potential-filled outlook. For perhaps the first time in the Laboratory's more than 100-year history, a significant number of neighbors in the Village of Laurel Hollow are disturbed by various matters relating to the Laboratory. Some are concerned by "dollars and cents" issues such as the cost of scientists' children attending local schools while living in tax-exempt housing. Others question the degree of Laboratory support for the Village budget and use of Village services. Such concerns are understandable at this time when school budgets everywhere are under pressure and with Village taxes here among the highest on Long Island. It should be possible to resolve these questions by reasoned discussion, better communication, and a willingness to be fair. More difficult is concern by some residents about the growth of the Laboratory and future use of the property. In recent years, the Laboratory has become substantially larger in number of buildings and employees, but so too has the Village of Laurel Hollow. The Laboratory has managed its growth with great care and concern for the environment, and today the campus grounds and architecture are widely regarded as among Long Island's most beautiful. It is hard to imagine anyone having a greater vested interest than the Laboratory itself in the continuation of that beauty over the next 100 years for the enjoyment and inspiration of our staff, our visitors, and our many neighbors who support us so generously.

Some believe, however, that the Village should limit future use of the campus, and at their behest, a temporary moratorium has been instituted on new non-residential construction. Renovation of the ground floor of the DeForest Stables as a day-care center for children of Laboratory employees has been put on hold after 2 years of study and planning with consultants and the appropriate state and local agencies. The Village has required a site-specific and generic environmental impact study; meanwhile, there is much disappointment and inconvenience for those who have been counting on the availability of child care.

To remain at the forefront of its field, the Laboratory must maintain its legal right to use its property in a manner consistent with its role as a world class research and education institution. Hopefully, reason and dialogue will prevail, and it will be possible to proceed into the next century with the spirit of cooperation and mutual respect that has benefited both the Laboratory and the Village for so long.

G. Morgan Browne

BUILDINGS AND GROUNDS

The Buildings and Grounds Department was again kept busy in 1993. With major projects from the previous year nearing completion and new projects started, the Buildings and Grounds staff could hardly catch its breath. The first half of the year was the busiest; the McClintock renovation was coming to completion and the DNA Learning Center was being renovated. The last half of the year was marked by many minor alterations and by the retrofitting of the sewage treatment plant and installation of a sewage pipeline two and a half miles long.
McClintock Laboratory

As 1993 opened, the Mcclintock renovation was moving along with the installation of the third-floor penthouse, all new interior walls, and new mechanical equipment. The next step was to install the laboratory benches, desks, fume hoods, shelves, etc., and to complete the mechanical system, which included the cooling tower for the air-conditioning system and a building to house the system north of the Cairn Laboratory. The last stage was to complete the trim, hang doors, install computers and telephone wires, clean the building, and landscape the exterior. David Beach’s laboratory, with all of its equipment, was moved from the Beckman building to the completed McClintock building. McClintock now has a wonderful third-floor office area with a spectacular view of Cold Spring Harbor. We moved a very large boulder to the entrance of McClintock Meadows to be used for a plaque in memory of Barbara Mcclintock.

DNA Learning Center

Major changes during the first half of 1993 were carried out at the DNA Learning Center after the Smithsonian Exhibition, which had occupied most of the building since 1987, was removed. We gutted the interior of the entire main floor and removed the main entrance steps. We then built some new exhibit rooms, along with a state-of-the-art auditorium in the east wing for the multimedia program donated by Cablevision Corporation. The auditorium became a highly technical room complete with multiple projectors, videos, and sound equipment, all computer coordinated. The west wing now holds a new computer laboratory which is linked to the "wet" laboratory next door. We also installed a fire sprinkler system and completely rebuilt the main entrance, including a ramp for the handicapped.

Sewage Treatment Plant

For 18 years, the Laboratory has maintained a sewage treatment facility in the middle of the Laboratory grounds, and the staff has always operated the plant in full compliance with all regulations. However, as the plant aged and the mechanical equipment became in constant need of repair, it became painfully clear that soon the plant would have to be completely rebuilt. The Laboratory therefore explored the possibility of pumping all of the sewage to Nassau County’s Cedar Creek Treatment Plant. After an engineering study and numerous applications, the Laboratory received permission from the Department of Conservation and Nassau County to construct a pump station and pipeline to connect to the county sewer system. In October, contracts were signed to install both the pipeline and pump station, and both contractors set to work planning and ordering materials. As the year ended, the two and half mile long pipeline was started and the existing treatment plant saw the beginnings of its conversion. Work will proceed through the winter of 1994, with completion scheduled for April 1, 1994.

Renovations, Alterations, and Other Projects

Throughout the year, numerous projects keep the Buildings and Grounds staff moving ever forward, from alterations to renovations to new and old construc-
tions. These projects can be as small as moving a desk or as large as demolition a house.

Bronze Helix: In May, a 15-foot sculpture of the double helix was presented to Dr. Watson during the Symposium meeting. We had the privilege of assisting Charles Reina, the artist, transport "Bronze Helix" from his home and install it in the lobby of Grace Auditorium. This beautiful sculpture is so huge that we had to anchor it to the wall!

The President's House: As April rolled around, we began demolishing the Northview house to prepare the site for the President's House. By June, the foundation was in and construction of the house was under way. As 1993 came to a close, the building was up, the windows were installed, and the roofing and stucco work were started. We also started the 300 linear feet of riprap seawall. We hope to complete all interior work on the house during the winter and start the exterior work in the spring.

Demerec Laboratory: As soon as the McClintock Laboratory was finished, many scientist's laboratories were relocated there. Demerec experienced most of the changes. The rooms on the ground floor of the south wing became support rooms, as the cell culture area was moved to a larger laboratory, and the old cell culture laboratory became a microinjection room. A number of the laboratories on the second floor were refurbished, and three of the cold rooms were completely updated with new mechanical equipment.

Hershey: Fiberoptic cabling was installed, and one of the offices was reworked into a computer laboratory.

Delbruck Laboratory: An energy management computer system came to Delbruck in 1993. This ongoing project is making its way throughout the Laboratory and is now showing results in both energy savings and fewer mechanical failures. Some structural roof repair was also done to Delbuck's teaching laboratory. A combination of age and type of construction had caused the roof to sag to a great extent. After the structural repairs were completed, we installed a new roof.

Beckman Laboratory: Two mechanical systems were improved. The first was the addition of a plate heat exchanger that uses free outdoor cooling and eliminates the need for costly mechanical cooling during the winter months. The second improvement was the addition of supplemental heat to enhance comfort levels in some of the office areas.

Olney Barn: The barn became the headquarters for the Grounds Department and will be used year-round. Some of the improvements included the addition of heating and bathroom facilities and an office for the Grounds Foreman. We also repaired the exterior in preparation for painting in the spring of 1994.

Hooper House and the Firehouse: The years had taken their toll on the roofs of these two buildings as evidenced by falling tiles and unsightly appearance. We installed new roofs on both buildings and look forward to years of trouble-free service.

Upper Cabins: We built a stairway from the lower set of cabins to the upper cabin site. We also constructed a spacious, round wood deck for our visitors at the top of this stairway.
Uplands Farm: Scientists at the farm use corn and Arabidopsis as scientific tools, and they need to grow the Arabidopsis in an enclosed growth room. Buildings and Grounds therefore designed and built a growth room 24 feet long and 12 feet wide specifically for these plants. Timed fluorescent lighting and a climate-controlled environment provide these plants with all they need for healthy growth. Credit for the success of this complex room goes to our tradesmen, who spent many weeks in its construction.

Banbury Center: Dan Miller, with the aid of one full-time helper and one part-timer, does a great job maintaining the 40 acres comprising the Robertson Estate. This team keeps moving right along without too much help from the main Buildings and Grounds office.

In March of 1993, the Laboratory experienced a fierce storm out of the northeast, which caused only minor damage. However, the round houseboat anchored in the harbor for more than 20 years lost its moorings and drifted onto Laboratory property. After spending numerous hours making many phone calls, the owner reluctantly agreed to dismantle the craft and haul it away. We can honestly say, Good riddance!

As the year comes to a close, the Buildings and Grounds Department can look back at all of its accomplishments and be very proud of its work. Our staff has worked very hard and with great skill to maintain the working environment of the Laboratory.

Jack Richards

PUBLIC AFFAIRS AND DEVELOPMENT, AND THE LIBRARY

Combined Effort

So often had the efforts of the Public Affairs and Development Departments dovetailed that in 1993 they were merged to align functions and provide a new environment to deal with the challenge of a project-oriented fund-raising effort. Raising funds for the clearly defined Second Century Capital Campaign, which had a monetary goal, a beginning, and an end, is much different from the day-to-day fund-raising needs of this complex organization. Funding plans and events must be coordinated with the already packed Laboratory-wide calendar. We also must prepare for the return of our chief fund-raiser, Jim Watson, from sabbatical in July 1994. As Laboratory president, he will concentrate on the various funding priorities set forth for the new department. We all look forward to the challenge.

This year of completion, expansion, and transition has been punctuated by great successes. The Infrastructure Fund, begun during the Second Century Campaign, was completed and will be named in honor of Trustee William R. Miller, who so successfully led this effort. The annual contributions grew, an
achievement largely attributable to the efforts of the Cold Spring Harbor Laboratory Association (CSHLA) and its volunteers, led enthusiastically and tirelessly by Mary Lindsay. Similarly, the fledging Corporate Advisory Board, chaired by Douglas Fox, broadened its base of Long Island corporate support for the Laboratory's DNA Learning Center and sharply increased the amount of unrestricted funds raised this year.

DEVELOPMENT

The William R. Miller Infrastructure Fund

The Infrastructure Fund, one of the last remaining funds established during the Second Century Campaign and spearheaded by Trustee William R. Miller, completed the arduous task of raising over $4 million, enabling the Laboratory to complete the buildings and facilities needed for our meetings program. The kitchen and dining areas in Blackford Dining Hall were modernized and the visitor cabins were completed. Fund-raising is difficult; finding funding for the underpinnings of an organization is nearly impossible—but not for Bill Miller, who rallied pharmaceutical industry support for the expanded facilities so that we might better serve our meetings attendees.

Major Gifts

Several major gifts were received in 1993. Generous gifts from William and Irene Miller, the David Koch Foundation, Dr. David Pall, the Perkin Fund, Sherburn Becker, the Daphne Seyboldt Culpeper Foundation, Fannie E. Rippel Foundation, Booth Ferris Foundation, and Oncor, Inc. provided over $4 million toward the Cancer Research Fund.

We were also fortunate to have received other major gifts during the year. John and Rita Cleary, Dr. James Watson, and Owen Smith, Esq., contributed to the Genetic Diseases Fund with a specific emphasis on muscular dystrophy. George and Mary Lindsay contributed unrestricted funds, and Henry Wendt and Dr. Maxwell Cowan gave for program support in Neurobiology. The Fort Hill Foundation provided funds toward the William J. Matheson Chair and the Bannbury Fund supported the Charles S. Robertson Chair.

We also received several planned gifts in 1993. Bob Gardner established a Charitable Remainder Trust (CRT) which named the Laboratory and two other institutions as equal beneficiaries; the trust was funded with appreciated securities. Townsend and Elise Knight established a CRT naming the Laboratory as the sole beneficiary; this trust was funded with proceeds from the sale of a house owned for many years by the Knights and donated to the Laboratory. In both cases, the donors added significantly to the Laboratory's endowment while creating a high-yield life income for themselves, avoiding capital gains taxes on the transfer of appreciated property, receiving a charitable deduction, and reducing estate taxes. In a similar fashion, Edward Palmer named the Laboratory as beneficiary of a life insurance policy he had received as a corporate director, and William Osborn, who sadly died in November, made a bequest of an insurance policy to be paid at a date in the future. Two other bequests were received from the estates of Taggart Whipple and Charles Henry Leach II. All of these gifts add to the Laboratory's endowment, helping to ensure the future of the science program at the highest level.
Annual Contributions

The Laboratory's Annual Fund consists of several programs: the CSHL Association Annual Fund, the Corporate Sponsor Program, the Student Scholarship Funds (which include both the Undergraduate Research Program and Partners for the Future), and the DNA Learning Center Annual Fund. Miscellaneous annual contributions also come from memorials and a few restricted annual gifts. These programs contributed a total of $1,468,093 to the Laboratory in 1993.

The CSHLA Annual Fund, the major source of unrestricted gifts to the Laboratory, reached a new high in 1993 at $526,241. Membership in the Cold Spring Harbor Laboratory Association rose to 889 members. Through the hard work of Mary Lindsay, Carol Large, and the other Association officers, directors, and members, the CSHLA's contribution to the Annual Fund was increased by $100,000 over the 1992 level. Two successful benefits, the Blue Hill Troupe and the "Secret of Life" garden party, raised $43,980. The "Secret of Life" party also added 140 people to the Association's membership.

Association dollars are well spent. Dwindling federal funds make it increasingly difficult for the scientists to find complete funding for their laboratory's operating costs. The Annual Fund provides fellowships for young scientists, start-up funding for new laboratories, equipment, and supplies, and other unrestricted monies to ease the budget burden. Providing these funds directly affects the success of basic research into the causes of cancer, the nature of learning and memory, the understanding of neurodegenerative diseases such as Alzheimer's, aging, and other genetic diseases such as muscular dystrophy.

The Corporate Sponsor Program, which supports the meetings program at the Laboratory and at the Banbury Center, generated $737,750 from 30 companies in 1993.

Corporate Advisory Board DNALC Annual Fund, composed of outstanding business and professional leaders from the Long Island business community, grew significantly during 1993. Led by Douglas Fox of Times-Mirror, members raised $66,650 in unrestricted funds in 1993, an increase of more than 200% from the previous year. Members also contributed their time and ideas to the Corporate Advisory Board, and three committees were formed to support future outreach and fund-raising efforts. The Education Committee, focusing on genetics workshops and seminars for the non-scientific community, is co-chaired by Gary Frashier of Oncogene Science and Rocco Barrese of Dilworth and Barrese. The Golf Tournament Committee will plan and execute the first Cold Spring Harbor Laboratory Golf Tournament in spring 1994.

In the fall, Advisory board members were proud to show off the newly expanded and renovated DNA Learning Center at Education Night, where they treated close friends and business associates to a hands-on visit in both the teaching and computer laboratories and to a viewing of Long Island Discovery; a Cablevision-sponsored multimedia presentation about Long Island. A delicious harvest meal capped the evening.

Undergraduate Research Program/Partners for the Future are highly selective programs that make up the Laboratory's Student Scholarship Funds. The Undergraduate Research Program (URP) is a 10-week intensive summer program that encourages college juniors and seniors to consider careers in basic research. Each student from the international group of about 20 works on a specific project with a mentor from senior Laboratory staff. Similarly, each Long Island
high school may nominate a single senior to be one of five Partners for the Future. These youngsters spend 10 hours a week after school, between October and March, working with a scientist/mentor. Funds for these two programs have been received from a variety of sources and total $119,771 in 1993. The lion's share of these funds were raised for the 34-year-old URP program, which started its own endowment effort in the Second Century Campaign. The Shakespeare URP internship became the first to be fully endowed this year when its donor added $50,000 to his original gift of that amount. More funds must be found to ensure the continuation of the vital Partners for the Future program.

Reorganization and Staff Changes

Following the completion of the Second Century Campaign, jobs were realigned to reflect the new organization of the development effort. Gordon “Skip” Hargraves became Director of Planned Giving to reflect the Department's emphasis on planned giving as the major mechanism for increasing the Laboratory’s endowment. Jill Clark, who came to us from the Foundation for Child Development with a background in nonprofit financial consulting, was appointed Associate Director of Development with responsibility for Foundations and Corporate relations. In her short time here, Jill's handiwork can be seen in the professional preparation of formal foundation proposals, the establishment and successful beginning of the new President's Council, and her initiative in planning a variety of departmental projects. Joan Pesek continues as the Associate Director of the CSHL Association Annual Fund. This year, Joan developed the new array of Association materials for the membership drive. Claire Fairman has responsibility for raising funds for the Student Scholarship Funds and coordinating most of the events for the Department. Deb Mullen, administrative assistant, on whom we all rely, keeps our lives in order with a can-do willingness seen in very few. Deb took responsibility for creating our fundraising database using the new software, Raiser's Edge from Black Baud. Gisela Jennings will continue to assist Deb until her temporary appointment ends in March 1994.

Plans for 1994 include continued emphasis on planned giving with the preparation and direct mailing of a planned giving brochure. Every effort will be made to further the success of the Association by developing marketing tools that expand the demographics of membership and broaden the base of annual unrestricted support. Foundation support will be sought to sustain the various programs that must be funded; these include the Cancer Research Fund, scientific program support, Student Scholarship Funds, the Education Fund, the Child Care Center, fiber optic cable system for high-speed computer networking, and the Library Fund. We hope to expand the role of the Corporate Advisory Board whose efforts presently go to raise unrestricted annual funds for the DNA Learning Center.

PUBLIC AFFAIRS

Major Events

Each year brings an increased number of events, and each year we imagine there could never be more. Certainly that could be said for 1993, a year that marked so many passages for Cold Spring Harbor Laboratory. Celebrations were
held to mark the 40th anniversary of the discovery of the double helix, Dr. Watson's 25th year as organizer of the Symposium, and his 25 years as director. We lauded 20 years of Robertson Research Fund support, applauded our newest Nobel Laureate, Richard Roberts, and rededicated two of our oldest buildings, Blackford Dining Hall and McClintock Laboratory.

The Public Affairs Department joined forces with Jan Witkowski to plan every aspect of the DNA 40th Anniversary celebration, March 1-3, 1993. We prepared a reprint of the original articles printed in Nature in 1953, which announced one of the most important scientific discoveries of our time. Sculptor Charles Reina was commissioned to design small double helix models; a gold one was presented to Francis Crick who was here for the 40th Anniversary. At the same time, we rededicated an expanded Blackford Hall with the preparation of a brochure that documented the history of the Hall from its inception in 1907 to its present modernization. At the dedication, a silver model of the double helix was given to William R. Miller, who led the fund-raising effort that, among other things, supported the Blackford expansion. A dinner was held in Clarkson Dining Hall for the contributors and special guests.

The last 20 years have been the most secure in the Laboratory’s more than 100 years, and the Robertson Research Fund, established in 1973, formed the original endowment. On June 11, the Robertson family and friends were feted with a presentation by one of the Robertson Fund-sponsored Outstanding Junior Fellows, Carol Greider, and by David Beach, both of whom described their cutting-edge research. Dr. Watson extolled the virtues of this important gift and presented the members of the family with a leather-bound commemorative volume charting the history and success of the fund. Bill Robertson spoke thoughtfully on behalf of the family. A wonderful dinner capped the evening.

The rededication of the McClintock building drew an interesting assemblage. Barbara McClintock's niece, Marjorie Bhavnani, and nephew, Scott McClintock, joined contributors, members of 1 in 9 from the Long Island Breast Cancer Coalition, and staff to hear Al Hershey describe sharing this laboratory with Dr. McClintock in the 1950s. The keynote address was given by David Beach, who discussed his work using yeast to understand control in the cell cycle. The day was marked by a dedication brochure featuring a reproduction of Eleanor Malamed's towering portrait of Barbara McClintock and an intriguing history of the building and its occupants.

Other Events

Many other events rounded out our year. The three Lloyd Harbor Seminars were introduced by village trustee George Toumanoff. In the spring, Dr. Francis Roberts, Superintendent, District 2, presented a talk on American schools in the 21st Century, and Meg Cleary of the Nature Conservancy spoke on the Conservation Initiative on Long Island. A seminar on eugenics was presented in the late fall by David Micklos, Director of the DNA Learning Center. Twice yearly, during a spring and fall meeting, performers from Young Concert Artists International come to the Laboratory to delight our meeting attendees. In 1993, Alex Slobodyanik, pianist, and Scott St. Johns, violinist, performed.

A new series of clinical lectures for the public, arranged in conjunction with Huntington Hospital, was presented in Grace Auditorium. Topics discussed included stroke, heart disease, and colon, prostate, and breast cancer. The popularity of these programs has encouraged us to continue them in 1994/1995.
Again we held our four quarterly Saturday morning public tours, introducing 117 visitors to the magic of Cold Spring Harbor. Special talks and tours were arranged for various groups throughout the year. Some of these groups included SPLIA; the New York Institute of Technology; Lion’s Club; SUNY Stony Brook science forum, Nassau Community College; and the Association of University Women. These tours and talks are successful because our scientists are willing to meet with visitors and outside groups.

The Association kept us busy arranging more than a dozen different events, including art exhibits, concerts, an operetta, garden parties, lectures, and meetings. We produced new stationery which includes two sizes of letterhead, thank you and note cards, donor and memorial cards, and the annual fund brochure. The Association President’s report appears on page 407.

Our involvement with holiday parties is kept to a minimum because of the good offices of Art Brings, who, along with his energetic staff, installs giant Christmas trees, wraps Christmas gifts, designs coffins for Halloween, arranges magic shows, and generally makes this a better place to be. This year’s staff events included the first Lab-wide summer staff picnic on the sandspit, a welcome get-together for staff and their families.

Seventy staff and their families sat down together for a shared Thanksgiving. Blackford Hall staff prepared turkey and trimmings, and the families provided the side dishes and dessert. After a game of football on the lawn, everyone enjoyed the international food and extended family. At the children’s holiday party, Santa presented 114 kids from the laboratory with gifts selected by helpers Joan Pesek and Grace Stillman. At our annual tree lighting, Old Bethpage Village Brass Band accompanied our caroling, and Jim Hope prepared great holiday treats for staff and neighbors.

It should be noted that events do not happen without the cooperation of many people. Safety and security handle the parking and crowd control with tremendous skill. The carpenters hang artwork and install brass plaques and are always on hand to fix something at the last minute. The computer staff makes light work of our mailing lists and in-house printing needs. The electricians work tirelessly to fix phones, install wiring, and ensure lighting. The custodial staff is called on to set up and take down tables and chairs daily. They keep the facilities clean and offer the human power necessary to move an event. The grounds are beautifully groomed. The Meetings office arranges housing for our overnight visitors, and the Blackford staff prepares all the food and drink.

Printed Materials

Harbor Transcript continues to expand its content. This year, our summer issue was a whopping 16 pager featuring special coverage of the very special events held this year by the CSHL Association. Each issue in 1993 recorded the many milestones of the Laboratory. We are distributing this newsletter to nearly 7000 scientific alumni, foundations, contributors, association members, neighbors, and friends. Rising costs will make it necessary to pare our mailing list in the future and offer it long-term to only those who are members of the Laboratory’s extended family.

Production of materials for the Laboratory is a cooperative effort of science writer Nathaniel Comfort and our multitalented art director, Margot Bennett. The addition of a new computer with design software makes the work faster and more efficient. We can now see what a piece will look like, color and all, directly on the
monitor, and this allows us to make minor changes instantaneously. With the capacity to work more efficiently, we have been called on to produce more material with no increase in staff. We prepared 11 formal invitations, 6 event programs, 11 pieces for the Association, a brochure for the new President’s Council, and, as always, the 3 meetings posters and the Nature Study brochure. Margot Bennett’s tasteful design of the dedication pieces, the Robertson book, and our new brochure, A Closer View (due early 1994), deserves special mention. Each design fit the occasion and each uniquely represents the Laboratory. Additionally, Margot Bennett, ably aided by Ed Campodonico, Herb Parsons, and Scott McBride, succeeds in keeping up with the growing demand for photographic services.

Nathaniel Comfort researched and wrote the three important brochures that have eloquently contributed to the documentation of the Laboratory’s history: The Blackford Dedication, the McClintock Dedication, and the Robertson Research Fund 20th Anniversary. He also wrote many press releases, foundation applications, and the text for A Closer View. Certainly, Nathan’s year could be characterized as one of change and commitment. He married, began his Ph.D. in the history of science, bought a home, completed the science history portion of a special research project for the Laboratory’s application to the National Register of Historic Places, prepared by Elizabeth Watson, and contracted with the CSHL Press to co-author a book with Dr. Bentley Glass on the Laboratory’s scientific history. It is our pleasure to find that Nathan’s career path has been influenced by the rich history of this institution.

**Partners for the Future**

Along with handling the day-to-day operation of a very busy Public Affairs office, Lynn Hardin is the administrator for the Partners program. Each year in March, the 151 schools on Long Island are asked to submit their candidates for the following school year. The applications are narrowed to ten candidates by a committee of scientists; in June, the committee interviews each of the finalists and the five successful Partners are notified on July 1. The fact that this program runs flawlessly can be attributed to Lynn’s adherence to timetables and execution of the rules for this competitive program. The 1993/1994 students are profiled in the Highlights section of this report.

**Donor Recognition**

Brass plaque recognition of our donors is also Lynn Hardin’s responsibility. Five major plaques were put in place in 1993. Among these were the giant Second Century Campaign plaque and the plaque marking the first 20 years of the Robertson Research Fund, each installed in Grace Auditorium lobby. In 1993, Lynn found a Long Island vendor who was able to do work of better quality for half the price and in half the time required by the previous vendor.

**Media**

The Department handled press questions that resulted in nearly 400 mentions in the print media, television, and radio. Two big events, the 40th Anniversary of DNA and the receipt of the Nobel prize by two former staff members, Richard Roberts and Phillip Sharp, dominated the coverage. The Department got special support from intern Elizabeth Cooper, who gave her Columbus Day weekend to
reproduce all the materials for the Press. Additionally, we had requests regarding other Nobel recipients who have been participants in the Laboratory’s meetings and courses, and questions relating to the promotions of Jim Watson to president and Bruce Stillman to director. Likewise, there were other notable features in 1993. The Economic Times of Long Island, in its series “Women in Technology,” profiled Carol Greider and her telomeres research. Bob Cooke of Newday wrote an excellent companion piece to the announcement of this year’s Nobel prize characterizing the Laboratory as “a fertile seedbed for biological research.” The Long Islander wrote “CSH lab sends experiment to space,” discussing the fact that the space shuttle Discovery carried a protein payload from David Barford’s laboratory. The experiment examined the effect of growing crystals under microgravity conditions.

Audiovisual

Ever present and willing to meet all the audiovisual needs of the Laboratory, Herb Parsons and Ed Campodonico bring new meaning to personal service and attention to detail. These men and their able student aides provide all audiovisual services for the meetings and courses, Banbury Center programs, and all special events inside and out. It is their responsibility to keep all seminar rooms in working order. We cannot ask more of this small dedicated staff.

LIBRARY SERVICES

Under this umbrella are the Library and its staff, who continue to serve and support the information needs of their many constituencies. The scientists receive prompt service and up-to-date bibliographic support of their projects through the availability of a variety of computer resources. Public Affairs requires major support when preparing the history or design for projects ranging from dedication booklets to commemorative reprints. The Development Department is provided with a range of services from art curation, commissioning, and framing to the acquisition of commemoratives. The Library also maintains Development’s collection of foundation and corporate annual reports and reference materials.

The Library staff continues to operate efficiently in ever-deteriorating conditions. Materials, machines, and machine readables contribute to the growth, whereas water problems, crowding, and noise weaken the resolve.

Reference and Bibliography

Patron access of MEDLINE, Current Contents, and Science Citation Index on computers increased 94% in 1993. Computer-based reference capability has grown to include Linscott’s Manual and Grolier’s Encyclopedia on CD. Patrons are assisted in discovering resources on the Internet, such as American Type Culture Collection catalog (ATCC), FlyBase, and the Arabidopsis Research Companion. Continuing problems with the CD-ROM towers computer, which is heavily used, put us out of commission for a while, but free searching was done by the staff to mitigate the inconvenience to researchers. These mechanical problems should be resolved early in 1994 with an upgrade of the system.

Seventy-one scientists use the Library’s table of contents service, which provides desktop access to the contents of 81 journals. The number of interlibrary
loans processed for the scientific staff increased 78% over last year. Wanda Stolen, senior library assistant, obtains articles quickly and efficiently using online access while keeping costs down by utilization of our membership in the New York State Hospital Program which supplements regional libraries.

A survey of the use of journal titles held by the Library and its branches produced excellent results when it revealed that 11 titles were not being used and could be canceled and replaced by 11 new titles required by the scientific staff. Inactive journals and accumulated duplicates were sold to make room for our expanding collection of books, journals, and tapes.

New Equipment
A new Xerox photocopier has replaced the tired, old IBM. The new copier does reduction and enlargement and has a photo mode, auto contrast, margin shift, stapler, and other special features. The use of the Library's photocopy service has increased 73% this year. As has been done in a growing number of buildings on grounds, a security access system has been installed to provide after-hours access by authorized personnel.

Service with a Smile
Superlative service to growing numbers of scientists in overcrowded conditions can only be achieved by a willing and dedicated staff. Margaret Henderson and her staff meet this challenge with great patience and intelligence. Wanda Stolen, Kelly Kasso, and Leigh Johnson ably handled the Library during Margaret's summer maternity leave.

Archives and Art
The Library archives were exploited in 1993 because of the convergence of several landmark events occurring at the Laboratory. Clare Bunce has made an indelible mark on archive service, accomplishing volumes of work for the torrent of requests. Not only does archives provide research and photographic service, but it also handles artistic reproduction and framing Lab-wide.

Research and photographs were provided for the Robertson Research Fund 20th Anniversary book, the McClintock Laboratory's rededication brochure, and the current forthcoming Laboratory overview, A Closer View. Photographs were also provided for the Annual Report and Harbor Transcript.

Outside use of the archival collection came from a diverse clientele consisting of book publishers, film producers, newspapers, museums, and educational facilities. Students of all ages are visitors to the archives for class projects on the history of molecular biology and genetics. Biographical information on and photographs of our Nobel Laureates, Alfred Hershey, Barbara McClintock, James Watson, and now Richard Roberts and Phillip Sharp, are routinely requested.

There was heightened interest in James D. Watson when the 40th anniversary of the discovery of the structure of DNA coincided with his 25th year as director of the Laboratory. Along with the original drawing of the double helix, archives provided various illustrations as the inspiration for the 15-foot 'Bronze Helix,' which stands in the lobby of Grace Auditorium. In addition, photographs were provided for the montage that hangs in the lobby. Both of these were gifts from colleagues of Jim Watson and were presented during the annual Symposium marking his first 25 years at the Laboratory.
Special Skills and Financial Support

Laura Hyman, marking her 20th year with Cold Spring Harbor Laboratory, is my greatest asset. She lends support to all aspects of my many-faceted department. Among her many 1993 accomplishments, Laura identified the artists, commissioned the works, and monitored the progress of the "Bronze Helix," the portrait of Dr. McClintock that hangs on the top floor of the laboratory building that bears Barbara's name, and the tray-table for Dr. and Mrs. Watson. Laura selects frames, commemoratives, artwork, photography, and furnishing for myriad projects Lab-wide. Her artistic talent and eye for excellence enable her to put many finishing touches on the Laboratory's aesthetic landscape.

As business manager, Laura is responsible for preparing the preliminary budgets for all three departments, overseeing expenditures throughout the year, and handling special projects. In 1993, she managed the records retention committee, determining needs, finding appropriate space, and planning the move. By the summer of 1994, the Records Center of the Laboratory will move into a permanent, long-term storage space in the basement of Sammis Hall. During these 20 years, no one has served the Laboratory any better or more willingly. She has my respect and gratitude.

It is gratifying to review the year of accomplishments and know that many people contributed to the success of the newly combined department. It is my pleasure to team-build with a dedicated staff, cooperative departments, and energetic volunteers.

Susan Cooper
Top row: T. Koothan; P. Kearney; C. Huang; K. McCloy

Center: M. Delay (President of Axon Institute), R. Malinow, A. Shirke

Bottom row: H. Zhou; S. Waga; M.-S. Jung, M. Regulski; C. Bautista
The Tumor Virus Section continues to use a variety of viruses to study the mechanisms of gene replication and expression. These studies follow the central dogma of biology, extending from control of DNA replication and transcription to mRNA processing and translation. In earlier years, research in this section relied heavily on DNA tumor viruses, especially adenovirus, papillomavirus, and SV40. More recently, the research has expanded to include other viruses such as herpes simplex virus and human immunodeficiency virus and nonmammalian organisms, in particular yeast. This diversification, however, has not weakened the network of interactions that have been a mainstay of this section. Indeed, the reports that follow attest to the vitality of this program now in its 23rd year.

During the past year, our studies on the replication of DNA in eukaryotes have progressed on many important fronts. The primate virus, simian virus 40, can replicate its DNA in human cells and has been the mainstay of our efforts toward understanding the enzymology of DNA replication in eukaryotes. This vigorous experimental system has been valuable primarily because of the ability to replicate SV40 DNA in a cell-free extract from human cells. In this past year, we have achieved a long-term goal of being able to replicate DNA containing the SV40 replicator with highly purified and characterized DNA replication proteins. This has resulted in a detailed understanding of the mechanism of DNA replication. Concomitant with studies on SV40 DNA replication, we continue to study the replication of chromosomal DNA using the yeast Saccharomyces cerevisiae as a model system. Progress during the last year in this rapidly expanding area has been substantial, including the recognition that initiation of DNA replication and transcriptional regulation are intimately connected.

One of the key events in the control of DNA replication is the binding of an initiator protein to a replicator located in the template DNA. The replicator is a genetically defined element in the DNA that controls the initiation of DNA replication and contains the binding site for the initiator protein. SV40 encodes its own initiator protein, the SV40 T antigen. This protein functions not only to recognize the replicator, but also as a DNA helicase and to load DNA polymerase α/DNA primase (polα/primase) onto the DNA at the replication origin. The binding of the initiator protein promotes initiation of DNA replication at the SV40 ori.

In collaboration with Dr. Daniel Simons and his colleagues (University of Delaware), we have studied the activities present in mutant forms of T antigen and have found that the DNA-binding domain con-
tributes to many of the T-antigen-associated DNA-replication activities. In particular, some mutants of T antigen distinguish between the ability of T antigen to bind to the replicator sequences at the SV40 ori and its ability to initiate unwinding and subsequent DNA synthesis functions. One of these functions may be a primosome-loading function of T antigen. SV40 T antigen promotes the assembly of the polα/primase complex onto single-stranded DNA coated with the single-stranded DNA-binding protein RPA (replication protein A). In many ways, this function of T antigen is analogous to that of the primosome assembly proteins that load DNA primase onto the template DNA during replication of the bacterial chromosome, and thus we have designated T antigen as a primosome-loading protein.

T antigen from SV40 supports DNA replication from the SV40 ori in primate cells but not in murine cells. Other investigators have shown that a correlation exists between the ability of SV40 T antigen to support DNA replication in these various cells and the ability of T antigen to bind to the polα/primase complex. In the past year, in collaboration with Dr. J. Kadonaga and R. Kamakaka (University of California, San Diego), we have demonstrated DNA replication in cell extracts prepared from Drosophila melanogaster embryos and tissue culture cells that is SV40-T-antigen- and SV40-ori-dependent. Furthermore, initiation of DNA replication can occur at the SV40 ori in the presence of highly purified T antigen, Drosophila polα/primase, and Drosophila RPA. In this case, the human RPA cannot support DNA replication, further emphasizing the role of protein-protein interactions between SV40 T antigen, RPA, and polα/primase in correctly initiating DNA replication.

As noted in last year's Annual Report, a number of new DNA replication proteins have been identified, and the replication of DNA from the SV40 ori can occur with highly purified human proteins and SV40 T antigen. To characterize these proteins more thoroughly and to facilitate production of large amounts of these proteins, we have cloned several cDNAs encoding human DNA replication proteins. cDNAs encoding the large subunit of replication factor C (RFC) have been isolated from human and mouse cells (the latter in collaboration with Dr. Gunter Schutz and his colleagues, Cancer Center, Heidelberg, Germany). A region in the human RFC 140-kD subunit has extensive similarity to the other human RFC subunits (cloned in Dr. J. Hurwitz's laboratory, Memorial Sloan-Kettering Cancer Center); it also contains an extensive similarity to a small region in the bacterial DNA ligases and mammalian poly(ADP)-ribose polymerase. The common feature between these proteins is their ability to recognize DNA in a structure-specific manner. We have also cloned cDNA encoding a portion of the MF1 5' to 3' exonuclease and have discovered that it is identical to a recently characterized endo- and exonuclease characterized by Dr. Micheal Leiber and his colleagues (Stanford University).

Because the human cell proteins required for replication of SV40 DNA in vitro have been purified, the role of these proteins in DNA replication can now be studied. In the last year, we have shown that three new DNA replication factors, MF1, DNA ligase I, and ribonuclease H1 (the latter is required as demonstrated by Dr. R. Bambara and his colleagues, University of Rochester) are required for complete replication of SV40 DNA, particularly for completion of the discontinuously synthesized lagging strand. In previous years, characterization of the mechanism of DNA replication has demonstrated that polα/primase primes DNA synthesis at the SV40 ori, and then DNA polymerase δ, together with the proliferating cell nuclear antigen (PCNA) and RFC, produces the continuously synthesized leading strand at the DNA replication fork.

Now that complete replication of both strands at the replication fork can be reproduced in vitro, we re-examined the role of the various DNA replication proteins. When either polδ, PCNA, or RFC was omitted from the complete DNA replication reactions, as expected, synthesis of the leading strand was prevented, but surprisingly, replication of the lagging strand at the fork was also abnormal. This and other results suggested that polδ and its accessory proteins PCNA and RFC are required for synthesis of the Okazaki fragments on the lagging-strand template. The idea was examined by constructing a synthetic lagging-strand DNA template and testing the requirement for various DNA replication proteins in the completion of Okazaki fragment synthesis and ligation of the fragments. The results demonstrated that PCNA and RFC cooperate to prevent polα/primase from extensively elongating a DNA primer and that they load polδ onto the primer DNA to complete the Okazaki fragment. Thus, there is a switch from polα to polδ during the synthesis of every Okazaki fragment (see Fig. 1). We have previously demonstrated that a similar switch from polα to polδ occurs at the
Cell Chromosome Replication


In parallel with the studies on the replication of SV40 DNA, we have for quite some time worked on the replication of cell chromosomal DNA in the yeast _S. cerevisiae_. A number of DNA replication proteins have been purified from this yeast and the genes encoding them have been identified. Most recently, the five genes encoding the _S. cerevisiae_ RFC subunits have been cloned. One of these genes that encodes the large subunit of RFC is related to the large subunits of human and mouse RFC and contains similarity to the DNA ligase and poly(ADP)-ribose polymerase mentioned above. Furthermore, the gene encoding the large subunit of _S. cerevisiae_ RFC is identical to the _CDC44_ gene. Mutations in this gene have a cell division cycle (cdc) arrest phenotype that is consistent with a role for this protein in DNA metabolism; in addition, there appears to be an effect of the mutations in this gene on the entry into mitosis, suggesting that the RFC might have a role in linking DNA replication and/or repair to mitosis controls.

The dependence of the SV40 system on the many functions of T antigen precludes insight from this system into the mechanism of initiation of cell chromosome replication. To begin to study this important problem, we have focused our attention on the DNA sequences that are required for initiation of DNA replication and the proteins that interact with these sequences.

The DNA sequences in the _ARS1_ and _ARS307_ replicators that are required for supporting autonomous replication of an artificial plasmid chromosome have been defined. Each replicator contains an essential A domain that contains within it a match to an 11-base-pair consensus sequence found in every yeast replicator. In addition, these two replicators contain B1 and B2 elements that are functionally conserved, even though there is little sequence similarity between them. _ARS1_ also contains a B3 element that is the binding site for the Abf1 protein.

Other investigators have demonstrated that only a subset of yeast sequences that function as replicator sequences in a minichromosome (plasmid) context actually function in the chromosome in their natural location to initiate DNA replication. For this reason,

SV40 _ori_ during initiation of leading-strand synthesis. These studies suggest that a eukaryotic DNA replication fork has two molecules of DNA polδ synthesizing DNA on both the leading and lagging strands (see Fig. 1). In contrast, polα/primase seems to function only during synthesis of a primer RNA/DNA both at the replication origin and for initiation of every Okazaki fragment.
we have investigated whether the four DNA elements required for ARS1 function in a plasmid context function in the chromosome. Linker scan or point mutations were introduced into the ARS1 locus on chromosome IV, and the presence or absence of an origin of DNA replication was assayed by two-dimensional electrophoresis. As expected, mutations in the A element abolished replicator activity. Mutations in any one of the three B elements had a small effect on the activity of the replicator, but double and triple mutations in the B element severely reduced or eliminated initiation of DNA replication at the ARS1 locus. These results are similar to those obtained employing the plasmid-based assay and demonstrate that a chromosomal replicator consists of a modular array of DNA sequence elements, some of which are in large part not absolutely necessary when mutated individually.

In the course of these experiments, a yeast strain was constructed that contained two copies of the ARS1 sequences separated by 4.6 kb of DNA. Interestingly, only one of these replicators (left) was functional in producing a replication origin and the other (right) was inactive. Thus, sequences outside the replicator DNA can influence the selection of identical replicators. The dominance of the left replicator over the inactive right replicator depended, however, on the active replicator. When the essential A element in the active left replicator was mutated, the right replicator was fully active. This suggests that the dominance of one replicator over another requires initiation of DNA replication.

In two previous Annual Reports, we have described the purification and characterization of the origin recognition complex (ORC). This protein complex contains subunits of 120, 72, 62, 56, 53, and 50 kD in mass. ORC binds in an ATP-dependent manner to the essential A element in the replicator and protects from nuclease digestion the A and B1 region of ARS sequences. We have demonstrated that the B1 element also contributes to the specificity of ORC binding to the replicator since mutations in the B1 element, but not the B2 or B3 elements, affect ORC DNA binding in vitro.

To characterize the ORC protein complex further, peptides from the ORC proteins were sequenced in collaboration with Dr. R. Kobayashi (Protein Chemistry Laboratory), and this information was used to obtain the genes encoding all six subunits. All of these gene products are essential for yeast viability. The peptide sequences derived from the ORC2 and ORC6 proteins were found to be encoded by genes that had been characterized by other investigators. Drs. J. Li and I. Herskowitz (University of California, San Francisco) cloned the ORC6 gene using a genetic screen for proteins that interact with the ARS consensus sequence. They demonstrated that overproduction of the ORC6 protein did not affect a wild-type strain, but in cdc6 and cdc46 mutant strains, overproduction of ORC6 was incompatible with cell viability. This suggested that ORC interacts with the CDC6 and CDC46 proteins, both of which are required for entry into S phase of the cell cycle.

In collaboration with Dr. J. Rine and his colleagues (University of California, Berkeley), we have identified temperature-sensitive mutants in the ORC2 and ORC5 genes that arrest at the nonpermissive temperature with a cdc phenotype, consistent with a role for these proteins in the initiation of DNA replication. Furthermore, these strains cannot maintain plasmids that contain only one replicator, even at the temperature where the cells are viable. Analysis of the DNA content by fluorescence-activated cell sorting suggests that ORC is required for correct initiation of DNA replication, but the data also suggest a role for ORC in preventing inappropriate DNA synthesis.

An unexpected finding from characterization of these orc2 and orc5 mutants was the observation that they are defective for transcripational silencing of the silent mating-type loci on chromosome III in S. cerevisiae. At the permissive temperature, both mutants failed to keep the normally silent HML and HMR genes from being transcribed. We have demonstrated that ORC binds to the DNA sequences that flank these silent genes that act as cis-acting silencer sequences. Within these silencers, the ORC-binding site cooperates with the binding sites for the Abf1 and Rap proteins. These results demonstrate a direct relationship between the initiation of DNA replication and the initiation or maintenance of a transcriptional state that is inherited to daughter cells.

PUBLICATIONS


In Press


MOLECULAR BIOLOGY OF BOVINE PAPILLOMAVIRUSES

A. Stenlund M. Berg J. Sedman
T. Sedman R. Kluttig

The papillomaviruses infect and transform the basal epithelium in their hosts, inducing proliferation of the cell at the site of infection. The resulting tumors are in most cases benign and will usually regress after some time, but certain types of human papillomaviruses (HPVs 16, 18, 31, 33, and 39) give rise to tumors that are prone to progress toward malignancy, especially frequently cervical carcinoma. In total, HPV DNA can now be found in biopsies from approximately 80% of all cervical carcinomas.

This very good correlation between papillomavirus infections and human carcinomas has intensified the research on the biology of these viruses over the past decade. One of the key impediments to this work has been the inability to define a simple in vitro cell culture system for HPVs, largely due to the fact that these viruses normally require specialized differentiating cells that only with great difficulty can be generated in cell culture. One way to circumvent this problem has been to study related animal viruses where at least a part of the viral life cycle can be reproduced. In particular, a bovine papillomavirus (BPV-1) has become something of a prototype virus for the papillomavirus group since a convenient cell culture system exists for this virus, where viral gene expression, oncogenic transformation, and viral DNA replication can be studied. To date, more than 60 different papillomavirus genomes have been characterized and cloned, and all of these show a remarkable degree of similarity in their genome organization. This similarity originally formed the basis for the assumption that what was learned from one virus could be applied to another. This assumption has largely been verified; many of the phenomena that originally
were described for BPV have subsequently been found also in the human viruses.

The DNA replication properties of these viruses show some unique and interesting characteristics. As part of their normal life cycle, papillomaviruses can exist in a state of latency which is characterized by maintenance of the viral DNA as a multicopy plasmid in the infected cells. The copy number of the viral DNA appears to be tightly controlled, and the viral DNA is stably inherited under these conditions. This system therefore provides a unique opportunity to study plasmid replication in mammalian cells.

In previous years, we have reported the development of a short-term replication assay that has enabled us, for the first time, to define the viral components that are required for viral DNA replication and to initiate detailed studies of the viral DNA replication properties of this group of viruses. In the past year, we have continued this work with most of our effort directed toward detailed biochemical analysis of the replication process, with specific emphasis on the respective function of the viral and cellular proteins that take part in this process. To this end, we have refined the expression and purification of the two viral proteins (E1 and E2) that are required for viral DNA replication, as well as continued the analysis of the initiation complexes that are formed on the Ori. We have also adapted and modified a cell-free replication system that was originally developed in M. Botchan's laboratory. In addition, we have begun (in collaboration with T. Melendy in B. Stillman's laboratory) to identify the cellular factors that are required for viral DNA replication.

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### Formation of Initiation Complexes

T. Sedman, A. Stenlund

The minimal Ori sequence that is required for replication in vivo is approximately 60 nucleotides long and contains three sequence elements. Two of these elements have known functions, consisting of binding sites for the viral E1 and E2 proteins. The third element has been termed the A+T-rich region. Our earlier genetic experiments have demonstrated that the presence of these elements is necessary for replication activity. However, the position and sequence of one of these elements, the E2-binding site, is not critical for replication activity of the Ori. The sequence of this site is not important per se and can be changed as long as these changes still allow binding of E2. Furthermore, the E2-binding site can function from various positions relative to the E1-binding site and A+T-rich region. During the past year, we have developed a series of assays to study the binding of E1 and E2 to the Ori sequence to determine the requirements for binding and for formation of productive replication complexes. By a combination of DNase protection assays, interference assays, and gel-retardation assays, we have been able to establish that E1 is capable of binding to the Ori in at least two different forms: either together with E2 to form an Ori+E1+E2 complex or by itself to form an Ori+E1 complex. The Ori+E1+E2 complex is formed by cooperative binding of E1 and E2 to Ori and can form at low concentration of E1 provided E2 is present. The formation of this complex also requires the presence of an E2-binding site adjacent to the E1-binding site. The Ori+E1 complex requires higher concentrations of E1 and forms on the same Ori sequence but does not require E2 or E2-binding sites for its formation. Results from interference analysis indicated that overlapping but not completely identical sequences are involved in binding of the two complexes. To determine which of these complexes is important for replication in vivo, we have generated mutations designed to affect the formation of the two complexes differentially. Mutations in the E2-binding site reduce the ability to form the E1+E2 complex and also reduce replication. Mutations that increase the spacing between the E1- and E2-binding sites concomitantly reduce the ability to form the E1+E2 complex and the ability to replicate. The sum of these results indicates that the ability to form the E1+E2 complex is essential for replication activity in vivo, whereas the ability to form the E1 complex is not. However, the ability to form the E1 complex clearly is sufficient for replication in vitro (see below), indicating that more than one pathway exists to generate functional replication complexes.

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### DNA Replication In Vitro

J. Sedman, A. Stenlund

A major effort during the past year has been to perfect the overexpression and purification of the two
viral proteins involved in viral DNA replication. The E1 and E2 proteins have been expressed in soluble form in *Escherichia coli* and have been purified to apparent homogeneity by a combination of conventional chromatography and affinity chromatography. Both proteins are site-specific DNA-binding proteins, and this activity can readily be measured in vitro. E1 has several additional biochemical activities. A number of analyses have been carried out to characterize the biochemical functions of the E1 polypeptide. In addition to site-specific DNA-binding activity, the protein also has a DNA-dependent ATPase activity and a 5' to 3' DNA helicase activity. E1 can also function to unwind a supercoiled plasmid that contains the Ori sequence. Thus, E1 appears to have the required activities associated with other known initiator proteins such as SV40 large T antigen. The presence of the E2 polypeptide in these assays has little or no effect on the activities of E1. During the past year, we have adapted an in vitro replication system that was developed in M. Botchan's laboratory at Berkeley. This system, which consists of a whole-cell extract from the mouse cell line FM3A, initiates DNA synthesis from the BPV origin of replication. In agreement with the original studies, the presence of viral E1 protein is sufficient for Ori-specific replication activity. These results are consistent with the biochemical activities observed for the E1 protein. The in vitro system thus displays several important differences compared to replication in vivo. First, although the E2 protein is absolutely required for replication in vivo, in the cell-free system, only a moderate stimulation is seen upon addition of E2 (Fig. 1). Second, in vivo replication is also absolutely dependent on a binding site for the E2 protein at the Ori. In vitro, neither E1-dependent replication nor slight E2 stimulation is dependent on the presence of an E2-binding site. Third, the A+T-rich region, which is essential for replication in vivo, appears to be completely dispensable for replication in vitro. The basis for these differences between replication in vivo and replication in the cell-free system is not clear; however, it is obvious that in vitro replication fails to reproduce certain aspects of replication in vivo (Fig. 2). Several possible explanations for this difference can be envisioned. One is that the in vitro and in vivo systems in fact represent different types of replication. Another perhaps more likely possibility is that the two types of complexes are present on the same pathway, eventually leading to the same functional initiation complex. The modification of this cell-free system continues in order to generate a system that corresponds more closely to the requirements for replication in vivo.

**Cellular Factors Required for Replication of BPV In Vitro**

J. Sedman, A. Stenlund [in collaboration with T. Melendy and B. Stillman, Cold Spring Harbor Laboratory]

The SV40 system has for a number of years served as the paradigm for DNA replication in mammalian
Requirements for replication in vivo.
- E1 protein
- E2 protein
- E1 binding site
- E2 binding site
- A+T rich region

Requirements for replication in vitro.
- E1 protein
- E1 binding site

**FIGURE 2** Schematic figure illustrating the requirements for replication in vivo and in vitro. The contrasting requirements for viral proteins and cis-acting sequences in the two replication systems are listed. The composition of the two complexes is inferred from indirect measurements in various binding assays.

cells. This system relies on the viral initiator large T antigen for Ori recognition, Ori unwinding, and helicase activity. By using this system, a number of cellular factors involved in DNA synthesis have been purified and characterized. A minimal purified system, which contains all of the cellular factors that are required for SV40 replication, has been developed by B. Stillman and co-workers. Although all of these factors are clearly involved in cellular DNA synthesis, it is unclear whether additional cellular replication proteins exist. To determine if BPV uses the same set of cellular replication factors that are utilized for SV40 replication, we have tested the requirement for each of these factors in a BPV in vitro replication system. Preliminary data indicate that all of the factors identified in the SV40 system are also required for BPV replication. However, the degree of dependence on a given factor appears to be different, indicating that differences exist between the two systems.

Interaction between the E1 and E2 Proteins

M. Berg, A. Stenlund

Transcription factors have been implicated as having an auxiliary role in a number of different replication systems, but the actual function of these transcription factors in initiation of DNA replication is not clear. In most well-studied systems, including SV40, polyomavirus, and ARS elements from *Saccharomyces cerevisiae*, the requirement for transcription factors generally shows a low degree of specificity, i.e., activity can be supplied by a variety of transcriptional trans-activators provided the cognate binding site is present. From our previous work, it is clear that the requirement for the transcription factor E2 in the BPV system differs from that in these other systems in several aspects: The E2-binding site at the Ori cannot be replaced by various enhancers that function as replication enhancers in other systems. Replacement of the trans-activation domain of E2 with the activation domain from the herpes simplex virus activator VP16 yields a trans-activator that fails to function for BPV replication, demonstrating a requirement for a high degree of specificity in the BPV system. As we have previously demonstrated, the requirement for E2 in replication of BPV is consistent with a requirement for interaction between the E1 and E2 proteins at the origin of replication. This interaction can be detected as a cooperative binding of the two proteins to the Ori, when the respective binding sites are located in the correct position relative to each other (see above). To further study this interaction, we have adopted an approach using chimeric proteins. The E2 proteins are quite well conserved between different papillomaviruses and are likely to have a conserved structure. For example, the sequence specificity for DNA binding is identical for E2 from all papillomaviruses tested so far. However, when tested for their ability to bind cooperatively with BPV E1, some E2 proteins from other papillomaviruses fail to interact with E1 even though binding to the E2-binding site can be detected. To analyze what regions of E1 and E2 are required for this interaction, we have initiated construction of chimeras between BPV E2, on the one hand, and interaction-defective E2 proteins from other papillomaviruses, on the other hand. These chimeras can then be tested for their ability to interact with BPV
E1, and regions of the protein important for the interaction can be defined. This approach has the distinctive advantage that the overall structure of the protein can be maintained.

The results from these experiments so far indicate that multiple regions both from the amino-terminal trans-activation domain of E2 and from the carboxy-terminal DNA-binding/dimerization domain cooperate to generate a strong interaction with E1. These results serve to indicate that the interaction surface may be composed of determinants from several domains, presumably brought together by protein folding.

PUBLICATIONS


PROTEIN CHEMISTRY

D.R. Marshak  E. Araya  J. Kass
R. Kobayashi  G. Binns  N. Poppito
              N. Carpino  G. Russo
              N. Chester  M. Vandenberg

The members of the Protein Chemistry laboratory conduct 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. The use of such procedures allows 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 determination 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, N. Carpino, N. Poppito, M. Vandenberg

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. Therefore, we use mass spectrometric measurements to assess the molecular weight of the product to elim-
inate 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.

During the past year, we have introduced new chemistry to our laboratory that allows the synthesis of phosphorylated peptides. This is accomplished in a four-step process. First, they are assembled on solid supports using FMOC-protected N-$\alpha$-amino acids. Since the FMOC group is labile to base, acid treatment of the peptide is unnecessary during assembly. The hydroxyl moiety of the amino acid to be phosphorylated is left unprotected.

Second, the assembled peptide-resin is treated with a protected phosphoramidite compound that leads to the production of the peptide-resin phosphite. Third, the phosphite is oxidized to the phosphate using an organic peroxide. Cysteinyl sulfhydryl groups are protected from oxidation by steric hindrance of the S-trityl derivative. Fourth, the peptide is deprotected and removed from the resin using trifluoroacetic acid. The solubilized peptide is purified to homogeneity by multiple rounds of HPLC under different conditions. The availability of this chemistry has permitted the production of several phosphopeptides that can be used to prepare antibodies specific to phosphorylation sites on proteins. In addition, some kinases require target sites that are already phosphorylated at a site nearby the substrate hydroxyl, and these synthetic phosphopeptides can be used as quantitative probes of activity and as substrates for such kinases.

**Molecular Cloning and Expression of Casein Kinase II Subunits**

N. Chester, M. Vandenberg, J. Kass, D.R. Marshak

The enzyme casein kinase II (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, $\alpha$ and $\beta$, with molecular masses of 37-44 kD and 24-28 kD, respectively, and an apparent subunit composition of $\alpha_2\beta_2$. The $\alpha$ subunit is found in two different forms, known as $\alpha$ and $\alpha'$, 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 $\alpha$ and $\beta$ subunits, using an innovative variation of the polymerase chain reaction (Chester and Marshak 1993). This cloning has allowed us to insert the coding sequences for the CKII subunits into various expression vectors. Using these constructs, we have expressed the proteins in large amounts in bacteria. The purified, recombinant catalytic subunits are enzymatically active, and the activity is increased by the regulatory subunit, $\beta$. The catalytic and regulatory subunits combine in a functional complex whether they are mixed in vitro or coexpressed in the same bacterial cells. We have purified large amounts of the regulatory subunit for structural analysis. In collaboration with J. Horton and J. Pflugrath (CSHL), we have crystallized the $\beta$ subunit of CKII in forms that appear to diffract X-rays. We are now attempting to produce larger crystals to solve the three-dimensional structure of this protein. Although the catalytic subunits of CKII are homologous to other known protein serine/threonine kinases, the $\beta$ subunit is unique. We expect that the structure of this regulatory subunit will be a novel contribution to the field.
ding yeast, *Saccharomyces cerevisiae*, in which we genetically manipulated the homolog of mammalian p34\(^{cd2}\), known as Cdc28. In collaboration with A. Sutton (CSHL), we have demonstrated that the homologous serine is phosphorylated on Cdc28 in yeast as on p34\(^{cd2}\) in mammalian cells. To test the functional role of this site in yeast, we constructed a yeast carrying a mutation that results in the substitution of an alanine residue for the serine. The mutant plasmid was introduced into a yeast defective for wild-type Cdc28. The phenotype of the mutant is a small cell size, indicating a premature entry into S phase. This result suggests that the phosphorylation of the serine on Cdc28 in yeast (and by analogy on p34\(^{cd2}\) in human cells) allows the cell to extend G1. Further genetic and biochemical experiments are under way to demonstrate the mechanistic details of this process.

### Subcellular Pools of Casein Kinase II

N. Chester, D.R. Marshak

The CKII enzyme activity is found both in cytosol and in nuclei, and substrates have been 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, \(\beta\)-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 has a significant role in cell physiology. We have developed a large set of specific antibodies to synthetic peptide antigens that react with the individual subunits of CKII. These antibodies allowed us to continue our studies of the subcellular localization of the subunits of the enzyme during the cell division cycle of HeLa cells. Nick Chester has used the antibodies developed in the laboratory to do detailed immunoprecipitation and immunoblots of the CKII subunits. First, he found that the synthetic rate for the CKII \(\beta\) subunit is significantly longer than that of the catalytic subunits, whereas the degradation rates for all of the subunits appear to be similar. Second, using steady-state metabolic labeling, he identified different pools of CKII that vary in subunit stoichiometry and activity. He is currently attempting to see if the different pools of CKII are localized in different subcellular compartments and if they change their characteristics during the cell division cycle in HeLa cells.

### Structural Analysis and Phosphorylation of Nuclear Proteins

D.R. Marshak, M. Vandenberg, G. Binns

**ZEBRA PROTEIN**

The Epstein-Barr virus protein Zebra has been characterized in collaboration with G. Miller’s group at Yale (Kolman et al. 1993). The Zebra protein is phosphorylated at a unique site by CKII. This phosphorylation results in the inability of the Zebra protein to bind DNA. This suggests that physiologically, phosphorylation of Zebra may control its functions in controlling viral gene transcription and replication. Because of the structural similarity to c-Jun, the phosphorylation of Zebra may parallel other mechanisms in human cells.

**FOS AND JUN PROTEINS**

Our analysis of the phosphorylation of the c-Fos and c-Jun proteins were done in collaboration with T. Curran’s group at the Roche Institute (Abate et al. 1993). This year, we found that the phosphorylation of Fos and Jun proteins changes depending on whether they are dimerized and bound to DNA. The direction of the change in activity of the proteins as substrates is different for different protein kinases. The enzymes tested were CKII, p34\(^{cd2}\)/cyclin B, double-stranded, DNA-dependent protein kinase, and protein kinase C. The results provide some insight into the complex mechanism of regulation of these transcription factors. In a separate project in collaboration with G. Landreth at Case Western Reserve University in Cleveland, we identified a novel phosphorylation site on Fos. The phosphorylation of this site is increased upon nerve growth factor treatment of rat pheochromocytoma cells in culture (Taylor et al. 1993).
The Protein Chemistry Core Facility provides high-technology methods, equipment, and expertise for use by all of the scientists at Cold Spring Harbor Laboratory. The services provided include protein sequencing, peptide mapping by HPLC, peptide synthesis, HPLC and electrophoretic purification of proteins, mass spectrometry, and amino acid analysis. These methods can be used for structural analysis of the amino acid sequence and composition of a protein, as well as for determining post-translational modifications. Peptide synthesis remained approximately constant, whereas amino acid analysis returned to levels of 1990. Protein sequencing and peptide mapping have been aided by the addition of new equipment to the laboratory. In the past year, we have used funds from a shared instrumentation grant to obtain an Applied Biosystems 477 protein sequencer and a new Hewlett Packard 1090M HPLC, with the new workstation based on DOS operating software. This instrument is networked with the existing 1090M HPLC and is complementary to another 1090L HPLC used by R. Kobayashi. The equipment in the Core Facility is sufficient for isolating and sequencing peptides in the 0.2-2-pmole range. Next year, we hope to make modifications that will extend this range below 100 fmole. R. Kobayashi has produced an enormous amount of useful protein sequence information in collaboration with several groups at Cold Spring Harbor Laboratory. A summary of these projects appears in the Structure and Computation Section.

Our facility is active in the Association of Biomolecular Resource Facilities (ABRF). The purposes of the ABRF are (1) to promote and facilitate discussion and cooperation among facilities; (2) to provide research mechanisms for evaluation and improvement of the capabilities of facilities; and (3) to promote the education of facility staff, users, administrators, and interested members of the scientific community regarding facility functions. The ABRF exists because facilities have demanded an organization that will provide them with a mechanism for sharing information, maintaining state-of-the-art procedures, evaluating performance, and expanding to new areas. D. Marshak has completed a 2-year term on the peptide synthesis research committee of the ABRF. During that period, the committee performed a series of studies to evaluate the performance of peptide synthesis facilities. During 1993, Marshak has been appointed to a 3-year term on the Executive Board of the ABRF, and he will host next year's winter Board meeting. Involvement with the ABRF helps to maintain communication with other facilities around the world and improves our ability to maintain high standards of work.

**PUBLICATIONS**


Gene expression in human cells is controlled at a variety of levels. In our research, we continue to explore regulatory processes that operate at the levels of RNA and protein synthesis, concentrating in particular on three systems in which viruses participate in the regulatory events.

The first system operates at the translational level. Its key component is a cellular protein kinase that phosphorylates an initiation factor (as well as other proteins, less well understood), thereby affecting the rate of this critical step in protein synthesis. The kinase is part of the antiviral defense mechanism that is induced by interferon, and many viruses have elaborated countermeasures that at least partly neutralize it and ensure their survival.

The second system, which operates at the transcriptional level, concerns the relationship between oncogenesis and DNA replication. Synthesis of a replication protein known as PCNA (proliferating cell nuclear antigen) is stimulated by one of the adenovirus oncogenes, and this induction is mediated through a number of cellular components and PCNA promoter elements.

The third system involves the human immunodeficiency virus, HIV-1, the causative agent of AIDS. Synthesis of viral RNA is dependent on a viral protein called Tat, which exerts a novel type of control over the efficiency of RNA polymerases transcribing the viral genome. During the year, Stephen Brand, Michael Greenberg, and Benjamin Lee joined the laboratory; Christian Schmedt completed his Diplomarbeit for the University of Konstanz and returned to Germany as a graduate student; Paul Clarke and Simon Green finished their postdoctoral training and moved to independent positions; and two senior members of the group, Michael Lasbia and Gilbert Morris, left for faculty positions at Dartmouth College and Tulane University, respectively.

Translational Control

Y. Ma, D. Taylor, P. Clarke, C. Schmedt, S. Green, S. Brand, L. Manche, M.B. Mathews

Like many biological pathways, protein synthesis can be controlled by the phosphorylation of enzymes that mediate the process. One of the key regulatory steps in translation is catalyzed by the eukaryotic initiation factor eIF-2. Phosphorylation of this enzyme on its α subunit leads to the entrapment of a second initiation factor, GEF or eIF-2B, that is required for continued eIF-2 activity. Three kinases are known that are able to phosphorylate the critical Ser-51 residue of eIF-2α, one in yeast and two in mammals. One of the mammalian kinases, DAI, has an important role in combating viral infection and has recently been implicated in processes such as growth control and tumorigenesis. An acronym for the double-stranded RNA (dsRNA)-activated inhibitor of translation, DAI (also known as PKR, p68, P1, etc.), is widespread in cells and tissues. It is normally present at a low level and in an inactive state; its synthesis is increased by treatment with the antiviral interferons, and its activation is accompanied by autophosphorylation which takes place in the presence of dsRNA. Viruses have responded to this threat to their propagation by developing numerous countermeasures. One of these is the production by adenovirus of a short (160-nucleotide) RNA called VA RNA1, which blocks DAI activation by dsRNA.

Our objective is to understand the molecular switches that are thrown by dsRNA and VA RNA so as to control the activity of the kinase. To this end, we have studied the nature of the RNAs that activate and inhibit DAI, the structure of the enzyme itself, and the interaction between the protein and the RNAs. Earlier studies had indicated that activation
requires a dsRNA containing at least 30–80 base pairs of perfectly matched duplex, whereas VA RNA is highly structured but only partially duplexed. Accordingly, we anticipated that the genomic RNA of the hepatitis delta agent might also inhibit DAI activation. This RNA is circular, predominantly single-stranded but base-paired such that it forms a linear structure resembling a collapsed rubber band. Together with Dr. H. Robertson (Cornell University Medical School), we found that delta agent RNA forms complexes with DAI, interacting specifically with the enzyme’s RNA-binding domain. Surprisingly, however, the consequence of binding is activation of the enzyme rather than the opposite. Consistent with the structure of delta RNA, this activation was sensitive to digestion with a single-strand-specific RNase but was resistant to treatment with a dsRNA-specific nuclease. RNase III cleaves RNA molecules that contain stretches of duplex exceeding 20 base pairs in length but is unable to act on delta agent RNA, indicating that dsRNA contaminants are not responsible for the activation. These results suggest that DAI (or possibly another cellular protein with similar RNA-binding properties) may contribute to hepatitis delta pathogenesis, and they show that some RNAs that are not formally double-stranded can activate the enzyme, emphasizing the subtleties of the nucleic acid–protein interactions involved.

VA RNA serves to protect adenovirus against interferon by preventing DAI activation. Its action rests on two elements of structure: an extended stem, the apical stem, containing about 40 imperfectly paired nucleotides, and a complex structure, the central domain, containing paired and unpaired regions. Mutagenic analysis shows that the apical stem and central domain are both involved in the binding of VA RNA to DAI and that the central domain is critical for inhibition of DAI activation. These conclusions have been extended by a direct examination of the sites of interaction, using the protein to protect the RNA from attack by nucleases or chemical probes. The regions of VA RNA influenced by DAI are illustrated in Figure 1, superimposed on a new model of the RNA’s secondary structure.

The new structure takes into account recent data from three sources: mutagenesis, chemical probing, and phylogenetic comparisons. Previous comparative studies with seven VA RNAs suggested that two pairs of conserved tetranucleotides might have a part in maintaining base pairing in VA RNA. One pair (GGGU/ACCC), located in the central domain, led us to suggest a revision of the previous adenovirus 2 (Ad2) VA RNA model. This year, we have taken three steps to test this prediction. First, we used more physiological conditions to probe VA RNA structure. This resulted in changes in the sensitivity of certain nucleotides, supporting the new model. Second, we made a set of mutations of these tetranucleotides, changing GGGU to GUCU and ACCC to AGAC. The double mutant, containing both changes, should be a compensatory mutant if the new structure is correct. Indeed, this mutant was more active in a CAT expression enhancing assay than either of the single mutants alone. Third, we extended the phylogenetic
analysis to cover all of the human adenoviruses. Using degenerate oligonucleotides complementary to conserved adenovirus genes that flank the VA RNA sequences, we amplified the VA RNA gene region and sequenced the amplified polymerase chain reaction (PCR) fragments. The GGGU/ACCC tetranucleotide pair was highly conserved in all of the VA RNAs, confirming the importance of the proposed base pairing.

Despite its opposite responses to dsRNA and VA RNA, no DAI mutations have been isolated so far that distinguish between the binding of these two ligands. The RNA-binding domain contains two divergent copies of a repeated sequence about 65 amino acids in length, which is also characteristic of some other dsRNA-binding proteins. We have explored the roles of the two copies of the motif through mutagenesis and direct analysis of protein overexpressed in Escherichia coli. Mutagenic studies showed that the first motif has a more important role than the second motif, although both are required for efficient RNA binding. The two motifs do not seem to be equivalent, however, because mutations of equivalent amino acids do not necessarily produce equivalent effects on RNA binding. Physical biochemistry studies are under way to define the interactions in detail. Further investigations, carried out with Drs. S. Lee and M. Esteban (SUNY, Brooklyn) have identified a region adjacent to the RNA-binding domain as having an important role in DAI function. Like the RNA-binding motifs, this region is highly basic in nature, but it does not appear to influence RNA binding. Nevertheless, its deletion eliminates kinase function in assays based on a vaccinia virus system. Although the function of this region is not yet understood, it may be significant that it contains some autophosphorylation sites.

DAI is a serine/threonine kinase and its activation as an eIF-2 kinase is correlated with its autophosphorylation. We have begun to identify the location of the autophosphorylation sites and the mechanism of autophosphorylation of DAI. In collaboration with Dr. D. Marshak and G. Binns (Protein Chemistry Core section), the locations of several autophosphorylated sites have been identified through peptide mapping and sequencing. These sites may define an autoregulatory region, separating the enzyme’s RNA-binding and kinase domains. We have also identified phosphorylated serine and threonine residues located with the spacer region of the RNA-binding domain. This region is likely to be accessible to phosphorylation, as it is not contained within either of the two predicted α helices. It will be interesting to see if these sites affect kinase function through RNA binding.

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 Regulation of PCNA


Activation of the DNA replication machinery is central to the process of neoplastic transformation. The protein PCNA, the proliferating cell nuclear antigen, was originally discovered as an antigen in the autoimmune disease SLE (systemic lupus erythematosus). As the auxiliary factor of DNA polymerase δ, it functions in both leading and lagging-strand DNA synthesis during DNA replication. In accord with this function, cellular PCNA levels increase during the oncogenic transformation of rodent cells by the adenovirus E1A gene. The goal of this project is to explore the relationship between transformation and gene activation by elucidating the mechanism of E1A-mediated induction of PCNA gene expression.

The E1A 243R protein (243 residues long), translated from the E1A 12S mRNA, is able to stimulate PCNA transcription and to activate the PCNA promoter in the absence of other viral genes. Last year, we showed that trans-activation of the human PCNA promoter by E1A 243R is mediated through an element termed the PERE (PCNA E1A-responsive element) and that multiple domains of the E1A protein are required to induce expression of the wild-type PCNA promoter. We also showed that the p300 and p107/cyclin A-binding function of E1A correlates most closely with its ability to activate the PCNA promoter.

To further elucidate the function of E1A, we have developed a simplified assay for activities of E1A
mutants by preparing a construct, G5PCNA-CAT, in which the PERE and sequences upstream in the PCNA promoter are replaced by five sites corresponding to the DNA sequence bound by the yeast GAL4 protein. With the G5PCNA-CAT construct, the transcriptional activator is specified by the GAL4 fusion protein, thereby obviating effects of E1A on the components that bind the PERE. In contrast to our earlier results with the wild-type PCNA promoter, we found that an E1A mutant with a deletion of conserved region 2 can cooperate with a weakly activating fusion protein to trans-activate G5PCNA-CAT as effectively as wild-type E1A 243R. An E1A mutant with a deletion of the amino terminus failed to trans-activate G5PCNA-CAT, however. These data suggest that the p300-binding function of E1A is associated with its ability to trans-activate transcription and that the p107/cyclin A requirement for trans-activation of the wild-type promoter is associated with PERE function.

The PERE contains an activating transcription factor (ATF)-binding site, together with an adjacent sequence that does not correspond to any known protein-binding site. We have made good progress toward identifying the cellular transcription factors that associate with the PERE and presumably mediate trans-activation of the PCNA promoter by E1A 243R. Through a combination of Southwestern analysis and UV light cross-linking experiments, we have identified five proteins in crude HeLa cell nuclear extracts that interact with the PERE. On the basis of the size of one of these polypeptides, we suspected that one of the PERE-binding proteins might be ATF-1 and have now proven this contention. Antibodies raised against ATF-1 specifically disrupt PERE-protein complexes in gel-retardation assays. Moreover, ATF-1 synthesized in vitro can bind to the PERE in a gel-shift assay. We are currently in the process of identifying the other PERE-binding proteins by biochemical methods.

All of the above studies were conducted in HeLa cells, a human cervical carcinoma cell line. Paradoxically, in contrast to the HeLa cell results, E1A represses expression from the PCNA-CAT promoter in two types of rodent cells, baby rat kidney (BRK) and cloned rat embryo fibroblasts (CREFs). We have now shown that removal of sequences between positions −234 and −213, upstream of the transcription initiation site, relieves E1A-mediated repression of PCNA-CAT expression in these rodent cells. Within this sequence is an 18 out of 20 match to the consensus sequence bound by the tumor suppressor protein p53. Several observations suggest that this sequence in the human PCNA promoter can function as a p53-responsive element. First, a construct (−249) with the site intact bound a protein antigenically related to p53 in cell extracts from yeast that express wild-type, but not mutant, human p53, whereas a construct (−213) with the site deleted did not. Second, cotransfection of a construct encoding wild-type p53 activated CAT expression from the −249 PCNA-CAT construct in a cell line that fails to express p53 (SAOS-2 cells) but represses CAT expression from −213 PCNA-CAT. Third, as with the wild-type PCNA promoter, E1A repressed CAT expression in BRK cells from a PCNA-CAT construct with a well-characterized heterologous p53-binding site substituted for the related sequence in the PCNA promoter (Fig. 2).

![Diagram of PCNA-CAT Constructs](image)

**Figure 2** E1A 243R repression of the PCNA promoter via p53-binding sites. The constructs (top) were cotransfected into BRK cells with a plasmid that expresses E1A 243R or a control plasmid. Transcription from the mutant PCNA promoters was measured in CAT units (bottom). The data show that removal of sequences between −249 and −213 abolished E1A repression, which was restored by insertion of a heterologous p53-binding site from the ribosomal gene cluster (RGC 213).
Finally, we have also been examining the structure and antigenicity of PCNA itself. In a collaboration with Dr. R. Bernstein (University of Manchester, U.K.), we defined two classes of antibodies against PCNA found in serum from SLE patients. The major class was highly conformation-dependent, tolerating only minor changes in protein structure; the minor class was less conformation-dependent and could react with PCNA lacking about half of its sequence from the amino terminus. We went on to use gel filtration and glycerol gradient sedimentation to analyze the native structure and size of PCNA. PCNA from three sources was studied—from HeLa cells, purified in the wheat-germ cell-free translation system after its overexpression in bacteria, and produced in the wheat-germ cell-free translation system—as well as mutant forms of PCNA translated in vitro. In each case, full-length PCNA behaved as a trimer. Analysis of mutant proteins revealed a correlation between their ability to adopt the trimeric form and binding to the common type of human anti-PCNA autoantibody, suggesting that the antibodies are specific for the active form of the protein. These findings are consistent with the idea that auto-antibodies are generated as a response to native antigen and provide experimental support for the hypothesis that PCNA serves its processive function in DNA replication as a trimeric ring structure.

Transcriptional Regulation by the HIV-1 Tat Protein

A. Lozeau, P. Wendel, L.A. Sheldon, M. Lasbia

The genome of HIV-1 contains, in addition to the structural genes gag, pol, and env, several novel regulatory genes. One of these, Tat, is essential and encodes a powerful trans-activator that greatly stimulates HIV-1 gene expression. It does so by binding to an RNA element, known as TAR, present in the 5' untranslated region of all HIV mRNAs. This causes an increase in transcription directed by the promoter located within the long terminal repeat (LTR) of the virus. Previous studies showed that, in the absence of Tat, the level of HIV-directed transcriptional initiation is low and transcriptional elongation is inefficient, such that the number of transcriptional complexes declines with increasing distance from the site of initiation. We found that Tat works through TAR to increase initiation of transcription by RNA polymerase II and also improves the efficiency of elongation. Thus, Tat appears to be a unique transcriptional activator in that promoter binding is via a structured RNA, as well as in its ability to stimulate both transcriptional initiation and elongation. We wish to understand the mechanism by which Tat acts to stimulate HIV transcription, both as a paradigm for regulation of eukaryotic gene expression and because approaches that interfere with trans-activation may have therapeutic potential for the treatment of AIDS.

The molecular mechanism by which Tat produces these dual effects on HIV-1 transcription is unclear. In particular, little is known about the role of cellular factors in trans-activation. A detailed understanding of the mechanism of trans-activation is most likely to emerge from the identification and purification of cellular components of the transcriptional machinery that interact with Tat. Therefore, we have pursued our analysis of trans-activation by Tat in a cell-free system. In an in vitro transcription system consisting of a DNA template and HeLa cell nuclear extract, the addition of purified, bacterially expressed Tat stimulates transcription from the HIV-1 promoter but not from heterologous promoters nor from the HIV-1 promoter when TAR is mutated. An examination of the effects of Tat on HIV-1 transcription rates in vitro has indicated that Tat increases promoter-distal transcription rates without similarly increasing promoter-proximal transcription rates, suggesting that in vitro, Tat principally stimulates transcriptional elongation.

TAR is the only promoter element known to be absolutely required for trans-activation by Tat. TAR RNA folds into a structure consisting of a 60-nucleotide paired stem with a trinucleotide bulge (+23CU+25) and an apical loop (+30CUUGGA+35). Tat binding to TAR RNA in vitro is abolished by mutations in the bulge, but mutations in the apical loop do not reduce the binding of Tat to TAR RNA. On the other hand, mutations that disrupt the stem or change the sequence in either the bulge or the loop greatly reduce trans-activation in vivo. To assess the structural requirements of TAR RNA in trans-activation, we examined the ability of Tat to stimulate transcription from HIV templates containing mutations in TAR. Deletion of the trinucleotide bulge or alteration of the loop sequence from +31UGGG+34 to CAAA greatly diminished trans-activation in vitro. Interestingly, a +23U to A transversion, which abolishes the ability of Tat to bind to TAR RNA in gel-shift experiments, supported stimulation by Tat in transcription assays at a reduced level.
We further analyzed the role of TAR RNA in *trans*-activation by Tat by examining the ability of synthetic competitor TAR RNA to inhibit *trans*-activation by Tat in vitro. Wild-type and mutant TAR RNAs were synthesized in vitro and purified. The addition of 1 mM wild-type TAR RNA, corresponding to nucleotides +1 to +83, strongly inhibited *trans*-activation by Tat, whereas the addition of 8 mM mutant TAR RNA containing a deletion of nucleotides +35 to +38 failed to inhibit *trans*-activation. In addition, synthetic TAR RNA with a mutation in either the bulge or the apical loop failed to inhibit *trans*-activation. These results indicate that TAR functions as an RNA element and that the integrity of the stem and the sequence of both the trinucleotide bulge and apical loop are essential for TAR RNA to inhibit *trans*-activation by Tat. Thus, the trinucleotide bulge is necessary but not sufficient for competitor TAR RNA to inhibit *trans*-activation, suggesting that Tat may bind to TAR cooperatively with a cellular protein that has specificity for the apical loop. We are examining whether inhibition of Tat responsiveness by synthetic TAR RNA is due to competition for the binding of Tat or a cellular protein(s). Preliminary studies indicate that synthetic TAR RNA does not function as a competitive inhibitor of Tat since inhibition cannot be overcome by increasing the concentration of Tat. Thus, inhibition by TAR may require the binding of a cellular protein, and we are currently addressing this possibility using nuclear extracts fractionated by conventional and affinity approaches to overcome inhibition.

An additional approach we are utilizing is to attempt to identify cellular proteins that bind to Tat that may be involved in *trans*-activation. Utilizing purified, bacterially expressed, "epitope-tagged" Tat, we have preliminary evidence that a cellular protein(s) that binds to Tat is required for activated but not basal levels of transcription in vitro. This is based on the finding that immunoprecipitation of Tat from nuclear extracts incubated with wild-type epitope-tagged Tat renders the extracts incapable of supporting *trans*-activation in vitro. We are currently attempting to reconstitute the activity of extracts depleted by Tat with nuclear extracts fractionated by conventional and affinity approaches. We are also using the two hybrid system, a genetic selection in yeast, to identify human proteins that interact with Tat in vivo. Through characterization of the interactions between Tat and the cellular transcriptional apparatus, we should come to a greater understanding of the biochemistry of transcriptional activation by Tat which hopefully will assist in the development of strategies that interfere with HIV-1 gene expression in patients with AIDS.

**Regulation of HIV**

**Gene Expression**

P. Nahreini, S. Gunnery, M. Greenberg, P. Wendel, B. Hofmann, M.B. Mathews

Activation of HIV-1 transcription, which is dependent on the viral Tat protein and its target RNA-binding sequence, TAR, is indispensable for the virus to exit from latency and initiate a productive infection. This suggested that the proximity of the integrated HIV provirus to a DNA replication origin may affect viral promoter activity and pathogenicity. To explore this possibility, we have examined the influence of replication origins on the transcriptional activity of the HIV-LTR promoter, using transient expression assays, and have begun to investigate the activity of the HIV-LTR promoter integrated into the human chromosomal DNA.

In transient expression assays, the transcriptional activity of the HIV promoter is influenced by the origin of SV40 DNA replication. When the SV40 origin was situated immediately upstream or downstream from an LTR-CAT reporter cassette, the basal transcription rate increased severalfold. Moreover, the origin enhanced the effects of HIV-Tat, which were an increase in transcription initiation rate and elongation processivity. A deletion in the core origin, which disrupts the SV40 T antigen binding and abolishes replication, abrogates the origin’s effects on HIV-LTR promoter activity. The DNA replication inhibitor hydroxyurea, which terminates nascent DNA chains without blocking initiation, reduced the effects of the origin but did not eliminate them. These studies suggest that the increase in DNA template number due to replication is not the only factor contributing to increased basal HIV-LTR promoter activity and the enhancement of transcription initiation and elongation by Tat. Possibly, incomplete replication of the DNA template containing the LTR-CAT, as achieved in the presence of hydroxyurea, imparts similar effects, albeit at a reduced level.

To address the second objective, we are exploit-
ing the ability of recombinant adeno-associated virus (AAV-1) to transfer and stably integrate a reporter gene cassette into human chromosome 19 in a site-specific fashion. We have constructed several chimeric AAV vectors containing the LTR-CAT reporter cassette. In some of these vectors, a selectable marker gene (for neomycin resistance) was inserted to facilitate the isolation of clones containing the integrated HIV gene cassette. The recombinant AAV vectors were used to infect HeLa cells; in some instances, the recombinant virus was coinfected with wild-type AAV to provide rep function and impart site-specific integration of the recombinant virus. Thus far, we have isolated 25 independent clones of HeLa cells containing the integrated LTR-CAT recombinant and have monitored the activity of the chromosomally integrated HIV-LTR promoter using CAT enzyme and RNase-protection assays.

In the absence of the HIV Tat protein, few clones expressed CAT activity. In the presence of HIV-Tat, however, CAT activity was detected in most of these clones. The level of CAT activity differed significantly among the Tat-responsive clones, and a few clones showed no CAT activity even though the linked neomycin resistance gene was biologically active. We plan to determine the integration site of the recombinant proviral DNA and to assess whether the flanking chromosomal DNA might account for the observed variation among these clones. In future studies, we hope to examine the influence of cellular replication origins on the activity of the integrated HIV-LTR promoter.

To explore the mechanism whereby Tat activates transcription from the HIV-1 promoter, we have exploited a cell-free transcription system that faithfully reproduces Tat stimulation. Together with D. Ostapenko (Weizmann Institute, Israel), we showed that the level of trans-activation increased significantly when transcription was measured at later times during the reaction as a result of two distinct processes. First, preincubation reduces the level of basal transcription for reasons that are not yet understood. Second, when the addition of Tat to preincubated reaction mixtures was delayed, the level of trans-activation decreased markedly, indicative of a lag in the formation of Tat-activated transcription complexes. Pulse-chase analysis revealed that this lag is not due to the rate-limiting binding of Tat to the TAR element. Rather, it seems that preincubation of Tat with nuclear extract is required for the assembly of Tat with a cellular cofactor whose nature we are currently investigating. We have also developed a solid-phase transcription system, in which a template bearing the HIV-1 promoter is immobilized on agarose beads through a biotin-streptavidin bridge. Tat stimulated transcription in this solid-phase system as in the soluble reaction. Although washing of the immobilized pre-initiation transcription complexes did not affect basal transcription, it abolished Tat-mediated stimulation, indicating that a cellular cofactor of Tat is removed by the washing. These results suggest that the immobilized transcription system will enable further analysis of cellular proteins which participate in trans-activation.

We have also exploited the Tat/TAR system to show that RNA polymerase III is capable of producing RNA that can be translated as messenger RNA. Eukaryotic cellular mRNA is synthesized by pol II, whereas pol I produces long rRNAs and pol III produces 5S rRNA, tRNA, and other small RNAs. To determine whether this functional differentiation reflects an underlying mechanistic specialization, such that each polymerase is restricted as to the kind of RNA that it produces, we examined the translational potential of a pol III transcript. The coding region of the HIV-1 Tat gene was placed under the control of the adenovirus-2 VA RNA<sub>g</sub> gene promoter, a strong pol III promoter. The resultant plasmid, pVA-Tat, was transcribed in vivo and in vitro to generate the predicted RNA, which was insensitive to low concentrations of α-amanitin as expected for a pol III transcript. Its translational activity was examined by cotransfection of pVA-Tat with a reporter construct, pHIV-LTR-CAT, which is trans-activated by the Tat protein. pVA-Tat elicited an 80-fold increase in CAT activity, showing that it generates functional mRNA (Fig. 3). Neither a control plasmid pVA that lacks the Tat-coding region nor pVA anti-Tat that contains the coding region in an antisense orientation elicited any effect in the trans-activation assay.

To verify that the translated RNA is indeed produced by pol III, we produced a series of mutations in pVA-Tat sites that are specific for pol III transcription. Site-directed mutations in the essential elements of the pol III promoter, the A and B boxes, down-regulated VA-Tat RNA production both in vivo and in vitro and dramatically reduced CAT trans-activation. Furthermore, the insertion of a pol III termination signal within the Tat-coding sequence led to the production of truncated RNA and reduced trans-activation.
FIGURE 3 Synthesis of Tat from a pol III chimera. The HIV-LTR-CAT reporter plasmid was cotransfected into HeLa cells with the pVA-Tat chimera (lane 5), in which the VA RNA promoter drives Tat RNA synthesis. As a positive control, Tat RNA was produced from a pol II promoter (pCMV-Tat, lane 6). Negative controls include cells lacking the reporter plasmid (lane 1), containing this plasmid alone (lane 2), or cotransfected with plasmids expressing VA RNA (pVA, lane 3) or a partial antisense copy of Tat (pVA-anti Tat, lane 4). The plasmids are shown schematically (top) and results of CAT assays are shown below.

As expected for a pol III transcript, VA-Tat RNA was neither polyadenylated at its 3′ end nor capped at its 5′ end. Like mRNA, the uncapped VA-Tat RNA was associated with polysomes in a salt-stable manner. We conclude that the Tat activity is generated by translation of an RNA produced by pol III. These findings imply that functional mRNA synthesis is not restricted to pol II, that pol III transcripts can be transported within the cell and assembled with the translational components, and that the 5′ cap structure is not obligatory for translation. The implications of these findings for the transcription and translation processes are being explored further, for example, to determine whether a pol III transcript can be spliced and to study the initiation of protein synthesis on an uncapped mRNA.

PUBLICATIONS


## RNA SPlicing

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D. Horowitz

### MECHANISMS OF MAMMALIAN PRE-mRNA SPlicing AND SPlice SITE SELECTION

RNA splicing is a required step in the expression of most eukaryotic protein-coding genes. 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. Both constitutive and alternative splicing mechanisms involve multiple protein components as well as RNA components that are part of small nuclear ribonucleoprotein (snRNP) particles. RNA-RNA base-pairing interactions among snRNAs and between snRNAs and pre-mRNA are important determinants of splice site recognition and, in addition, probably participate in the formation of the active sites for catalyzing the *trans*-esterification reactions within the spliceosome. The role of spliceosomal proteins is not well understood, although protein-RNA and protein-protein interactions are also essential for pre-mRNA splicing. Several required protein-splicing factors are thought to have important roles in spliceosome assembly, splice site selection, and conformational rearrangements coupled to ATP hydrolysis. Other possible roles include regulation of active site accessibility, turnover of snRNPs, and positive and negative regulation of alternative splice site selection, for example, through posttranslational modification. Some protein factors might also contribute chemical groups to the *trans*-esterification active sites. Our laboratory has focused on the identification, purification, and molecular characterization of protein factors that are necessary for the catalysis of splicing and/or for the regulation of alternative splice site selection in vitro.

### STRUCTURE, FUNCTION, AND EXPRESSION OF HUMAN SPlicing FACTORS SF2/ASF AND hnRNP A1

Human pre-mRNA splicing factor SF2/ASF has an activity required for general splicing in vitro and promotes utilization of proximal alternative 5′ splice sites in a concentration-dependent manner. The latter activity results from antagonistic interactions with the protein heterogeneous nuclear RNP (hnRNP) A1, which stimulates use of distal 5′ splice sites. J. Cáceres introduced selected mutations in the amino-terminal RNA recognition motif (RRM) and the carboxy-terminal arginine/serine (RS) domain of SF2/ASF and assayed the resulting recombinant proteins for constitutive and alternative splicing in vitro and for binding to pre-mRNA and mRNA (Figs. 71.
1 and 2). Mutations were designed on the basis of known RRM tertiary structures in such a way as to minimize structural or folding perturbations. These experiments showed that mutant proteins inactive in constitutive splicing continued to affect alternative splice site selection, demonstrating that these activities involve distinct molecular interactions. Specific protein-RNA contacts mediated by solvent-exposed Phe-56 and Phe-58 in the RNP-1 submotif of the SF2/ASF RRM were found to be essential for constitutive splicing, although they are not required for RRM-mediated binding to pre-mRNA. The RS domain is also required for efficient constitutive splicing activity, and both arginine and serine residues are important for biochemical activity. Analysis of domain deletion mutants demonstrated strong synergy between the RRM and a central degenerate RRM repeat in binding to RNA. These two domains are sufficient for alternative splicing activity in the absence of an RS domain (Fig. 2).

A related mutagenic analysis of hnRNP A1 structure and function was carried out by A. Mayeda in collaboration with Steve Munroe (Marquette University). The ability of hnRNP A1 to promote use of certain distal alternative 5' splice sites requires specific protein-RNA contacts mediated by pairs of conserved phenylalanine residues (analogous to those of SF2/ASF) in the RNP-1 submotifs of each of the two RRMs, although these residues are not required for general RNA binding. The glycine-rich carboxy terminus of hnRNP A1 is also essential for alternative splicing activity. These mutants were also analyzed for their effects on the RNA annealing activity of hnRNP A1. Previous work on hnRNP A1 showed 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. More recently, intrinsic high-affinity binding sites for hnRNP A1 have been identified by G. Dreyfuss and colleagues. A. Hanamura and I. Watakabe are currently identifying high-affinity SF2/ASF-binding sites by selection and amplification from random RNA libraries. The functional significance of binding to sites of different affinities by wild-type and mutant SF2/ASF and hnRNP A1 proteins will then be evaluated.

Collaborative experiments between I. Eperon's laboratory (University of Leicester) and our laboratory showed that U1 snRNP can bind simultaneously to two alternative 5' splice sites on a single pre-mRNA molecule. When this occurs, the downstream 5' splice site is selected, and the effect of SF2/ASF appears to be to enhance multiple 5' splice site occupancy by U1 snRNP. Ongoing experiments will address the role of hnRNP A1 and the mechanism by which SF2/ASF stabilizes U1 snRNP binding.
We have proposed that the intracellular ratios of SF2/ASF and hnRNP A1 may control the specificity of splice site selection and that in vivo regulation of one or both of these activities may have an important role in the tissue-specific or developmental regulation of alternative splicing. A. Hanamura measured the levels of SF2/ASF and hnRNP A1 polypeptides in different rat tissues by quantitative immunoblotting with a monoclonal anti-SF2/ASF antibody (generated in collaboration with C. Bautista and M. Falkowski, CSHL Monoclonal Antibody Facility) and a monoclonal anti-hnRNP A1 antibody (provided by G. Dreyfuss). Substantial variation in the molar ratio of SF2/ASF to A1 was observed, well above the range required to elicit splice site switching in vitro.

Altered electrophoretic mobilities that would reflect differences in posttranslational modifications were not observed in rapidly lysed samples. In addition, small but reproducible changes were also observed among different cell lines, for example, in response to transformation (in collaboration with J. Cáceres and with R. Franz Jr., CSHL), which might explain some of the alterations of alternative splicing patterns that are commonly seen in transformed cells.

**ROLE OF SF2/ASF IN SPlicing REACTIONS DEPENDENT ON EXONIC SPlicing ENHANCERS**

A novel function for SF2/ASF was uncovered in collaborative studies with F. Rottman and colleagues.
Their laboratory had previously identified a purine-rich cis-acting element in the last exon of bovine growth hormone (bGH) pre-mRNA which activates alternative splicing of the bGH last intron in vivo and in vitro. A 35-kD protein factor that interacts specifically with the exonic splicing enhancer (ESE) turned out to be identical to SF2/ASF. UV cross-linking followed by immunoprecipitation with an SF2/ASF-specific monoclonal antibody confirmed that SF2/ASF binds to the ESE in splicing extracts. Competition experiments showed that sequence-specific binding of SF2/ASF to the ESE is an intrinsic property of the protein and can occur in the absence of other components. Addition of purified SF2/ASF enhanced in vitro splicing of the last intron in wild-type bGH pre-mRNA but not in mutant bGH pre-mRNA lacking the ESE. A related SR protein, SC35, failed to bind to the bGH ESE and did not activate splicing of the last intron. Thus, SF2/ASF functions as a sequence-specific exonic enhancer recognition factor to activate the splicing of the preceding intron. hnRNP A1 counteracted the SF2/ASF-mediated enhancement of bGH splicing, although it bound the ESE only in a nonspecific manner.

**FIGURE 3** SF2/ASF activation of enhancer-dependent and -independent splicing events. (**Top**) Based on the bGH system, SF2/ASF binds sequence-specifically to a purine-rich exonic splicing enhancer (ESE). The resulting complex activates splicing of the preceding intron. Splicing is dependent on the ESE because of the presence of weak splicing signals in the intron. **(Bottom)** Constitutively spliced pre-mRNA with strong intronic splicing signals. Although SF2/ASF, or another SR protein, is required for splicing of this substrate, the nature and location of the functionally relevant binding site(s) are presently unknown. The 5' and 3' splice sites (ss), branchpoint (bp), and poly-pyrimidine tract (py) are indicated.

**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. Using selective inactivation and biochemical complementation strategies, D. Horowitz identified two novel activities required for cleavage at the 5' splice site and lariat formation. These activities have been chromatographically separated, and their purification is in progress. T.-L. Tseng is searching for proteins with RS domains in budding and fission yeast, with the ultimate goal of studying the structure and function of RS domain-containing splicing factors by genetic methods. The rationale is based on our previous observation that very few known proteins in the databases contain significant repeats of interspersed arginine and serine residues, and virtually all of these proteins have been directly or indirectly implicated in constitutive or regulated splicing.

B. Dong purified a 54-kD HeLa cell polypeptide, initially based on cross-reactivity with antibodies raised against a yeast splicing factor, although this cross-reactivity turned out to be fortuitous. This protein is in the nuclear fraction and can be cross-linked to RNA and was therefore termed p54

The sequence of p54

cDNA clones revealed the presence of two RRMs and significant sequence similarity to a number of RNA-binding proteins and mammalian splicing factors. In particular, the 471-amino-acid-long p54
shares a 320-amino-acid central region of 71% identity with the 712-amino-acid-long human splicing factor PSF (Fig. 4). The same region is 42% identical to a central region of a 700-amino-acid-long *Drosophila* protein known as NONA, DISS, or BJ6. Previous work in other laboratories showed that this fly protein is ubiquitously expressed and has pleio-
tropic functions in the nervous system, such that mutations in its gene impair normal visual acuity and male courtship song and give rise to abnormal electroretinograms. On the basis of the significant homology with known and putative splicing factors, we have suggested that NONA may affect alternative splicing of pre-mRNAs encoding ion channels or other proteins in such a way as to affect the expression of one or several isoforms that function in specific neural tissues. A variety of approaches is being used to study the RNA-binding properties of p54nr, its likely involvement in pre-mRNA splicing, and its functional relation to PSF.

CONSERVED MOTIFS IN PROTEIN SPLICING FACTORS
E. Birney (now at Balliol College, Oxford), and S. Kumar (in R.J. Roberts’ laboratory at New England Biolabs) carried out a detailed sequence analysis of conserved motifs in metazoan protein-splicing factors and related RNA-binding proteins. This analysis included the characterization of RRMs, the construction of a comprehensive RRM database, and statistical analysis of arginine-serine and glycine-rich repeats found in RNA-binding proteins. An alignment of 70 RRMs (Fig. 5) was constructed placing special emphasis on tertiary structural requirements, modeled after two available structures. The most conserved positions in this alignment correspond precisely to residues that form the hydrophobic core of the U1-A RRM1 tertiary structure, as previously noted by J. Keene and colleagues. Analysis of the RRM alignment and the model tertiary RRM structure led us to propose the following consensus structural core sequence for RRMs: UxUxxLxx[x-0_6]Z[x]xxxLxx Fxxx[x]GxUx[x]Zxxxxx[x-0_21]UxVxF[x]xxxxx ZxxA, where x = any residue; U = unchanged residues, L,I,V,A,G,F,W,Y,C,M; Z = U + S,T; and + indicates that loop 3 may be expanded further.

This is a degenerate consensus that is too permissive to be used as the sole criterion to identify RRMs, but which should be matched by all RRMs with the correct tertiary fold, including those with atypical RNP-1 submotifs.

Using profile analysis based on an alignment slightly larger than that in Figure 5, an RRM-enriched database was generated. Examination of each entry for a match to the above structural consensus yielded a comprehensive RRM database. The RRM database is periodically updated by E. Birney and is accessible through the Internet (send E-mail to RRM@molbiol.ox.ac.uk with the words SEND RRM in the body of the message). As of this writing, the RRM database contains 151 proteins with 289 RRMs. 17 proteins were found to contain 22 previously unreported RRMs, which strongly suggests that they are
FIGURE 5 Alignment of 70 selected RRM.s. Selected RRM.s were aligned manually with special emphasis on tertiary structural requirements modeled after two available structures. Alignment gaps are indicated by dashes. For sequences with more than one RRM, each one is numbered from the amino terminus, with the number given in parentheses after the sequence name. Beginning and ending residue positions within the parent protein are given for each RRM at the left and right of the alignment. The alignment positions are numbered above and below; conserved positions are highlighted by vertical shading. Black shading indicates positions in which a single residue occurs in at least 75% of the sequences. Gray shading at the same positions represents conservative substitutions. Elsewhere, gray shading indicates positions in which residues belonging to a single conservative grouping are present in at least 50% of the sequences. When a single conservative grouping represents at least 75% of the sequences, this is denoted by gray shading in boxed columns. Acceptable conservative groupings were I=V=L=F=Y=W,Q=N,R,K=E,S=T. Three additional positions at which the consensus is split between two residues are also shaded: positions 6 and 53g (G=N) and position 36 (A=G). The consensus structural core residues are shown below the alignment (U = uncharged residues: I,L,V,A,G,F,W,Y,C,M; Z = U + S,T), along with the position of the RNP-1 and RNP-2 submotifs. Secondary structure, modeled primarily after the humU1A(1) tertiary structure except for $\alpha_5$, which was based on the humHNRNPC secondary structure, is given above the alignment. In humU1A(1), $\alpha_5$ extends another two residues toward the amino terminus. Most positions of the RRM lack a firm consensus. The range of sequence similarity between two otherwise unrelated RRM.s is 10–20% identity.
RNA-binding proteins. Additional homologies with known splicing factors were also found. One example of this analysis is the finding of striking homology between metazoan SR proteins that contain a central atypical RRM and the *Saccharomyces cerevisiae* protein NPL3/NOP3. NPL3 mutants have pleiotropic phenotypes, but this protein so far is not known to be involved in splicing. The homology includes a perfectly conserved heptapeptide, SWQDLKD, which is an invariant signature for SR proteins that contain a central atypical RRM and is not found in any other proteins in the databases. Another finding from the above analysis is the presence of two unreported RRs in *Schizosaccharomyces pombe* mei2, a protein involved in meiosis. The presence of these RRs strongly suggests that RNA binding is involved in the function of mei2.

**PUBLICATIONS**


**TRANSCRIPTIONAL REGULATION**

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D. Aufiero  
M. Cleary  
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**A. Wilson**

We study the mechanisms of transcriptional regulation in eukaryotes. To probe these mechanisms, we use both cellular and viral regulatory factors, in particular two cellular POU-domain proteins, Oct-1 and Oct-2, and a herpes simplex virus (HSV) protein, VP16. Although the cellular proteins Oct-1 and Oct-2 possess the same intrinsic DNA-binding specificity, their transcriptional regulatory properties are different. For example, both proteins recognize the octamer sequence ATGCAAAAT found in a variety of promoters, but the efficiencies with which they activate such promoters differ. These differences result from the activities of promoter-selective transcriptional activation domains that can discriminate among promoters. The viral protein VP16 is a component of the HSV virion that, after infection, associates with Oct-1 and a second cellular factor we refer to as HCF to form a multiprotein-DNA regulatory
complex. By its association with Oct-1, VP16 alters the transcriptional regulatory properties of Oct-1 in two ways: It recruits Oct-1 to new DNA sites that are not recognized effectively by Oct-1 alone, and it provides a potent transcriptional activation domain to activate the HSV immediate early promoters, a type of promoter that is normally resistant to activation by Oct-1.

This year, our studies focused on three issues: (1) How does a eukaryotic transcription factor recognize a diverse and yet specific set of DNA sequences? (2) How is a DNA site-specific multiprotein-DNA regulatory complex, as exemplified by the VP16-induced complex, assembled? (3) What are the rules for how transcriptional activation domains and the basal transcriptional apparatus communicate with one another? Additionally, we study promoter structure by analyzing the natural variation that occurs in the human immunodeficiency virus (HIV) promoter isolated from infected individuals.

Below are descriptions of accomplishments we made during the past year.

Crystal Structure of the Oct-1 POU Domain Bound to an Octamer Site: DNA Recognition with Tethered DNA-binding Modules

R. Aurora [in collaboration with J. Klemm, M. Rould, and C. Pabo, Massachusetts Institute of Technology]

Oct-1 and Oct-2 are members of the POU subfamily of homeodomain proteins. These proteins are distinguished by a bipartite DNA-binding domain, consisting of an amino-terminal POU-specific (POUS) domain linked to a carboxy-terminal homeo (POUH) domain by a hypervariable linker. We showed last year, in collaboration with N. Assa-Munt, R. Mortishire-Smith, and P. Wright (The Scripps Research Institute), that the POUS domain is structurally similar to the DNA-binding domains of the bacteriophage λ and 434 repressors and 434 Cro protein. These DNA-binding domains, like homeodomains, contain a helix-turn-helix (HTH) motif; unlike homeodomains, however, which contain a single α helix in addition to the HTH structure, the POUS and bacteriophage DNA-binding domains contain two additional α helices.

Although the POUS and bacteriophage DNA-binding domains are structurally very similar, they differ in the region that is normally most highly conserved, the HTH structure itself. In the POUS structure, the first helix and turn of the HTH structure are both extended. These extensions compensate for one another such that glutamines that lie at the beginning of the two HTH α helices in these proteins are identically positioned in relation to one another. In the bacteriophage DNA-binding domains, these glutamines were known to make critical sequence-specific contacts. The structural similarity between the POUS and bacteriophage DNA-binding domains led us to suggest that the POUS domain glutamines contact DNA in a similar manner. The crystal structure of the Oct-1 POUS domain bound to an octamer site shows that this prediction was indeed correct.

Figure 1 shows a schematic of the Oct-1 POUS domain bound to DNA. The most striking feature of the structure is the structurally independent association of the two POUS subdomains with the DNA. The POUS and POUH domains bind on opposite sides of the DNA, without evident contacts between them. This "independent" association highlights the importance of the linker that tethers the two domains: By apparently increasing their relative concentration, the linker is largely responsible for the interdependence of the two DNA-binding domains. The linker, how-
ever, is likely to be structurally flexible, because it could not be seen in the cocrystal structure. The structure of the DNA-bound Oct-1 POU domain suggests how this DNA-binding domain is well suited to recognize a degenerate set of DNA sequences, because the two domains should be able to bind to differently oriented half sites, and to associate with a protein like VP16, because the two domains are very accessible.

Flexible DNA Sequence Recognition by the Oct-1 POU Domain

M. Cleary

The Oct-1 POU domain is capable of binding to a number of sites that bear little octamer similarity. For example, the TAATGARAT sequence, which is found in the promoters of the HSV immediate early genes and which serves as a target of activation by VP16, is a weak but specific binding site for the Oct-1 POU domain in the absence of VP16.

We have studied the mechanisms by which the POU domain achieves sequence recognition flexibility by focusing on the contribution of the POU$_S$ domain to the overall binding ability of the entire POU domain. By analyzing the separate and combined effects of amino acid substitutions in the POU$_S$ domain and base changes in the Oct-1-binding sites, we have uncovered two mechanisms for DNA-binding flexibility. First, an amino acid side chain in the POU$_S$ DNA recognition helix that makes critical DNA contacts can adapt to DNA sequence alterations at its sites of interaction. Alanine substitution of this residue, an arginine, greatly destabilizes the POU domain on DNA; base changes at the DNA contact sites of the arginine, however, only have a minor disruptive effect. This finding suggests that although the arginine is necessary for DNA contact, its DNA sequence requirements are not rigid, allowing the Oct-1 POU domain to recognize sites that diverge from the octamer sequence. In the second mechanism, the two subdomains of the POU domain can apparently reorient themselves on different sites to accommodate differential positioning of the POU$_S$ and POU$_H$ domain-binding sites. Our studies indicate that binding of the Oct-1 POU domain to the TAATGARAT site involves a translocation of the POU$_S$ domain from the left-hand side of the POU$_H$ domain, as it is shown in Figure 1, to the right-hand side.

VP16-induced Complex Formation

J.-L. Lai, C. Huang, M. Cleary

After HSV infection, transcription of the HSV immediate early genes is activated by a multiprotein-DNA complex containing one viral and two cellular proteins. The viral component is the virion protein VP16 and the cellular components are Oct-1 and a less-well understood cellular factor we refer to as HCF. Figure 2 shows a cartoon of this VP16-induced complex. The complex forms on specific sites within the immediate early promoters that conform to the consensus sequence TAATGARAT (R = purine). These VP16-responsive sites are sometimes associated with an overlapping octamer sequence, which serves as a binding site for Oct-1, but other TAATGARAT sites lack any strong similarity to an octamer sequence; these sites, called (OCTA-)TAATGARAT, are low-affinity binding sites for Oct-1 on their own.

When VP16 is first delivered to the cell, it forms a heterodimeric complex with HCF. This complex is then able to associate with Oct-1 on TAATGARAT sites, through interactions with the Oct-1 homeodomain and the GARAT segment of the TAATGARAT motif. Although Oct-1 and Oct-2 are very closely related, Oct-2 fails to associate effectively with VP16 because of a single-amino-acid difference on the exposed surface of the DNA-bound Oct-1 and Oct-2 homeodomains.

We have extended our studies on the sequence requirements within both Oct-1 and VP16 for VP16-induced complex formation. Previously, our studies
have focused on the involvement of the Oct-1 homeodomain in VP16-induced complex formation; the contribution of the POUs domain has not been evident. By testing the activity of a series of Oct-1 POUs domain mutants, we have found that the POUs domain contributes to complex formation by stabilizing Oct-1 on the DNA. There are residues in the POUs domain, however, that contribute more directly to VP16-induced complex formation; mutation of these residues has a more dramatic effect on formation of the VP16-induced complex than on Oct-1 binding to a TAATGARAT site on its own.

Analysis of VP16 has revealed multiple regions involved in complex assembly. These studies have been aided by the recent elucidation by other investigators of the sequence of VP16 homologs in other herpesviruses. Sequence comparison reveals at least five conserved regions, and mutations within these regions affect VP16-induced complex formation. One small region of VP16, near the carboxy-terminal transcriptional activation domain, is of particular interest because individual mutations within this region disrupt complex formation by alternatively affecting VP16 association with either HCF, Oct-1, or DNA. The interaction of a small region of VP16 with all of the other components of the VP16-induced complex suggests that, unlike the structure of the VP16-induced complex shown in Figure 2, VP16, Oct-1, HCF, and the DNA are all in close proximity to one another in the complex.

**Molecular Analysis of the VP16-Accessory Protein HCF**

A. Wilson, R. Freiman, D. Aufiero, W. Herr [in collaboration with K. LaMarco and G. Peterson, Tularik, Inc.]

HCF, the second cellular factor in the VP16-induced complex, has unusual properties in stabilizing the VP16-induced complex. It binds to VP16 and enables it to associate more effectively with Oct-1 on TAATGARAT sites. The normal cellular function of HCF is unknown, although it is likely to be important, because HCF activity is found in animals as diverged from humans as nematodes and insects.

Last year, we described the purification of HCF and the isolation of cDNA clones encoding HCF. Figure 3A shows samples of purified human HCF; it is a collection of polypeptides ranging in size from 110 to 300 kD. The cDNA clones revealed that HCF is encoded by a 2035-codon open reading frame and is initially expressed as a large precursor protein of about 300 kD. Figure 3B shows a schematic of HCF, highlighting elements observed in the deduced HCF amino acid sequence. HCF bears little resemblance to other known proteins, but centrally located within the HCF sequence is a prominent repeat of a 26-amino-acid sequence that we refer to as the HCF repeat. A consensus sequence of the HCF repeat is shown in Figure 3C.
carboxy-terminal halves, which remain noncovalently associated with each other. The HCF repeats direct this unusual form of proteolytic processing. The purpose of the proteolytic processing of HCF is not known. The amino-terminal segments, however, are sufficient to associate with VP16 and induce complex formation with VP16 and Oct-1.

To investigate the cellular function of HCF, we have analyzed, in collaboration with B. Trask (University of Washington) and J. Parrish and D. Nelson (Baylor College of Medicine), the genomic location of the HCF gene and its expression patterns. The gene encoding HCF, HCF-1, is located within the q28 region of the X chromosome. This region of the human genome contains many genes and is associated with many human diseases such as hemophilia and color blindness. We have not as yet, however, identified a human disease that might correspond to mutations in the HCF-1 gene. The HCF-1 gene is highly expressed in fetal tissues and tissue culture cells, but in adult tissues, it is generally not highly expressed, suggesting a role in cell proliferation. HCF is, however, highly expressed in the adult kidney where it may be involved in a function separate from cell proliferation. Curiously, the brain, the site of HSV latency, contains very little HCF protein.

Regulatory Mechanisms of Transcriptional Activation Domains

G. Das, C. Hinkley, W. Tansey

Little is known about how transcriptional activation domains stimulate transcription or about their structure. Activation domains rarely display high levels of sequence similarity, such as that observed among DNA-binding domains, suggesting less stringent structural requirements. We have taken three approaches to study the structural and functional properties of activation domains: (1) We have performed an "alanine-scanning" mutagenesis of a small 18-amino-acid activation domain; (2) we have assayed the influence of basal promoter elements on the activity of activation domains; and (3) we have studied the role of sequences on the surface of the TATA-box-binding protein TBP in responding to activators.

We have previously described a multimerization approach, developed by M. Tanaka, to analyze the structure and function of transcriptional activation domains. In this approach, a short, otherwise inactive, segment from within an activation domain is reiterated in tandem, creating a larger and now functional activation domain. In this manner, two separate 18-amino-acid segments from within a 66-amino-acid glutamine-rich activation domain in Oct-2, called Q18II and Q18III, were shown to possess activation domain potential.

This approach is well suited to dissect activation domain structure and function by mutagenesis, because the effects of mutations are amplified by the multimerization process. We therefore mutated individually all 17 nonalanine residues in the Q18III segment to alanine and assayed their effects on transcriptional activation. To our surprise, most of the mutations disrupted activation. Thus, although not displaying strong sequence similarity to other activation domains, this 18-amino-acid activation domain apparently possesses important structural properties that are easily disrupted by point mutagenesis.

The activation domains of Oct-1 and Oct-2 display different abilities to activate transcription from snRNA and mRNA promoters. To explore the different activities of promoter-selective activation domains in more detail, we have assayed the activity of a large collection of activators, all carrying different activation domains fused to the same yeast GAL4 DNA-binding domain, in different promoter contexts. Figure 4 shows three of the promoter contexts and the interaction of activators with basal factor complexes.
texts we have tested: an mRNA promoter (c-Fos), along with two snRNA promoters, one directing transcription by RNA polymerase III (U6) and one directing transcription by RNA polymerase II (U2). The major difference among these three promoters is the architecture of the basal elements situated near the transcriptional start sites. These elements direct formation of different DNA-bound TBP-containing complexes. The TATA box found in mRNA promoters, such as that in the c-Fos promoter, binds the TBP-containing TFIIID complex, whereas in snRNA promoters, a conserved proximal sequence element, the PSE, binds a TBP-containing complex called SNAP. One snRNA promoter, that for the U6 snRNA, directs RNA polymerase III transcription and contains, in addition to a PSE, a TATA box that binds TBP.

Among the activator collection tested were the very potent and acidic transcriptional activation domain from the herpesvirus protein VP16 and a glutamine-rich activation domain from the well-characterized transcription factor Sp1. As with acidic activation domains in general, the VP16 activation domain can activate mRNA promoters in all eukaryotic species tested, including such diverse species as yeast and human cells. In contrast, the human Sp1 activation domain is not active in yeast cells. Although not active in as many species, however, the Sp1 activation domain we tested is more universally active with respect to promoter type than the VP16 activation domain; whereas the snRNA promoters did not respond to the VP16 activation domain, the Sp1 activation domain was active on both the mRNA and snRNA promoters tested, although it displayed considerably more activity on the snRNA promoters. These results suggest that activation domains discriminate among factors bound to different sets of basal elements and emphasize the importance of the composition of basal elements in defining the activity of activators.

To study how basal factors respond to activators, we have assayed the ability of mutated human TBP molecules to activate transcription in vivo. To bypass the activity of endogenous human TBP, we adapted to human cells the altered TBP DNA-binding specificity strategy developed by K. Struhl and colleagues (Harvard University) in yeast cells. In this strategy, the activity of a mutant TBP molecule with relaxed DNA-binding specificity can be measured on a promoter containing a mutated TATA box that is not recognized by the wild-type endogenous TBP. By use of this assay, we have made two important findings. First, although Sp1 does not activate transcription in yeast cells, yeast TBP responds to Sp1 in mammalian cells, indicating that the defect in yeast is not due to TBP itself. Second, individual sets of mutations on different surfaces of TBP do not interfere with the ability of TBP to respond to activators in vivo, but combinations of these mutations can have dramatic effects. These results suggest that separate surfaces of TBP cooperate in responding to activators and that these surfaces of TBP can compensate for each other’s defects. Thus, although TBP has been very highly conserved during evolution, it is very resistant to mutagenesis for response to activators of RNA polymerase II transcription in vivo.

Analysis of VP16 Activation Domain Subsegments in Yeast

M. Tanaka

Transcriptional activators often contain multiple and separable activation domains that cooperate with one another to activate transcription. To identify an elementary unit for this synergistic action, the VP16 activation domain was analyzed in the yeast Saccharomyces cerevisiae using a multimerization approach. I chose multiple 12–13-amino-acid-long segments within the 78-amino-acid-long acidic VP16 activation domain, individually reiterated as two to six tandem copies and fused them to the GAL4 DNA-binding domain. They were tested for the ability to activate transcription of a GAL4-responsive reporter gene in yeast cells.

Among the segments tested, all of which contained abundant acidic and hydrophobic amino acid residues, two strongly activated transcription; even subsegments as short as four or eight amino acids activated transcription when reiterated. Their transcriptional activity increased dramatically with an increasing number of reiterations. Thus, an activation domain can be reconstituted from an extremely small repetitive module, which cooperates to function efficiently.

Analysis of these GAL4-VP16 fusion activators expressed in yeast cells has identified the hsp60 chaperonin as a factor that can interact with func-
tional but not with nonfunctional VP16-derived activation domains. Indeed, the relative level of association with hsp60 correlates directly with the relative level of activation domain activity. Although it remains to be determined if hsp60 is involved in nuclear transcription, the observation that it specifically associates with functional activation domains is intriguing, because hsp60 is thought to interact with exposed hydrophobic surfaces of folding intermediates of a number of proteins. Therefore, an important feature of an activation domain may be a hydrophobic surface being exposed in folded proteins, whereas acidic amino acid residues may be required to expose the region to solvents while avoiding intramolecular aggregation. The interaction between a transcriptional activation domain and a chaperonin also suggests that a target molecule of an activation domain during activation process can, like chaperonins, interact with divergent sequences, not containing specific secondary or tertiary structures.

Sequence Rearrangements in the HIV-1 LTR Isolated from Infected Individuals

B. Lee, D. Aufiero [in collaboration with A. Wiznia, Bronx-Lebanon Hospital, New York]

We have an ongoing project to study the structure of transcriptional regulatory sequences in the HIV-1 long terminal repeat (LTR) isolated from infected individuals. We have previously described a region of the HIV-1 LTR that is a hot spot for the appearance of tandem sequence duplications or TSDs. This TSD hot-spot region lies just upstream of the HIV-1 enhancer core sequences that bind the transcription factor NF-κB. We originally identified these rearrangements in lymphocytes from infected adults, but viral LTRs containing the rearrangements were always accompanied by unrearranged forms. This year, we analyzed samples from infected children in the Bronx and found that, in contrast to the earlier results, many of these infected individuals only carried rearranged LTRs with TSDs. These results show that TSD-containing viruses on their own can produce productive infections. We are currently studying the nature of the trans-acting factors that recognize the TSD elements.

PUBLICATIONS


In Press


Laboratories in this section study various aspects of eukaryotic cell biology, particularly those relating to disease, cell signaling, transcription, and structure.

- Dr. Skowronski’s laboratory (Accessory Genes of Human Immunodeficiency Virus) is focused on the mechanisms by which HIV, the virus responsible for AIDS, causes immunodeficiency. They have determined that expression of the nef gene down-regulates CD4 antigen in T cells. CD4 antigen is required for normal T-cell function. Mechanisms for this down-regulation are being explored.

- Dr. Franza’s laboratory (Quantitative Regulatory Biology) has continued their studies of HIV and has laid groundwork for the inhibition of certain viral proteins with oligonucleotides.

- Dr. Garrels’ laboratory (Quest Protein Database Center) has continued software development to allow more convenient and widespread use of 2D gel analysis and protein databases. They have expanded their application to the yeast *Saccharomyces cerevisiae* in order to enrich the effort, ongoing elsewhere, to sequence the genome of that organism.

- Dr. Wigler’s laboratory (Mammalian Cell Genetics) studies signal transduction in yeasts and mammalian cells, concentrating on the RAS pathway and associated protein kinases, such as RAF. Both RAS and RAF are oncoproteins, capable of inducing malignant transformation in normal cells. Additionally, they are in the midst of discovery of genetic lesions in malignant cells.

- Dr. Bar-Sagi’s laboratory (Transmembrane Signaling) studies signal transduction through the RAS pathway in mammalian cells, in particular its mode of regulation by growth factor receptors and its interaction with the RAF serine/threonine protein kinase. These studies build upon evidence gathered in her laboratory that receptor activation leads to a reorganization of the subcellular localization of components of the RAS pathway through SH2 and SH3 protein motif domains.

- Dr. Tonks’ laboratory (Protein Tyrosine Phosphatases and the Control of Cellular Signaling Responses) has produced the first X-ray crystal structure of a protein phosphatase in collaboration with David Barford. In addition, this laboratory has identified a protein phosphatase that dephosphorylates MAP kinase and blocks its signaling.

- Dr. Gilman’s laboratory (Nuclear Signal Transduction) explores the mechanisms whereby cellular signaling by growth factors and hormones leads to alterations in gene transcription. In a seminal series of experiments, this group has demonstrated that growth factor receptor activation leads to the tyrosine phosphorylation and subsequent activation of a set of transcription factors. This pathway is distinct from the pathway that alters gene expression through the participation of RAS, demonstrating a redundancy in signal transduction circuitry. Other work in this laboratory is directed to understanding how the same signal elicits different responses in different cells.

- Dr. Spector’s laboratory (Cell Biology of the Nucleus) has addressed questions about functions within the nucleus using predominantly microscopic techniques. His laboratory has recently demonstrated the recruitment of splicing factors from nuclear storage sites to sites of active transcription.

- Dr. Helfman’s laboratory (Molecular Cell Biology) continues the study of tropomyosins and tissue-specific splicing. Over 16 different forms of tropomyosins, derived from four different genes, are found distributed in a tissue-specific manner. This diversity arises in part from tissue-specific differential splicing.
ACCESSORY GENES OF HUMAN IMMUNODEFICIENCY VIRUS

J. Skowronski  R. Mariani  S. Salghetti

AIDS immunodeficiency is invariably associated with depletion of a regulatory subset of T cells that express CD4 antigen on the cell surface. These CD4+ T cells are preferentially infected by human immunodeficiency virus (HIV), but neither the mechanism(s) that subverts the normal function of the infected cells nor the identity of viral genes involved is clear. Besides the gag, pol, and env genes, found in all retroviruses, immunodeficiency viruses encode several additional "accessory" proteins. Some of these accessory genes are not required for the viral life cycle in established cell lines, and their function has been difficult to assess. Remarkably, several lines of evidence indicate that these "nonessential" genes have an essential function in vivo, or in primary cells, for efficient virus replication and pathogenesis. Research in this laboratory focuses on the function and mechanism of action of these accessory proteins of immunodeficiency viruses.

Our recent work has focused on understanding the function and mechanism of action of the HIV-1 nef gene. nef is dispensable for the viral life cycle in vitro, but it is strictly required for high viral load and CD4+ T-cell depletion in animal models for AIDS. Our laboratory has been using approaches in vivo, in transgenic mice, and in vitro to address the consequences of Nef expression on normal cellular functions and its interaction with cellular regulatory mechanisms. We have shown previously that (1) HIV-1 Nef alleles isolated directly from peripheral blood of HIV-1-infected individuals decrease expression of CD4 antigen on the surface of human T cells and (2) HIV-1 Nef alters activation and blocks development of a CD4+ T-cell subset when expressed in T cells in transgenic mice. These effects of Nef expression in transgenic T cells correlated with abnormally low CD4 antigen expression on the cell surface. These results have broad implications, because CD4 antigen is involved in many aspects of biology of CD4+ T cells and also is a cell-surface receptor that mediates HIV infection. During the last year, we have focused on cellular mechanisms impacted by nef. We have also taken the first step toward elucidation of molecular interactions between Nef and CD4.

CD4 DOWN-MODULATION BY NEF REQUIRES A MEMBRANE PROXIMAL REGION IN THE CD4 CYTOPLASMIC TAIL

CD4 is an integral membrane glycoprotein expressed primarily on immature thymic T cells and mature helper T lymphocytes. The extracellular domain of CD4 binds to a nonpolymorphic region of major histocompatibility complex (MHC) class II molecules expressed on antigen-presenting cells. These events are associated with activation of a nonreceptor Src-like protein tyrosine kinase p56lk, associated with CD4 via its cytoplasmic tail, which couples CD4 to cellular signaling (Fig. 1). CD4 is required for the normal ontogeny of the regulatory helper subset. In mature T cells, CD4 is involved in T-cell activation. In addition, under some experimental conditions, CD4 is involved in specifying programmed cell death and/or G1 block in the cell cycle progression of CD4+ T cells.

As a first step to address the interaction between Nef and CD4-p56lk complexes, we defined the region in CD4 that is required for down-modulation by Nef. We constructed a set of mutant CD4 proteins that were subsequently expressed in the 171.22 T-cell hybridoma that does not express the endogenous CD4 genes. These derivative cell lines were transduced with NA13 nef or with a control vector, and CD4 antigen expression on the cell surface was analyzed by flow cytometry. A summary from this analysis is shown in Figure 1. Results from initial experiments with wild-type and mutant CD4 proteins, where the cytoplasmic tail was either deleted or replaced with that of the CD8 transmembrane protein which is not responsive to Nef, indicated that down-modulation by Nef requires the intact cytoplasmic domain of CD4. Subsequent experiments with a set of carboxy-terminal deletions in the cytoplasmic tail of CD4 indicated that the last 15 amino acids of the cytoplasmic tail of CD4, including C420 and C422, which that are essential for CD4-p56lk association, had no detectable effect. In contrast, more extensive truncations past T419 almost completely abolished Nef-induced CD4 down-modulation. The observation that the region membrane-proximal to the T419 was criti-
FIGURE 1 CD4 down-modulation by HIV-1 Nef required a membrane proximal region in the CD4 cytoplasmic tail. Compilation of results from flow cytometry analysis of mutant CD4 proteins. Amino acid sequences of the cytoplasmic tails of mutant CD4 proteins are aligned on the left with that of the wild-type human CD4. Dots indicate amino acid identities with the wild-type protein, dashes indicate the extent of internal deletions, and letters identify amino acid substitutions in a single-letter code. Following staining with Leu3A α-CD4 monoclonal antibody, the fluorescence of cells expressing various CD4 proteins was 50-60 channels (on the 256-channel logarithmic scale) higher than that of the parental 171.22 cells that do not express CD4. The extent of change in CD4 expression following treatment of cells with phorbol ester (PMA), or following transduction with HIV-1 NA13 Nef expression vector (Nef), is indicated by (+++), (++), (+), (+/-), and (-), which reflects a decrease in the fluorescence intensity of 35-55, 20-35, 10-20, 5-10, and less than 5 channels, respectively.

NEF DISRUPTS CD4 ASSOCIATION WITH THE P561ck PROTEIN TYROSINE KINASE

In T cells, CD4 is associated with the p561ck protein tyrosine kinase via its cytoplasmic tail, and this interaction is required for signal transduction via CD4. The region required for Nef-induced modulation (positions 407 through 418) is in a close proximity to cysteines 420 and 422, which are required for CD4 association with p561ck. Therefore, it seemed plausible that Nef may interfere with CD4 association with p561ck. The ability of p561ck to remain in a complex with CD4 in the presence of Nef was analyzed by immunoprecipitating CD4 from detergent lysates prepared from 171.22 cells coexpressing human CD4 and NA13 Nef, and the immune complexes were immunoblotted with the p561ck antiserum for determination of CD4-associated p561ck and, as a control, with CD4 antiserum. Two additional sets of controls were used in these experiments: (1) CD4 immune complexes were prepared directly from the parental cells expressing the respective CD4 proteins but not Nef and (2) CD4 immune complexes were also isolated from both the parental and Nef-expressing cells following treatment with phorbol ester, which induces CD4 endocytosis and disrupts CD4-p561ck association.

As shown in Figure 2, coexpression of wild-type CD4 and NA13 Nef resulted in an approximately two- to threefold lower steady-state level of total CD4 (compare lanes 8 and 10 with lanes 1 to 6, lower panel). This decrease most likely reflects the previously observed increased rate of the CD4 turnover and lysosomal degradation induced by functional HIV-1 and SIV nef alleles. The CD4 detected by immunoblot analysis must reflect the intracellular pool, because no detectable staining with anti-CD4 antibody was detected by flow cytometry analysis of Nef-expressing cells. A similar reduction of the steady-state level of CD4 was also observed following treatment of cells with phorbol ester (Fig. 2, lane 9). Immunoblot analysis with p561ck antiserum revealed an approximately 20-fold decrease in CD4-
FIGURE 2 Effect of HIV-1 NA13 Nef or phorbol ester on the association of p56\textsuperscript{Ick} with CD4 protein in 171.22 T cells. 171 cells expressing the wild-type human CD4 (lanes 8, 9) and from a derivative population transduced with HIV-1 nef (lanes 10, 11) were incubated for 1 hr in the presence (lanes 9, 11) or absence (lanes 8, 10) of 50 ng/ml PMA. For each experiment, lysates were prepared from 2 x 10\textsuperscript{7} cells and immunoprecipitated with OKT4 α-CD4 monoclonal antibody. Western blots of immune complexes were probed with antiserum specific for p56\textsuperscript{Ick} (α-Ick) or human CD4 (α-CD4). Twofold serial dilutions of protein extracts prepared from COS7 cells transiently expressing p56\textsuperscript{Ick} or human CD4 were used as standards for quantitation, respectively (lanes 1–6). Aliquots of an extract prepared from COS7 cells that were not transfected were used as a negative control for specificity of immunoblot analysis (lane 7).

associated p56\textsuperscript{Ick} in Nef-expressing or phorbol-ester-treated cells (compare lanes 8, 9, and 10 with lanes 1 to 6, lower panel). Thus, the intracellular pool of CD4 is by and large not complexed with p56\textsuperscript{Ick}.

Additional experiments have shown that disruption of p56\textsuperscript{Ick} complexes by Nef does not involve activa-

MAMMALLAN CELL GENETICS

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Our laboratory has been involved in three distinct activities: studying aspects of signal transduction; developing methods for the discovery of small molecules with desirable chemical properties; and applying a new system for genomic analysis for the discovery of genetic lesions in human cancers. This year has witnessed important progress emerging from four breakthroughs that were described in last year’s Annual Report: the discovery of RAS targets; the definition of a conserved protein kinase signal transduction cascade; the development of a molecular indexing system for complex combinatorial chemical synthesis; and the invention of a powerful method for comparative genomic analysis. We have used heavily the
two-hybrid genetic system developed by Fields and Song (Nature 340: 245 [1989]) that allows the detection of complex formation between any two proteins expressed in the yeast Saccharomyces cerevisiae.

RAS in Yeasts

R. Ballester, H.-P. Xu, E. Chang, L. Van Aelst, S. Marcus, V. Jung, K. Sen, A. Polverino, M. Barr

RAS homologs in the budding yeast, S. cerevisiae, and the evolutionary distant fission yeast, Schizosaccharomyces pombe, have highly diverged functions. In the budding yeast, RAS proteins are essential for growth. They regulate adenyl cyclase and probably another as yet unidentified target. In the fission yeast, ras1 is required for sexual differentiation and pheromone-inducible responses as well as an elongated cell shape. As we previously described, one of the target effectors for ras1 in S. pombe is the byr2 protein kinase required for mediating pheromone responses. Complex formation between RAS and byr2 was detected using the two-hybrid genetic systems of Fields and Song (Van Aelst et al. 1993). Mutations in the effector loop of RAS abrogate this interaction. The byr2 protein kinase is part of the conserved MAP kinase module, described in last year’s Annual Report and in more detail below. Additional regulators of byr2 have been found (see later section).

byr2 does not mediate the effects of ras1 on cell morphology (Wang et al., Mol. Cell. Biol. 11: 3554 [1991]), and hence ras1 must have additional mediators. Mutant hunts unearthed two, scd1 and scd2, both required for elongated cell shape and conjugation. scd1 and scd2 are homologs of S. cerevisiae CDC24 and BEM1, respectively, each involved in morphogenic events in that organism, as is RSR1, a RAS homolog (Miyamoto et al., Gene 54: 125 [1987]; Bender and Pringle, Proc. Natl. Acad. Sci. 86: 9976 [1989]; Miyamoto et al., Biochem. Biophys. Res. Commun. 181: 604 [1991]; Chenevert et al., Nature 356: 77 [1992]). In the budding yeast, the CDC42 protein, a member of the RHO family of small GTPases, is also functionally linked with CDC24 (Bender and Pringle, Proc. Natl. Acad. Sci. 86: 9976 [1989]; Zheng et al., J. Biol. Chem. 269: 2369 [1994]). Likewise, we found that the S. pombe CDC42 homolog, cdc42sp, is functionally linked with ras1, scd1, and scd2. Genetic studies led to the conclusion that ras1 and scd2 act in concert upon scd1, which interacts with cdc42sp. Biochemical studies indicate that scd1 and scd2 interact directly. Studies with the two-hybrid system confirm this and further indicate that scd1, scd2, cdc42sp, and ras1 can all form a complex: scd2 binds cdc42sp; ras1, in its GTP-bound state, induces scd1 to bind cdc42sp; and scd2 induces scd1 to bind ras1. It seems likely that the morphogenic module we have described in fission yeast will have been conserved in evolution, perhaps even in metazoans.

We have continued our studies of the new interfering mutants of RAS found in S. pombe, first mentioned in last year’s Annual Report. In collaboration with Dan Broek’s group at the University of Southern California, we have confirmed that these mutants (ras1Tyr62 and HRASTyr57) block nucleotide exchange function by binding virtually irreversibly to exchange factors of the CDC25 type. ras1Tyr62 blocks all ras1 function, beyond that dependent on the ste6 exchange factor, which is required for conjugation but not cell shape (Jung et al. 1994). We therefore infer that ras1Tyr62 blocks other regulatory molecules involved in morphogenesis. One candidate protein has been identified through two hybrid interactions and is now being characterized genetically.

RAS in Mammalian Cells

L. Van Aelst, M. White, A. Polverino

As reported last year, we detected complex formation between RAS and RAF in the yeast two-hybrid genetic system (Van Aelst et al. 1993). RAF is itself an oncoprotein (Rapp et al., Proc. Natl. Acad. Sci. 80: 4128 [1983]), a protein kinase capable of activating MAP kinase through a MAP kinase kinase (Dent et al., Science 257: 1404 [1992]; Howe et al., Cell 71: 335 [1992]; Kyriakis et al., Nature 358: 417 [1992]), and able to bypass dominant negative mutants of RAS to transform mammalian cells (Bonner et al., Nucleic Acids Res. 14: 1009 [1986]; Cia et al., Mol. Cell. Biol. 10: 5314 [1990]). Genetic evidence for the direct interaction of RAS and RAF comes from mutational studies. We first made a mutagenized library of RAS and then screened for mutations of RAS that could not complex with RAF, yet still formed complexes with byr2. One, HRAS Gly37, was chosen for
further study. We reasoned that if RAS and RAF inter-act directly, we could find mutants of RAF that restored interaction with HRAS\textsuperscript{Gly37}. Several mu-tants were found. The compensating mutations in RAF all mapped to the conserved CR2 domain in the RAF regulatory domain. These studies give evidence of direct physical contact between the effector loop of RAS and this domain of RAF.

The mutant HRAS\textsuperscript{Gly37} was activated to an HRAS\textsuperscript{Val12,Gly37} double mutant. The double mutant failed to induce transformed foci in NIH-3T3 cells. However, when the RAFs with mutations in the CR2 domain were cotransfected with HRAS\textsuperscript{Val12,Gly37}, transformed foci were readily observed. The mutant RAFs were unable to induce foci on their own but synergized with the mutant HRAS\textsuperscript{Gly37} greater than tenfold better than did wild-type RAF. These are the first studies to show that interaction between RAS and RAF is necessary for transformation.

Evidence has been presented in previous Annual Reports, and in this one as well, that RAS has multiple target effectors in yeasts. We have long speculated, therefore, that RAS would have multiple target effectors in mammalian cells. To test this idea, a second type of mutant RAS was sought, one that bound RAF but failed to bind byr2. We reasoned that such a mutant might fail to bind other effectors essential for RAS transformation of mammalian cells. Such a mutant, HRAS\textsuperscript{Ser35}, was found. As expected from its failure to interact with byr2 in the two-hybrid assay, HRAS\textsuperscript{Ser35} fails to replace ras1 function in \textit{S. pombe}. This in itself is rather astonishing since serine is a conservative substitution for threonine, the wild-type residue at position 35. Gratifyingly, HRAS\textsuperscript{Val12, Ser35} fails to transform NIH-3T3 cells but efficiently induces transformed foci when cotransfected with HRAS\textsuperscript{Val12,Gly37}. This result argues very strongly that HRAS transforms cells by interacting with two different target effectors.

Using the two-hybrid system again, we have screened mammalian cDNAs for those encoding proteins capable of interacting with RAS. Among those so found were cDNAs encoding members of the RAF family of protein kinases, a guanine nucleotide exchange protein for RAL (Albright et al., \textit{EMBO J.} 12: 339 [1993]), and a number of other as yet unidentified cDNAs. We should be able to determine which of these candidates are actually required for the transforming potential of RAS by utilizing the type of approach we have already taken for RAF.

Regulation of the Conserved MAP Kinase Module

H.-P. Xu, L. Van Aelst, S. Marcus, A. Polverino, M. Barr

We described in last year's Annual Report the realization that a cascade of protein kinases, which we call the MAP kinase module, was conserved in evolution. The first examples come from the pheromone signaling pathways in yeasts: in \textit{S. cerevisiae}, STE11, STE7, and FUS3; in \textit{S. pombe}, byr2, byr1, and spk1 (Neiman et al. 1993). These protein kinases are now known to have homologs in vertebrates: FUS3 and spk1 are homologous to the mitogen-activated protein kinases (MAPKs or ERKs); STE7 and byr1, to the MAP kinase kinases (MEKs); and STE11 and byr2, to MEK kinases (MEKKs) (Neiman et al. 1993). In addition, this kinase cascade motif is repeated multiple times within other signaling pathways within yeasts (Errede and Leven, \textit{Curr. Opin. Cell Biol.} 5: 254 [1993]), and one has to presume that this module is a "good approximate solution" to some recurring problem of adaption. Our interest in this cascade began when we and other investigators discovered evidence for the involvement of RAS in its regulation in \textit{S. pombe} and in mammalian cells (see Nieman et al. 1993; Van Aelst et al. 1993). We have been interested in the control and integration of the MAP kinase module, both in \textit{S. pombe} and in \textit{S. cerevisiae} (even though, in the latter, RAS does not appear to be involved).

byr2 and byr1 are required for the basal level expression of the pheromone receptor gene mam2 (Xu et al. 1994). Interestingly, spk1 is not required for this, either because the kinase cascade branches prior to activation of spk1 or because spk1 has redundant kinase function. The latter is a distinct possibility, given that multiple genes encoding MAP kinase homologs are found in other organisms, but spk1 is essential for many aspects of sexual differentiation. We conclude that it is probable that byr1 has functions other than the activation of spk1.

The \textit{S. pombe} kinase module appears to require both ras1 and the pheromone sensing apparatus (Neiman et al. 1993). Disruption of ras1 or gpa1, encoding the \(\alpha\) subunit of the heterotrimer mediating receptor occupancy, abolishes basal level expression of mam2 in \(h^{90}\) (homothallic) \textit{S. pombe}. Epistatic studies indicate that ras1 and gpa1 act in concert upon mam2 expression. High-level expression of gpa1 can induce mam2 even in the absence of ras1;
yet disruption of ras1 produces a depression of mam2 expression even in the absence of gpal in heterothallic S. pombe strains. From these results, we infer that the MAP kinase module is under independent but cooperative regulation by ras1 and the upstream mating pheromone response pathway (Xu et al. 1994).

We have identified a new component of the S. pombe MAP kinase module: ste4. ste4 was found in a two-hybrid screen of a library of S. pombe cDNAs encoding proteins capable of binding to byr2. It was previously known that ste4 was essential for conjugation and encoded a leucine zipper protein (Okazaki et al., Nucleic Acids Res. 19: 7043 [1991]). Using the two-hybrid system, we demonstrated that ste4 indeed forms dimers, binds to the regulatory domain of byr2, and causes byr2 to dimerize. Hence, we propose that ste4 has a role in activating byr2 by inducing autophosphorylation through dimerization. We suspect that a ste4 homolog may exist in S. cerevisiae, since we find that STE11 dimerizes in that organism. Although ras1 does not bind directly to ste4, overexpression of the byr2 regulatory domain induces complex formation between ras1 and ste4 detectable with the two-hybrid system. Hence, we are investigating whether this binding is cooperative. If so, this may partly explain the regulatory function of ras1.

One of the proteins of S. cerevisiae that participates in pheromone signaling is STE5. Genetic studies have placed STE5 upstream of the MAP kinase module (Hasson et al., Mol. Cell. Biol. 14: 1054 [1994]). Using the two-hybrid system, we demonstrated that STE5 binds all three of the protein kinases that comprise the MAP kinase module, STE11, STE7, and FUS3. The binding between STE11 and STE7 is not apparent unless STE5 is present. Hence, we have proposed that STE5 is a scaffolding protein that promotes interactions between the members of the cascade. In addition to possibly activating the pathway thereby, STE5 may serve to "insulate" the pathway from cross talk with other MAP kinase modules within the same cell that are dedicated to other signal transduction functions. We feel it likely that scaffolding proteins like STE5 may exist for other MAP kinase cascades given that we cannot detect interactions between MEKK and MEK and between byr2 and byr1 with the two-hybrid system.

Finally, we have found that the S. cerevisiae STE20 protein kinase (Leberer et al., EMBO J. 11: 4815 [1993]) may be a conserved element of the MAP kinase cascade. STE20 functions weakly in S. pombe as a suppressor of ras1null cells. Moreover, STE20 protein can induce the phosphorylation of MAP kinase in Xenopus oocyte cell-free extracts. We therefore sought homologs of STE20 in S. pombe and mammalian cells. In collaboration with Dr. M. Cobb's laboratory at the University of Texas Southwestern Medical School, we identified several clear STE20 homologs in both fission yeast and mammalian cells. Their properties are currently under investigation.

Combinatorial Chemistry

R. Swanson

As we began to describe in last year's Annual Report, we have collaborated with Clark Still and Michael Ohlmeyer of the Department of Chemistry at Columbia University to develop a general method for the molecular tagging of solid-phase synthesis beads used in "split" synthesis to generate chemical libraries of great complexity. In split synthesis, solid-phase synthesis beads are used as substrates to build molecules by iterative synthetic steps (Furka et al., Int. J. Pept. Protein Res. 37: 487 [1991]; Lam et al., Nature 354: 82 [1991]). By consecutive batching and segregating into different reaction vessels, huge libraries of diverse compounds are created, with each bead bearing a single predominant moiety according to its particular reaction history. We have devised a method of using tags to record the reaction history of each bead (Ohlmeyer et al. 1993). After beads containing a desirable moiety are identified, the composition of the moiety is inferred by reading the reaction history of the bead encoded by the tags. The tags are read, after detachment from the bead, by gas chromatography and electron capture. We have utilized this system to tag a combinatorial library of oligopeptides of greater than 10^5 complexity and demonstrated the correct identification of peptide epitopes reactive with a monoclonal antibody. Many conserved amino acid substitutions were allowed within the epitope, but to our surprise, we found that certain very conservative substitutions reduced the binding of the epitope to the antibody by several orders of magnitude. The tagging method is perfectly general and can be applied to libraries of organic molecules created on beads by virtually any synthetic
route. In this vein, Dr. Still and co-workers have used the method to tag libraries of nonlinear peptidic moieties containing structures that would be difficult to elucidate by other means (Borchardt and Still, J. Am. Chem. Soc. 116: 373 [1994]). In collaboration with other investigators, we are testing the application of this method for the discovery of chemical agonists and antagonists of components of signal transduction pathways.

Comparative Genomic Analysis

N. Lisitsyn, H.-P. Xu

We have developed a method for genomic difference analysis that we call representational difference analysis, or RDA, and are applying the method to clone probes that define genetic lesions occurring in cancer cells or are linked to inherited disease loci. This method allows the detection and cloning of specific DNA sequences ("difference" sequences) present in one population of DNA molecules ("tester") that are not equivalently present in a highly related second population of DNA molecules ("driver"). The RDA procedure is composed of two parts. In the first part, we make a reductive sampling of the two DNA populations that are to be compared, thereby deriving two new populations of DNA molecules, called representations. The sequence complexity of the representation is considerably lower than that of the DNA population from which it derived. In the second part, we utilize "sequence subtraction" and "kinetic enrichment" to clone the difference sequences. A detailed description of this powerful method was presented in last year's Annual Report and recently published in Science (Lisitsyn et al. 1993). At its core, our method detects small restriction fragments in one DNA population that are not present in another.

The method can be used to find probes linked to inherited disease in animals that can be bred (Lisitsyn et al. 1994). In collaboration with Eric Lander's group at MIT, we designed two protocols that exploit the ability of RDA to detect polymorphic differences between individuals or groups of individuals. The first protocol requires the use of congenic strains, in which a strain carrying an inherited disease is repeatedly back-crossed into a polymorphic strain. The second protocol, which is more general, merely requires the generation of F2 progeny from the cross of a disease-carrying strain to one that is disease-free. It is possible to generate thereby polymorphic probes linked to the disease locus for any experimental animal that can be bred. Preexisting genetic maps are not needed. Similar strategies may be applicable to humans.

The method can be applied to define the lesions in cancer cells in either of two ways. Using DNA from normal cells as tester and DNA from cancer cells as driver, we can isolate probes that are homozygously lost in cancers or that detect restriction endonuclease polymorphisms that are heterozygously lost in cancers. To be applied in this way, the cancer and normal must be from the same individual and the cancer DNA (driver) must be nearly free of contaminating normal DNA (tester). As starting material, we have used paired normal and cancer cell lines, derived in the main from renal cell carcinomas or colon cancers. We have also successfully implemented the protocol on cancer biopsies using nuclei fractionated by flow cytometry into aneuploid (cancer) and diploid (normal) fractions. In this manner, we have identified seven loci that undergo homozygous deletion in at least one cancer sample. Two of these loci were observed to undergo very frequent loss. Presumably, these are the sites of genes the loss of which contribute to the initiation or progression of cancer.

When cancer DNA is taken as tester, probes arising from several types of lesions can, in principle, be detected: point mutations, genetic rearrangements, gene amplifications, and pathogens. In practice, we have observed point mutations and amplifications. We can calculate that on the order of 1/5000 point mutations should create a detectable tester fragment. The presence of such fragments may indicate an increased frequency of point mutation in the cancer. The application of RDA to the detection of amplifications may be of substantial value, since amplification is a useful prognostic indicator for the disease. We have not yet documented examples of RDA probes that detect the other types of lesions.

PUBLICATIONS


In Press


**TRANSMEMBRANE SIGNALING**

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R. Buccafusca  
K. Degenhardt  
N. Gale  
L. Graziaidei  
S. Kaplan  
T. Pullerwitz  
S.-S. Yang

Our work continues to focus on the mechanisms of signal transduction by Ras proteins. Ras proteins are members of a large superfamily of low-molecular-weight proteins that bind guanine nucleotides. They are essential for the transduction of diverse extracellular signals that control cell growth, and abnormal activation of Ras proteins is thought to contribute to the development of several types of human cancer. Signal transduction through Ras proteins is tightly controlled by several cellular proteins whose function is to up-regulate or down-regulate Ras activity. Much of our effort during the past year has been directed at the functional characterization of these proteins. Our findings provide the first molecular description of the pathway by which cell-surface receptors activate Ras proteins. We have shown that the critical components of this pathway include the small adaptor protein GRB2 and the Ras guanine nucleotide exchange factor Sos. In addition to the mechanisms involved in the regulation of Ras activity, we are interested in understanding how Ras proteins interact with target molecules to induce a cellular response. Our work on this subject involved the biochemical characterization of the interaction between Ras and the serine/threonine kinase Raf. During this year, Nick Gale completed his Ph.D. re-
search, and Roberto Buccafusca and Todd Pulerwitz worked for three productive months as summer students.

### Activation of Ras by Receptor Tyrosine Kinases

N. Gale, T. Pulerwitz

It is well established that Ras proteins are critical intermediates in the signaling pathways initiated by receptor tyrosine kinases. The role of Ras proteins in transducing signals from receptor tyrosine kinases has been highly conserved: In *Drosophila*, activation of the *sevenless* receptor tyrosine kinase is required for the proper specification of R7 in the *Drosophila* eye, and activation of Ras is a crucial early event in this signaling pathway. Similarly, in *Caenorhabditis elegans*, it has been shown that a receptor tyrosine kinase encoded by the *let-23* gene and a Ras protein encoded by the *let-60* gene are required for the induction of vulval cell fate. Biochemical data from mammalian cells have shown that stimulation of receptor tyrosine kinases leads to an increase in the fraction of active GTP-bound Ras molecules. Work carried out in several laboratories including our own has indicated that stimulation of guanine nucleotide exchange is the predominant mechanism by which receptor tyrosine kinases control Ras activation. This suggested the presence of a coupling mechanism between receptor tyrosine kinases and a guanine nucleotide exchange factor for Ras. Until recently, identity of the guanine nucleotide exchange factor for Ras was unknown. Genetic studies in *Drosophila* have indicated that the protein encoded by the *Son of sevenless* gene (*Sos*) functions as a guanine nucleotide exchange factor for Ras.

Homologs of *Sos* have been identified in mammalian cells, and last year, we demonstrated by both genetic and biochemical techniques that the mammalian Sos protein is a specific guanine nucleotide exchange factor for Ras. The crucial insight into the mechanism by which receptor tyrosine kinases may communicate with Sos came from the identification of a similar proline-rich motif in a protein termed 3BP-1. In 3BP-1, the proline-rich motif was shown to constitute the binding site for the SH3 domain of c-Abl. This observation, combined with our earlier findings concerning the role of the SH2 and SH3 domain-containing protein GRB2 in linking receptor tyrosine kinases to the Ras pathway, prompted us to investigate the possibility that the proline-rich sequences in the carboxy-terminal of Sos mediates its interaction with the SH3 domain of GRB2. Using the coimmunoprecipitation approach, we have demonstrated a physical interaction between Sos and GRB2. This interaction was detected both in resting cells and in growth-factor-stimulated cells. We utilized the two-hybrid system to characterize this interaction further and to show that the Sos-GRB2 association is mediated by the carboxy-terminal domain of Sos and the SH3 domains of GRB2. Upon growth factor stimulation, the GRB2-Sos complex has been shown to bind to the activated growth factor receptor. This interaction has been shown to be mediated by the direct binding of the SH2 domain of GRB2 to a specific phosphotyrosine residue on the activated receptor. However, we detected no changes in the guanine nucleotide exchange activity of Sos after epidermal growth factor (EGF) stimulation. On the basis of these observations, we have proposed the following mechanism for the coupling of receptor tyrosine kinases to Ras activation (Fig. 1): Receptor activation induces the

![FIGURE 1 Transduction of signals from receptor tyrosine kinases to Ras. GRB2 binds to the activated receptors via the SH2 domain and to Sos via the SH3 domain. Thus, Sos is recruited to the activated receptor in the plasma membrane where Ras activation takes place. Activated Ras triggers a cascade of serine-threonine kinases that send signals to the nucleus.](image-url)
translocation of a constitutive complex between GRB2 and Sos to the activated receptor.

This translocation could link receptor activation to an increase in the guanine nucleotide exchange on Ras by increasing the local concentration of the exchange factor in the plasma membrane where Ras is located.

Regulation of the Sos by Phosphorylation

K. Degenhardt, N. Gale, R. Buccafusca

Upon growth factor stimulation, the Ras guanine nucleotide exchange factor Sos becomes phosphorylated on serine and threonine. To understand the functional significance of this phosphorylation event, we sought to identify the kinase responsible for this phosphorylation event. The carboxy-terminal region of Sos contains 16 potential MAP kinase sites. Therefore, we have investigated the possibility that MAP kinase is involved in the phosphorylation of Sos. To this end, we initially tested the ability of MAP kinase to phosphorylate Sos polypeptides in vitro using Sos immunoprecipitates from serum-starved cells or from growth-factor-stimulated cells. We reasoned that if growth-factor-induced phosphorylation of Sos occurs at MAP kinase sites, then Sos immunoprecipitated from growth-factor-stimulated cells would be a poor substrate for MAP kinase relative to Sos derived from serum-starved cells. Indeed, we found that recombinant MAP kinase efficiently phosphorylated Sos from serum-starved cells. In contrast, Sos derived from growth-factor-stimulated cells was a poor substrate for MAP kinase in vitro. These results are consistent with the idea that Sos is phosphorylated by MAP kinase in response to growth factor stimulation (Fig. 2). To prove directly the role of MAP kinase in Sos phosphorylation, we compared the patterns obtained from tryptic phosphopeptide mapping of Sos phosphorylated by growth factor in vivo or by MAP kinase in vitro. We have found that the growth factor stimulation results in the appearance of five major phosphorylated species, four of which correspond to phosphopeptides generated by MAP kinase phosphorylation of Sos in vitro (Fig. 2). We have determined that all of the MAP kinase sites are contained within the carboxy-terminal domain of Sos and studies are under way to identify the exact sites of MAP kinase phosphorylation. On the basis of these analyses, we plan to generate Sos mutants lacking the MAP-kinase phosphorylation sites. These mutants should enable us to investigate the functional significance of Sos phosphorylation. Specifically, it will be interesting to examine whether Sos phosphorylation has a role in regulating Sos catalytic activity, the interaction with GRB2, and Sos subcellular localization.

FIGURE 2 Phosphorylation of Sos by MAP kinase. (A) Phosphorylation in vivo was determined using Sos immunoprecipitates isolated from serum-starved or EGF-stimulated 32P-labeled cells. For the in vitro phosphorylation, Sos was immunoprecipitated from unlabeled serum-starved or EGF-stimulated cells, and the immunoprecipitats were incubated with purified recombinant MAP kinase in the presence of [γ-32P]ATP for 30 min. Mock reactions (minus) were performed under the same conditions but without the kinase. (B-D) Tryptic phosphopeptide maps of Sos after phosphorylation in vivo (B and C) and in vitro (D).
Functional Differences among Sos Proteins
S.-S. Yang

Mammalian cells contain two related but distinct Sos genes designated Sos1 and Sos2. It has been shown that these genes are widely expressed during development and in adult tissues, consistent with their role as positive regulators of the ubiquitously expressed ras genes. Alignment of the two Sos genes shows that they share a high degree of similarity (65% overall amino acid identity). However, at their carboxy-terminal ends, the homology between Sos1 and Sos2 is scattered and the conserved regions are mostly restricted to the proline-rich motifs. Since the carboxy-terminal region has been implicated in the regulation of Sos function, it is enticing to speculate that Sos1 functions in a regulatory capacity different from that of Sos2.

Using the two hybrid system, we have found that the interaction between GRB2 and the carboxy-terminal region of Sos2 is significantly tighter than that observed for GRB2 and the carboxy-terminal region of Sos1. Moreover, we have found that the carboxy-terminal tail of Sos1 interacts with Nck, an SH2- and SH3-domain containing protein implicated in mitogenic signaling via the EGF and platelet-derived growth factor (PDGF) receptors. These results suggest qualitative and/or quantitative differences between Sos1 and Sos2 in terms of their interactions with SH3 domain-containing proteins. The physiological significance of these differences is currently being investigated.

Regulation of Ras-Raf Interactions
S. Kaplan [in collaboration with T. Polverino and M. Wigler, Cold Spring Harbor Laboratory]

The stimulation of cell growth and differentiation by a variety of extracellular signals frequently involves the Ras-dependent activation of the mitogen-activated protein kinase (MAPK) cascade (Fig. 1). Moreover, it has been recently shown, using a combination

![Figure 3](image-url)
of biochemical and genetic techniques, that this activation is probably mediated through the direct interaction of Ras with the MAP protein kinase kinase kinase Raf. To characterize further the interactions between Ras and Raf, we have established an in-vitro-binding assay that is based on the ability of Raf proteins present in cell extracts to interact with immobilized GST-Ras fusion protein. The amount of Ras-bound Raf is determined by immunoblotting using specific anti-Raf antibodies. In agreement with earlier studies, the binding of Raf to Ras in our assay system required the Ras proteins to be in the active GTP-bound form. We have found that agonist-induced activation of Raf results in the significant reduction in its binding affinity for Ras (Fig. 3). In addition, Raf proteins display a dramatically reduced affinity for the oncogenic form of Ras protein (Ras$^\text{val12}$) in comparison with the normal form of the protein (Ras$^\text{gly12}$).

Finally, we have observed that treatment of cells with agents that increase the intracellular concentration of cAMP results in the inhibition of Raf activation and a concomitant reduction in the affinity of Ras for Raf. These observations suggest that a reduction in the affinity of Raf for Ras may constitute a negative feedback mechanism to prevent the overstimulation of the Ras pathway in the presence of agonists. Additionally, the modulation of the affinity of Raf for Ras may provide a mechanism by which Ras or Raf would be able to interact with other target molecules. The identity of these molecules is currently being investigated.

Identification of Exploitable Phenotypic Changes Associated with Abnormalities in the NF1 Gene in B Lymphocytes

M. Boyer [in collaboration with D. Gutmann, Washington University, St. Louis]

von Recklinghausen neurofibromatosis type 1 (NF1) is a common autosomal dominant disorder affecting approximately 1 in 3500 individuals. The NF1 locus has been identified, and all of the mutations identified so far within the NF1 gene are consistent with an inactivation mechanism, indicating that NF1 may function as a tumor suppressor gene. The protein product of the NF1 gene, neurofibromin, was shown to share a region of homology with negative regulators of Ras (GAPs). Moreover, it has been shown that the GAP-related domain of NF1 can function as a Ras-GAP, suggesting that neurofibromin may function as a tumor suppressor gene by virtue of its ability to modulate Ras negatively. We have been studying the role of neurofibromin in B lymphocytes. In these cells, surface immunoglobulin receptors (sIg) are rapidly redistributed following exposure to antibody or other multivalent ligands. This receptor redistribution event, termed sIg capping, initiates signals that lead to the activation of a B cell. Previously, we had demonstrated that Ras proteins co-cap with sIg in B lymphocytes. More recently, we have found that neurofibromin also co-caps with sIg. If the redistribution of neurofibromin is functionally significant, then a testable prediction can be made that cellular events linked to sIg redistribution will be affected by abnormalities in the NF1 gene. We are using two model systems to test this prediction: (1) B cells derived from mice heterozygous for targeted mutations in NF1 and (2) Epstein-Barr virus (EBV)-immortalized lymphoblast cell lines derived from NF1 patients. In both systems, we find that the level of expression of neurofibromin is approximately 50% reduced compared to normal cells. We are characterizing the NF1-deficient cells with respect to several Ig-mediated signaling events, including the induction of tyrosine phosphorylation, the kinetics of sIg capping and internalization, and cytoskeletal reorganization. If we find that these cells are quantitatively different from normal cells with respect to any of these aspects, we plan to utilize this difference toward the establishment of a quantitative assay for differentiating between normal and NF1-deficient B cells. This assay will constitute, we hope, the foundation for the development of a diagnostic test for NF1. The clinical manifestations of the NF1 disorder are extremely variable, even within the same family. At present, no approach exists that enables the molecular diagnosis of NF1 in patients who fail to meet the common diagnostic criteria of the disease. Thus, the availability of a diagnostic test that reflects a partial functional loss of neurofibromin would have a profound impact on the ability to diagnose patients with NF1 who lack a family history or in whom diagnostic criteria are not met.
SH3 domains are noncatalytic protein modules of approximately 50 amino acids. We have recently shown that the SH3 domain of PLC\(\gamma\) mediates the localization of this protein to the actin cytoskeleton. We have also shown that the SH3 domains of GRB2 are responsible for its specific localization in membrane ruffles. On the basis of these findings, we suggested that SH3 domain-mediated interactions could have a role in specifying the subcellular location of proteins. To examine the generality of this concept, we have extended our analysis to the role of SH3-mediated interaction in the differential localization of the amiloride-sensitive Na\(^+\) channel in epithelial cells. Earlier studies have demonstrated that this channel is localized at the apical membrane of epithelial cells. This localization is essential for proper channel function in Na\(^+\) transporting epithelia. The molecular mechanisms that determine the selective localization of the channel to the apical membrane have not been defined. The \(\alpha\) subunit of the channel was recently cloned and has been shown to contain two proline-rich sequences resembling the SH3-binding motifs found in Sos and 3BP-1. Biochemical binding studies have indicated that these proline-rich motifs can bind to SH3 domains of several proteins. When a recombinant fusion protein containing the proline-rich sequences was microinjected into polarized epithelial cells, the protein localized exclusively to the apical area of the plasma membrane as determined by confocal microscopy (Fig. 4). In contrast, a recombinant fusion protein corresponding to a region within the \(\alpha\)
subunit that lacks the proline-rich sequences remained diffuse in the cytoplasm when microinjected into these cells (Fig. 4). These results suggest that the SH3-binding region in the α subunit of the Na+ channel is a critical determinant in the apical localization of this channel.

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In Press


NUCLEAR SIGNAL TRANSDUCTION

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Our interest is in how extracellular signals are communicated to the nucleus to control the growth and differentiation of eukaryotic cells. Cells are presented with a complex spectrum of signals, in the form of soluble hormones and growth factors and direct cell-cell and cell-matrix contacts. They must be able to distinguish among these signals and to translate each signal into the pattern of gene expression required to execute the appropriate biological program. Our work focuses on two major aspects of how cells receive these signals, process them, and interpret them into specific patterns of gene expression.

First, we are interested in the mechanics of signal transduction: How are signals passed from cell-surface receptors to nuclear transcription factors? Recent progress in this area has allowed at least two distinct signal transduction pathways to be traced nearly in their entirety from membrane to nucleus. Our current effort focuses on one of these pathways, which leads to the direct tyrosine phosphorylation of a latent cytoplasmic transcription factor.

Second, we are interested in the specificity of these signaling pathways: How do cells know what signal they are receiving and how are they programmed to respond to a signal at the level of transcription? Here, we have gained our major insight from genetic and biochemical studies of proteins of the homedomain family, which we believe function to route signal transduction information to cell-type-specific target genes.

Our principal experimental focus is the proto-oncogene c-fos, which is rapidly and transiently activated at the transcriptional level within minutes of exposure to a spectrum of extracellular signals. c-fos transcription is activated by multiple intracellular signal transduction pathways. In the past, our work has...
focused on identification of the *cis*-acting regulatory elements in the *c-fos* gene that are connected to each of these pathways and on the DNA-binding proteins that interact with these elements. This year, we have concentrated on one of these nuclear signal transduction pathways, a pathway mediated by a family of DNA-binding proteins regulated by direct tyrosine phosphorylation. In addition, we have extended our analysis on the role of homeodomain proteins in targeting these nuclear signal transduction pathways to specific target genes. We have also begun to move our studies into more complex biological systems, including *Drosophila* and mice.

**The Serum Response Element as a Focus for Incoming Signals**

M. Gilman, C. Alexandre, G. Lee, H. Sadowski

The serum response element (SRE) is required for the response of the *c-fos* gene to at least three distinct signal transduction pathways, one dependent on protein kinase C (PKC) and at least two that are independent of PKC. In addition, the SRE is the target for the rapid repression of *c-fos* transcription that follows induction. The SRE is bound by a ubiquitously expressed 67-kD phosphoprotein, serum response factor (SRF). Extensive mutagenesis of the SRE indicates that binding of SRF to the SRE is absolutely required for all functions of the element, but increasingly we have come to realize that SRF binding is not sufficient for many—and perhaps all—of these functions. SRF DNA-binding activity is not detectably influenced by signaling events, nor does the pattern of proteins bound to the SRE in living cells change, suggesting that SRF is always bound at the SRE. Instead, we believe that SRF works principally by recruiting a set of different accessory proteins, each of which connects SRF and the SRE to a distinct signal transduction pathway.

One well-characterized group of SRF accessory proteins is a small family of Ets-domain proteins collectively termed ternary complex factor (TCF). TCF does not display strong binding affinity for either free SRF or SRE DNA. Rather, it specifically recognizes and binds to the SRF-SRE complex. Previously, we found that we could make mutations in the SRE that did not affect the binding of SRF but prevented the recruitment of TCF. These mutant SREs lost the ability to respond to PKC-dependent signals but retained wild-type response to PKC-independent signals. Thus, the PKC pathway targets the ternary complex of SRF and TCF. Indeed, recent work in several laboratories indicates that TCF is a direct physical target for these signals: TCF is phosphorylated by MAP kinase, and this phosphorylation switches on its transcriptional activation potential.

What is the target for the PKC-independent signals that continue to function in the absence of TCF? One target, we believe, is another small family of DNA-binding proteins, collectively designated SIF. These proteins, which we describe in more detail below, become activated at the level of DNA binding within seconds of growth factor treatment and bind to a sequence, the SIE, located approximately 25 bp upstream of the SRE. When mutations are introduced into the SIE, the remaining TCF-independent response of the *c-fos* gene to platelet-derived growth factor (PDGF) is eliminated. Thus, we can distinguish two nuclear signals transmitted by the PDGF receptor, one that reaches *c-fos* via TCF and one that arrives via SIF. Mutations that abolish the binding of SRF interrupt both signals, again suggesting that the functions of TCF and SIF are dependent on the binding of SRF.

When a doubly mutant promoter unable to bind either TCF or SIF is assayed following stimulation with whole serum, a complex cocktail of growth factors and hormones, it retains a reduced ability to respond. This observation suggests the existence of at least one additional pathway dependent on SRF. This signal either targets SRF alone or requires an as yet unidentified accessory factor. Thus, we believe that SRF works principally as a landing pad for other proteins, which are the actual physical targets for distinct signal transduction pathways. The *c-fos* gene, and presumably other immediate early genes, can thus be independently connected to these signal transduction pathways. The ability of each of these proteins to interact at the *c-fos* gene depends on the presence of two elements in the gene: a DNA sequence that is recognized directly by each protein and a DNA-bound SRF molecule. This model has two important ramifications. First, it suggests that the specification of which signal transduction pathways a gene can sense is directly encoded in regulatory DNA. Second, by determining where SRF is allowed to bind, a cell can exert global control over which genes are connected to signal transduction pathways.
Signal transduction to the nucleus by growth factor receptors with protein-tyrosine kinase activity is complex. The initial event in activation of these types of receptors is the cross-linking of receptors by ligand, resulting in the rapid and concerted trans-phosphorylation of the receptors on multiple tyrosine residues. Phosphorylation on tyrosine is absolutely required for receptor signaling. The next step is the recruitment of a series of cytoplasmic and membrane-associated adaptor/signaling proteins to the receptor where they may or may not be phosphorylated on tyrosine. Many of these proteins associate with phosphotyrosine residues on the receptor via protein domains termed SH2 (src homology region-2) domains. Each of these SH2-domain proteins is thought to elicit an independent intracellular signal transduction pathway.

Among these SH2-domain proteins involved in growth factor signal transduction is the family of DNA-binding proteins termed SIF. SIF proteins are members of a larger family, termed signal transducers and activators of transcription (STATs). These proteins undergo rapid phosphorylation on tyrosine in cells treated with a variety of polypeptide growth factors and cytokines. Phosphorylation of the STATs induces protein dimerization, DNA-binding activity, and nuclear translocation.

Using A431 cells as a model system, we have examined the mechanism of SIF activation by epidermal growth factor (EGF). Activation does not involve the well-characterized signal transduction pathways utilizing PKC, calcium, or cAMP but clearly requires tyrosine kinase activity as it is inhibited by cell-permeable tyrosine kinase inhibitors. SIF activation is extremely rapid at 37°C, detectable within 20 seconds of EGF addition, peaking within 4 minutes, and persisting for at least 30 minutes. Three distinct mobility-shift complexes (SIF A, B, and C) rapidly appear in nuclear extracts, whereas only the two more rapidly migrating complexes (SIF B and C) appear in the cytoplasm. When activations are performed at 0°C, where internalization of receptors and nuclear transport of proteins is inhibited, SIF activation is still readily apparent within 30 seconds, with apparent accumulation over time of the two faster-migrating complexes in the cytoplasmic fraction relative to the nuclear fraction. Together, these data suggest that latent SIF exists in the cytoplasm where it is rapidly activated by a receptor proximal event (kinetics just behind receptor tyrosine phosphorylation), after which DNA-binding-competent SIF is translocated to the nucleus.

Since the activation of SIF by EGF in A431 cells is quite robust and a receptor proximal event, we have used fractions from unstimulated A431 cells to develop a cell-free assay for SIF activation. In this assay, we observe time- and ligand-dependent activation of SIF, with a requirement for detergent-treated A431 membranes, cytoplasm (from virtually any cell line), ATP, Mg++, and Mn++. Antibody depletion experiments indicate that activation is absolutely dependent on the EGF receptor. Activation is blocked by antibody to phosphotyrosine, recombinant SH2 domains, and free phosphotyrosine, indicating that at least one phosphotyrosine-SH2 domain interaction is required.

The addition of either phosphotyrosine antibody or a highly purified protein tyrosine phosphatase to nuclear extracts from EGF-treated cells inhibits formation of SIF-DNA complexes. Thus, SIF polypeptides contain phosphotyrosine, and this phosphorylation is required for DNA-binding activity. Using a combination of UV cross-linking, and DNA-affinity precipitations of [35S]methionine-labeled cell extracts, we have detected four polypeptides in the 80–94-kD range that specifically bind to the SIE after treatment of A431 cells with EGF.

Several of the characteristics of SIF, including subunit size, DNA-binding specificity, and mechanism of activation, resemble those of the interferon (IFN)-activated transcription factors, particularly the 91K subunit selectively activated by IFN-γ (Stat1). Using a variety of experimental approaches, we have shown that the IFN-γ-activated factor complex (GAF), which contains only tyrosine phosphorylated Stat1, is identical to SIF-C and phosphorylated on the same tyrosine residue. In contrast, SIF-A does not contain the Stat1 protein, does not form on a GAS probe, and is not activated by IFN-γ. The SIF-A complex contains a distinct SH2-domain protein, Stat3. SIF complex B behaves as a composite of A and C, and it appears to be a heterodimer of Stat1 and Stat3. Stat1 and Stat3 differ in their DNA-binding specificities and in the ligands that activate them. Stat1 is preferentially activated by IFN-γ and binds selective-
ly to regulatory elements in IFN-γ-inducible genes. Stat3, in contrast, is preferentially induced by polypeptide growth factors, such as EGF and PDGF, and by certain cytokines, such as interleukin-6. Stat3 exhibits weak affinity for IFN-γ-inducible sites and binds preferentially to the SIE in the c-fos gene.

Recently, we have focused on the mechanism of SIF activation by polypeptide growth factors. Using a bank of cell lines expressing wild-type and mutant human B-PDGF receptors (constructed by Adam Keshishian and Jonathan Cooper, Fred Hutchinson Cancer Research Center), we are examining which portions of the PDGF receptor are required for SIF activation. At the same time, various phosphotyrosine-containing peptides and SH2 domains from different signaling proteins are being tested as competitive inhibitors in the in vitro activation assay. And, as detailed below, we are also trying to identify the protein tyrosine kinase responsible for phosphorylating the SIF proteins.

Regulation of SIF Activity by Tyrosine Phosphorylation

M.-L. Vignais

The c-sis-inducible element (SIE) of the c-fos promoter is the target for the two STAT proteins, Stat1 and Stat3. These two proteins undergo rapid phosphorylation on a single tyrosine residue when cells are exposed to polypeptide growth factors such as EGF and PDGF. Phosphorylation of Tyr-701 of Stat1 is required for protein dimerization, DNA-binding activity, and translocation of the protein to the nucleus. The SH2 domain of Stat1 is also required for activation. Stat1 and Stat3 can bind DNA as either homodimers or heterodimers, generating the three distinguishable DNA-protein complexes on an SIE probe (SIF A, B, and C) in gel-shift assays. Stat1 and Stat3 proteins are also activated by a number of cytokines, whose receptors, unlike those of EGF and PDGF, do not harbor intrinsic protein tyrosine kinase activity. Signaling by these receptors relies on non-covalently associated protein tyrosine kinases of the JAK family.

We are interested in establishing the mechanism of activation of Stat1 and Stat3 proteins by growth factor receptors. Three different models could account for how the STAT proteins become tyrosine phosphorylated. First, they could be recruited to the activated growth factor receptor via their SH2 domains and directly phosphorylated by the receptor kinase. Second, the STAT proteins could be phosphorylated by the same JAK kinases that apparently mediate their activation by cytokine receptors. Third, a distinct class of protein tyrosine kinases, such as the Src family, could be involved.

We have begun to address these questions in two ways. First, we have shown that Stat1 protein-produced reticulocyte lysate from a cDNA clone can be activated in vitro by EGF in the presence of A431 membranes. No cytosolic components beyond those supplied by the reticulocyte lysate are required. We can now exploit this assay to investigate the structural and functional domains of the STAT proteins required for various aspects of their regulation by tyrosine phosphorylation. The next step is to repeat these activation experiments with STAT proteins produced in Escherichia coli. If activation of bacterially produced protein can be achieved, then we will use conventional biochemical approaches, such as fractionation and depletion, to identify the membrane and/or cytosolic components required for STAT activation. The ultimate goal of these experiments is to reconstitute STAT activation with purified proteins.

Our second approach is genetic. We have obtained a number of cell lines that lack individual protein tyrosine kinases of the JAK and Src families and are testing these cell lines for the ability to support SIF activation by PDGF. In some cases, this has required the transfer of functional PDGF receptors into the appropriate cell lines. Using these cell lines, we will determine if any of these protein tyrosine kinases are required for STAT activation by PDGF in vivo.

Dual Modes of Control of c-fos Transcription by Calcium in T Cells

G. Lee

Many extracellular stimuli elevate intracellular calcium, and calcium ions can be potent second messengers. Cytoplasmic calcium pulses are particularly important in T lymphocytes, where they mediate one component of the activation signal triggered by the antigen receptor. A second signal activated by the antigen receptor results in the activation of protein kinase C (PKC). Neither signal is sufficient for T-cell activation, but together, activators of PKC and calcium ionophores cooperate synergistically to elicit
activation. This synergy between PKC activators and calcium ionophores is also evident at the level of c-fos mRNA induction. Neither stimulus alone significantly elevates c-fos mRNA, but both together yield significant c-fos induction.

We have investigated the mechanisms by which calcium interacts synergistically with PKC activators to induce c-fos mRNA. Induction of c-fos transcription by phorbol ester requires the serum response element (SRE). Mutagenesis of the SRE indicates that, as we have observed previously in fibroblasts, induction by phorbol ester requires the ternary complex of SRF and TCF. Calcium cooperates with phorbol ester in two distinct ways. First, calcium induces the activity of the c-fos promoter, as measured in reporter gene assays and by nuclear run-on analysis of endogenous c-fos transcription. Induction of promoter activity by calcium requires a conserved AP-1/CRE-like element located immediately 3' to the SRE. Second, calcium greatly enhances the efficiency with which nascent c-fos transcripts are elongated to completion. In the absence of a calcium signal, transcripts initiated at the c-fos promoter in response to phorbol ester appear to terminate near the boundary of the first exon and intron. In nuclear run-on assays, little transcription of downstream portions of the gene is observed. In the presence of a calcium signal, however, these transcripts proceed through the gene with greatly enhanced efficiency. The strong synergy between phorbol ester and calcium ionophore thus arises because these agents affect discrete steps in the production of c-fos mRNA: Phorbol ester promotes initiation of transcription, whereas calcium promotes elongation. Only when both signals are present do mature c-fos mRNAs accumulate. Furthermore, the enhanced elongation induced by calcium occurs for a very short period of time following signaling, even while the enhanced initiation of transcription persists. Thus, control of transcript elongation is the principal form of control over both the kinetics and the extent of c-fos mRNA accumulation under these treatment conditions.

Role of YY1 in the Organization of Protein-DNA Complexes on the c-fos Promoter

S. Natesan

YY1 is a zinc-finger-containing protein that can activate or repress transcription depending on the promoter context. YY1 is indistinguishable from a previously characterized SRE-binding protein termed p62DBF. YY1 binds to at least three sites in the mouse c-fos promoter including the SRE and sites positioned near -255 and -55. The -55 site is situated between the TATA box and the cAMP response element (CRE), which is also a major basal element of the promoter. The presence of YY1 sites within or adjacent to these essential promoter elements suggests that YY1 may have an important role in the regulation of c-fos expression. At each of these sites, YY1 binding induces DNA bending. Thus, YY1 has the potential to induce significant three-dimensional structure in the mouse c-fos promoter, suggesting that YY1 may facilitate interactions among proteins bound to different sites in the promoter.

To test this hypothesis, we carried out a series of transfection experiments with a simplified c-fos promoter carrying sequences from -71 to +109 fused to the bacterial CAT gene. These experiments suggest that YY1 binding to the -55 region represses promoter activity but that repression is not an intrinsic property of YY1 in this context, because its ability to repress transcription requires an intact CRE at -65. Repression by YY1 is also alleviated by changing the relative phasing of the factor binding sites on either side of YY1. Moreover, when the orientation of the -55 YY1 site is reversed, YY1 activates the promoter. These data suggest that the principal function of YY1 in this promoter context is to bend DNA to regulate contact between other proteins. In support of this view, we have shown that the product of male sex determination gene SRY encodes a protein that can bind to YY1 sites and induce DNA bending. In transfection assays, SRY can partially mimic the function of YY1. Therefore, YY1, and perhaps SRY as well, belongs to a novel class of transcription factors that affect promoter function by affecting DNA structure, rather than directly contacting the transcriptional machinery.

We are currently investigating the role of YY1 in the regulation of SRE function. Our data suggest that both affinity-purified DBF and bacterially expressed YY1 enhance the binding of SRF to SRE. Enhancement of SRF-binding activity requires the binding of YY1 to SRE. Given that YY1 bends DNA, including the SRE, we imagine that YY1 may enhance the binding of SRF to the SRE by inducing a DNA conformation that is favored by SRF. Furthermore, under certain assay conditions, we can detect stable com-
plexes in which YY1 and SRF co-occupy the SRE. Interestingly, both the enhancement of SRF binding and co-occupancy of the SRE with YY1 appear to require regions outside the DNA-binding and dimerization domain of SRF.

How can YY1 and SRF simultaneously occupy overlapping binding sites? YY1 is a member of the C2H2 class of zinc finger proteins. On the basis of established structures of related proteins, it is reasonable to assume that YY1 contacts the SRE predominantly in the major groove. The structure of the SRF-SRE complex is unknown, but modification/interference assays have been interpreted as evidence for major groove binding as well. To test whether these proteins indeed make major groove contacts in regions of the SRE in which their binding sites overlap, we made SRE probes in which adenine-thymine base pairs have been replaced with inosine-cytosine base pairs. These substitutions change protein contact groups in the major groove while leaving the minor groove essentially unchanged. We find that YY1 is unable to bind this substituted SRE, consistent with the hypothesis that it recognizes the SRE primarily in the major groove. In contrast, SRF does bind the substituted SRE, suggesting that SRF does not make major groove contacts, at least in the central region of the SRE. SRF may make minor groove contacts in this region or it may make no contacts at all. To distinguish between these possibilities, we used distamycin, a drug that binds to the minor groove of AT-rich DNA, for its ability to function as a competitive inhibitor of SRF binding. Distamycin effectively inhibited the binding of SRF to the wild-type SRE, whereas it did not affect the binding of YY1. Thus, at least in the central AT-rich core of the SRE, SRF binds predominantly in the minor groove and YY1 in the major groove, making co-occupancy possible.

SRF-Homeodomain Interactions and the Specificity of Signal Transduction to the Nucleus

D. Grueneberg

How do cells interpret incoming signals into an appropriate pattern of gene expression? This question has become particularly important with the observation that different growth factor receptors share a small number of common signal transduction pathways. For example, in the *Drosophila* eye, the *sevenless* receptor tyrosine kinase (RTK) transmits an inductive signal that specifies the development of the R7 photoreceptor cell. Genetic analysis of the *sev* signaling pathway indicates that this signal uses the *ras/MAP* kinase pathway. But other receptors in *Drosophila*, including the EGF receptor and the *torso*-encoded RTK, utilize the same signaling machinery and elicit completely distinct biological responses. Thus, these signals must be generic, and the unique biological response of an individual cell to such a signal must be due to interpretation of the signal at the transcriptional level into a cell-type-specific pattern of gene expression. We have proposed that this interpretation is carried out by complexes of proteins of the MADS box family, such as SRF, and proteins of the homeodomain family. Our work this year has focused on devising assays to test this hypothesis.

Earlier, we had used a genetic screen in yeast to identify potential accessory factors for SRF. From that screen, we isolated cDNAs encoding a novel human homeodomain protein, Phox1. Biochemical studies, using recombinant proteins produced in *E. coli*, confirmed that Phox1 interacts with SRF in vitro. Furthermore, we found that Phox1 enhanced the binding of SRF to the SRE. This activity resides within the homeodomain of Phox1 and is shared with closely related homeodomain proteins, such as the *Drosophila* Paired and Orthodenticle proteins, but not with more distantly related homeodomain proteins. The observation that proteins of the homeodomain family, with clearly documented roles in assigning cell identity, could interact physically with proteins like SRF, which connect genes to signal transduction pathways, suggested a simple model for how these processes might be linked. We speculated that one way in which homeodomain proteins impart identity to a cell is by routing incoming signals to cell-type-specific genes and that they do this by recruiting SRF to these sites.

To test this model, we have devised an assay to study the interaction between Phox1 and SRF in vivo. In HeLa cells, a simplified reporter gene carrying a single SRF-binding site is not responsive to serum growth factors. However, upon cotransfection of an expression vector producing the Phox1 homeodo-
main, the reporter becomes serum-inducible. As we observed in our in vitro assays, this activity is shared with related homeodomains, such as Prd, but not more distantly related homeodomains. Thus, homeodomain proteins have the ability to link a gene to the cellular signal transduction machinery. Moreover, the ability of Phox1 to impart serum inducibility of the reporter gene requires cooperation with endogenous cellular proteins, presumably including SRF, because a reporter gene carrying mutations that prevent the binding of SRF is not responsive to Phox1. Therefore, although we cannot form stable SRF/Phox1 complexes in vitro using purified proteins, we believe that this in vivo assay provides a measure of the formation of such complexes on the reporter gene.

We have used this assay in several ways. First, together with Ken Simon, we have extensively mutagenized the Phox1 homeodomain to determine which parts of the protein are required for this activity. We find that the ability of Phox1 to impart serum inducibility to the reporter gene requires the DNA-binding activity of the homeodomain. In addition, as described in more detail below, Phox1 activity also requires amino acid residues on the solvent-exposed surfaces of homeodomain helices one and two. This observation indicates a requirement for direct protein-protein interactions between Phox1 and some other component(s) in the complex at the SRE.

Second, together with Keith Brennan, we have examined the DNA sequence specificity of Phox1 in vivo. By randomly mutagenizing the AT-rich core of the SRE, we have derived sets of SRF-binding sites that differ dramatically in their abilities to be activated by Phox1 in vivo. This observation suggests that Phox1 can recruit SRF to only a subset of potential binding sites, consistent with the hypothesis that homeodomains recruit SRF to cell-type-specific genes. Interestingly, the ability of Phox1 to impart serum inducibility to these SRF-binding sites does not strictly correlate with the simple affinities of either Phox1 or SRF for these sites in vitro. Thus, the specificity of action of the SRF/Phox1 complexes in vivo is distinct from the DNA-binding specificity of the individual partners in vivo. This observation is consistent with a large body of data concerning the specificity of action of homeodomain proteins: Their exquisite biological specificity in vivo contrasts dramatically with their rather weak affinity and degenerate sequence specificity for DNA in vitro.

### Mutagenesis of the Homeodomain-SRF Interface

K. Simon

Homeodomain proteins, first characterized in the fruit fly *Drosophila melanogaster*, have important regulatory roles in embryonic development. Among these functions is the ability to specify the identity of individual body segments in the fly and presumably of the individual cell types within each segment. This ability of homeodomain proteins to assign cell identity appears to be conserved in metazoans, as well as in fungi. Homeodomain proteins bind DNA and act as transcription factors, but their DNA-binding specificity is generally poor and is not sufficient to explain their high degree of specificity in vivo. It is widely believed that the homeodomain must have additional activities, including perhaps the ability to interact with other proteins. As described above, we have amassed considerable evidence that certain homeodomain proteins have the ability to interact with SRF and other proteins of the MADS box family. To understand this interaction, we have begun a mutagenic analysis of the Phox1 homeodomain protein to determine which amino acid residues of the homeodomain are required for interaction with SRF.

To begin, we have systematically substituted amino acid residues predicted to reside on the solvent-exposed surfaces of Phox1 homeodomain helices one and two with alanine residues. Mutant proteins were assayed in the HeLa cotransfection assay described in the previous section. Many substitutions in helices one and two had little detectable effect on the ability of Phox1 to recruit SRF to the SRE reporter gene. Several substitutions did, however. These included residues on both helices one and two and in the loop joining these helices. We are continuing this analysis by testing the effect of different amino acid substitutions at these positions and by testing the mutant proteins in other assays.

Our goals for the upcoming year include a mutagenic analysis of SRF to determine what residues in this protein are required for interaction with Phox1. In addition, we plan to undertake a more detailed analysis of the structural organization of the SRF/Phox1 complex, focusing on the stoichiometry of the complex, the nature of the DNA contacts, and whether other proteins are present in the functional complex in vivo.
Isolation and Characterization of a Drosophila Homolog of SRF

A. Ryan

To test our hypothesis that SRF (and other proteins of the MADS box family) cooperates with homeodomain proteins to impart cell type specificity to signal transduction pathways, we would like to be able to study this interaction in an intact animal. One attractive choice is Drosophila. Embryogenesis in Drosophila has been actively studied at the genetic, morphological, and molecular levels. The role of homeodomain proteins in key regulatory events in the fly embryo is relatively well understood. In addition, powerful molecular tools are available for monitoring and manipulating embryonic development. As a first step in studying the role of potential SRF/homeodomain interactions in fly development, we have isolated an SRF homolog from Drosophila.

Using a partial sequence provided to us by Ron Prywes and Michael Levine, we isolated a polymerase chain reaction (PCR) product from reverse-transcribed Drosophila mRNA that encodes a protein closely related to human SRF. With this probe, we have isolated both genomic and cDNA clones corresponding to this gene. The gene encodes a protein very closely related to human SRF in the DNA-binding domain; amino acid identity is greater than 90% in this region. We find that this protein binds DNA with specificity essentially indistinguishable from that of the human protein. Moreover, the fly protein interacts readily with the human SRF accessory protein, Elk-1. Thus, the biochemical properties of the fly SRF protein are highly conserved.

The SRF gene maps to region 60C on the right arm of chromosome 2. We have identified two deficiencies in this region of the genome. A number of recessive lethal genes map to this area, one of which may correspond to SRF. By in situ hybridization of an SRF probe to developing fly embryos, we find that SRF mRNA is provided to the embryo maternally. It is ubiquitously distributed throughout early development. This ubiquitous distribution is consistent with the possibility that SRF is a cofactor for homeodomain proteins active in the early embryo. After germ-band extension, however, SRF mRNA disappears from most tissues and is found at high levels in a relatively small number of cells scattered throughout the embryo. This pattern of expression suggests that the function of SRF at later stages of embryonic development is restricted to a subset of cell types.

To complement our studies of SRF, we have generated a series of lacZ reporter genes under the control of a portion of the mouse c-fos promoter. This region, which includes the binding sites for SRF and several accessory factors, is sufficient to mediate a transcriptional response to receptor tyrosine kinases in mammalian cells. We are introducing these reporters into transgenic flies by P-element-mediated transformation. We hope to find that this reporter gene is expressed in regions of the embryo in which signal transduction pathways are active. We would then use such fly lines for studying the role of SRF, its accessory proteins, and homeodomains in determining the pattern of expression of the reporter gene.

Role of Nuclear Signal Transduction Pathways in Tumorigenesis in a Transgenic Mouse Model

J. lafrate

Our studies of c-fos regulation have allowed us to identify nuclear proteins that are the recipients of the signals generated by growth factor receptors. We believe it is likely that these proteins must also have a significant role in transmitting proliferative signals from activated oncoproteins in tumors. We plan to test this hypothesis in transgenic mice expressing activated oncoprobes under the control of the mammary tumor virus long terminal repeat (MTV LTR). These animals reproducibly develop mammary tumors, with varying degrees of latency and penetrance, depending on the oncogene. Our experiments are being carried out in collaboration with Dr. William Muller, McMaster University.

Our first goal will be a simple biochemical analysis of nuclear signal transduction proteins in cell lines derived from transgenic mouse tumors. We will examine the ternary complex factors to determine if they are constitutively phosphorylated by MAP kinase. In addition, we will examine the SIF proteins to determine if they are constitutively activated and phosphorylated on tyrosine. To verify the activation state of these proteins, we will transfect appropriate reporter genes into these cell lines to determine if they exhibit elevated transcriptional activity.

If we find that these proteins are activated in tumors, we will then determine whether they contribute functionally to tumorigenesis by expressing
mutant proteins in transgenic mice under the control of the MTV LTR. We expect that gain-of-function mutants might function as oncogenes in these animals, either alone or in cooperation with other oncogenes. We expect that dominant-negative mutants should inhibit or retard tumor development in animals bearing transgenic oncogenes.

In addition, we plan to explore the role of homeodomain proteins in mammary tumorigenesis. In much the same way that we think homeodomain proteins interpret incoming signals by routing them to the appropriate genes, we believe that homeodomains should also be responsible for routing signals from activated oncogenes to target genes required for cell proliferation. Thus, we predict that mammary tumors from transgenic animals must express homeodomain proteins that are normally expressed only early in the development of the mammary epithelium, during its proliferative phase, or tumors may ectopically express homeodomain proteins that are not normally expressed in mammary cells. Therefore, we plan to isolate homeodomain cDNAs from libraries prepared from transgenic tumors, examine their expression patterns during development of the mammary gland, and test them for the ability to act as oncogenes in transgenic mice.

PROTEIN TYROSINE PHOSPHATASES AND THE CONTROL OF CELLULAR SIGNALING RESPONSES

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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. Protein 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 study the expanding family of protein tyrosine phosphatases (PTPs) that, like the kinases, comprise both transmembrane, receptor-linked forms and non-transmembrane, cytosolic species. The structures of the PTPs indicate important roles in the control of processes such as cell adhesion, cytoskeletal function, and the cell cycle.

During the last year, Qing Yang left to take a position in the Gene Therapy Center at the University of North Carolina, Chapel Hill, and Arne Östman returned to a position in the Ludwig Institute for Cancer Research in Uppsala, Sweden. Yan-Fen Hu,

PUBLICATIONS


In Press

Receptor-like PTPs and Cell-Cell Contact

S.M. Brady-Kalnay, S.N. Mamajiwalla, C.A. Östman, Q. Yang, N.K. Tonks

A characteristic feature of the extracellular segment of many receptor-like PTPs is the presence of immunoglobulin (Ig)-like and/or fibronectin type III (FNIII) domains. These are motifs that are commonly found in cell adhesion molecules. We have focused on two such enzymes, PTPµ and DEP-1.

PTPµ is characterized by an extracellular segment containing one Ig-like and four FNIII domains and thus displays structural similarity to members of the Ig superfamily of cell adhesion molecules that includes neural cell adhesion molecule (NCAM). There is a single transmembrane domain and a large intracellular segment containing two PTPase domains preceded by a juxtamembrane segment that is 70 residues longer than the equivalent segment in other receptor-like PTPs and which displays homology with the intracellular domain of members of the cadherin family of cell adhesion molecules. Such homology is unique among both the receptor-like PTPs and members of the Ig superfamily.

On the basis of the structural similarity between the extracellular segments of PTPµ and NCAM, one would predict that this receptor PTP may participate in homophilic binding interactions. We have tested this prediction using an overexpression system, reconstitution of the binding reaction in vitro, and cells in which PTPµ is normally expressed. Using the baculovirus system, expression of full-length PTPµ, or mutant forms with an intact extracellular segment, induced Sf9 cells to aggregate. In addition, we have demonstrated that homophilic binding between molecules of PTPµ can be reconstituted in vitro. Fluorescent beads linked to baculovirus-expressed PTPµ were able to bind to the surface of petri dishes coated with GST/PTPµ (a GST fusion protein containing the extracellular segment of PTPµ purified following expression in Escherichia coli) but did not bind to GST alone. In addition, beads coated with the GST/PTPµ fusion protein self-aggregate, whereas those coated with GST alone do not. The PTPµ-induced aggregation was found to be independent of calcium and glycosylation. Furthermore, phosphatase activity and the adhesive function are not mutually dependent. We also demonstrated that PTPµ, as it is expressed endogenously on the surface of MvLu cells, retains the capacity to interact with baculovirus-expressed PTPµ linked to beads. The binding of PTPµ-coated beads to the MvLu cell surface is blocked by antibody to the extracellular segment of PTPµ but not by a control pre-immune antibody. These data establish homophilic binding between molecules of PTPµ, thus indicating that the ligand for PTPµ is another PTPµ molecule on the surface of an adjacent cell. We are currently trying to define the sequences in the extracellular segment of PTPµ that mediate homophilic binding interactions and to examine the effect of perturbing these interactions on cell growth and development.

As far as we can tell, aggregation, i.e., ligand binding to the extracellular segment, had no detectable direct effect on the activity of the intracellular PTP domains. Nevertheless, it is possible that such an interaction could serve a tethering role, controlling the activity of the PTP indirectly by restricting its spatial distribution on the membrane and thus restricting the spectrum of substrates with which it may interact. Preliminary immunocytochemical analysis has localized PTPµ to particular junctional complexes at points of cell-cell contact under physiological conditions of expression. We are characterizing the components of these junctional complexes that bind to PTPµ and examining the consequences of such interactions with respect to the function of this enzyme.

Our data with PTPµ reinforce the concept that receptor PTPs may have a role in transducing signals initiated by cell-cell contact. For example, normal cells display density-dependent arrest of cell growth. Thus, as cultures approach confluence, and adjacent cells touch each other, growth is inhibited. Since tyrosine phosphorylation, triggered by growth factor receptor PTKs, has been implicated in promoting cell growth and proliferation, PTPs, as the natural antagonists of PTK function, may exert a negative effect on such growth-promoting signals. We have isolated cDNA encoding a novel receptor-like PTP from a HeLa cell library. The cDNA predicts an enzyme consisting of an extracellular segment containing eight FNIII repeats, a single transmembrane segment,
and a single intracellular PTP domain. We have proposed the name DEP-1 (high-cell-density enhanced PTP) for this enzyme. Following expression of DEP-1 cDNA in COS cells, a glycoprotein of 180 kD was detected, and PTP activity was demonstrated in immune complexes using a carboxy-terminal peptide antiserum. Endogenous DEP-1 was detected in WI38 lung fibroblasts by immunoblotting, immunoprecipitation of metabolically labeled cells, and immune complex PTP assays. Immunoblot analysis of DEP-1 expression in WI38 revealed dramatically increased levels and activity of the PTP in dense relative to sparse cultures. In addition, DEP-1 activity, detected in PTP assays of immune complexes, was increased in dense cell cultures. In contrast, the expression levels of PTP-1B, a ubiquitously expressed cytoplasmic enzyme that is the major PTP in many tissues and cell lines, did not change with cell density. This enhancement of DEP-1 expression with increasing cell density was also observed in another fibroblast cell line, AG1518. The increase in levels of DEP-1 occurs gradually with increasing cell contact and is initiated before saturation cell density is reached (Fig. 1). These observations suggest that high-cell-density-induced expression of DEP-1, which is broadly distributed, may contribute to the mechanism of contact inhibition of cell growth by promoting net dephosphorylation of proteins in the membrane and countering the effect of growth-promoting PTKs. We are currently examining the link between cell contact and expression of DEP-1 and testing directly the ability of this PTP to antagonize cell growth.

**FIGURE 1** Analysis of DEP-1 expression at various cell densities. DEP-1 expression was analyzed by immunoblotting of lysates of WI38 cells. Photomicrographs of cultures at varying cell densities from which the lysates were derived are shown below.

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**MKP-1, a Tyr/Thr Phosphatase That Dephosphorylates, and Blocks Signaling Downstream from, MAP Kinase**


Recently, progress has been made by many laboratories in defining signaling pathways initiated by mitogenic stimuli. Growth-factor-induced autophosphorylation of tyrosyl residues in receptor-PTKs induces the formation of multiprotein complexes in the membrane that trigger conversion of Ras from an inactive GDP-bound form to an active GTP-bound state. Activated Ras then initiates a cascade of sequential phosphorylation events in which the serine/threonine kinase Raf phosphatases and activates MAP kinase kinase (also known as MEK), which is a dual specificity kinase that in turn phosphatylates both Thr-183 and Tyr-185 in p42 MAP kinase. Phosphorylation of both tyrosine and threonine regulatory sites is essential for activation. The MAP kinase family of enzymes has been implicated as common and essential components of signaling pathways induced by diverse mitogenic stimuli. Once activated, MAP kinase can phosphorylate a number of substrates, including transcription factors, that are essential for triggering the expression of genes required for the mitogenic response. Therefore, growth factor binding initiates a complex network of protein phosphorylation events that leads a quiescent cell to enter the cell cycle, undergo DNA replication, and ultimately divide. One of these immediate early genes that is activated rapidly and transiently in quiescent fibroblasts treated with serum growth factors is 3CH134, which encodes a dual specificity (tyrosine/threonine) phosphatase termed MKP-1. MKP-1 possesses intrinsic phosphatase activity that is highly specific for both the tyrosine and threonine regulatory sites in MAP kinases. In serum-stimulated fibroblasts, the kinetics of inactivation of MAP kinases coincides with the appearance of newly synthesized MKP-1 protein, and the protein synthesis inhibitor cycloheximide leads to persistent activation of MAP kinases. Expression of the phosphatase in COS cells leads to selective dephosphorylation of MAP kinases from the spectrum of phosphotyrosyl proteins visualized by immunoblotting with an antiphosphotyrosine antibody. MKP-1 blocks phos-
phorylation and activation of p42mapk-mediated by serum, oncogenic Ras, or activated Raf. Most striking is the observation that a catalytically inactive mutant of the phosphatase, Cys-258→Ser, augments MAP kinase phosphorylation under similar conditions. The mutant phosphatase forms a physical complex with the phosphorylated form of p42mapk and p44mapk (Fig. 2). Therefore, our findings suggest that MKP-1 is a physiological MAP kinase phosphatase that may feed back on the growth factor signaling pathway to dephosphorylate and inactivate MAP kinase, preventing uncontrolled growth and proliferation. To examine the effect of expression of MKP-1 on signaling responses downstream from MAP kinase, we employed a transcription assay in which MAP kinase activation was measured by its ability to trans-activate a promoter containing a serum response element (SRE). We have observed that the induction of SRE is mediated through the MAP kinase pathway and is sensitive to the expression of MKP-1. We have also established a microinjection assay to measure MAP kinase activation in single cells with use of the SRE reporter. We found that microinjection of either purified MKP-1 protein or MKP-1 expression plasmid blocks SRE induction mediated by TPA, EGF, and oncogenic Ras. We are currently using MKP-1 as a tool to define the role of MAP kinase activation in G1/G0-s-phase transition of the cell cycle in response to growth stimuli or oncoproteins such as Ras.

Analysis of the Structure, Regulation, and Function of PTP1B

A.J. Flint, K.R. La Montagne, N.K. Tonks [in collaboration with David Barford and Bob Franzia, Cold Spring Harbor Laboratory]

PHOSPHORYLATION OF PTP1B

PTP1B is a nontransmembrane PTP, the first member of the family to be isolated in homogeneous form. Its cDNA predicts a protein of 435 amino acids, of which the carboxy-terminal 114 residues have been implicated in controlling both the localization and function of the enzyme. Neel's group (Beth Israel Hospital, Boston) has shown that the extreme carboxy-terminal 35 amino acids, which are of a highly hydrophobic nature, are both necessary and sufficient for targeting the enzyme to the cytoplasmic face of membranes of the endoplasmic reticulum. Upon inspection of the sequence of the preceding 80 amino acids, which are predominantly hydrophilic, we noted several potential sites for phosphorylation by serine/threonine kinases. We have now confirmed that PTP1B is phosphorylated in a cell-cycle-dependent manner in vivo. In asynchronously growing HeLa cells, PTP1B is phosphorylated predominantly on Ser-378 by PKC and at two other minor sites. In mitotically arrested cells, these interphase sites are dephosphorylated, and PTP1B becomes phosphorylated instead on Ser-386 by p34cdc2 and on Ser-352 by a "proline-directed" kinase. These mitotic phosphorylation events coincide with a 30% inhibition of PTP1B activity.

The interaction between these phosphorylation sites has now been examined in greater detail following expression of point mutants in which individual serine residues were converted to alanine. The results suggest that (1) the phosphorylation of Ser-386 by p34cdc2 occurs independently of phosphorylation at another site, and (2) phosphorylation of Ser-352 promotes the dephosphorylation of the interphase sites, including Ser-378, that occurs in mitotic HeLa cells.
A number of approaches have been taken to identify the kinase responsible for phosphorylating Ser-352, and current data point to a MAP kinase-like enzyme. However, the precise identity of this kinase remains to be defined.

DETERMINATION OF THE CRYSTAL STRUCTURE OF PTP1B

The families of receptor-like and nontransmembrane PTPs are related through a conserved catalytic core structure of approximately 240 residues. PTP1B was the first PTP to be isolated in homogeneous form. It was purified from human placenta as a 321-residue protein of 37 kD that primarily comprises the catalytic domain and which is derived from the full-length protein by proteolytic removal of the regulatory carboxy-terminal segment. In collaboration with David Barford, from the crystallography group at Cold Spring Harbor, we have determined the crystal structure of this 37,000 form of PTP1B which we hope will serve as a model for understanding structure-function relationships and the catalytic mechanism of the PTPs. The enzyme was expressed in E. coli in a stable and soluble form, the activity of which is indistinguishable from that of the molecule purified from human placenta. The structure was determined by single isomorphous replacement and anomalous scattering methods using sodium tungstate as the heavy atom derivative. This was important because sodium tungstate is an effective inhibitor of PTP1B (IC₅₀ = 10 μM) that binds at the catalytic site and presumably functions as an analog of phosphate. Thus, the structure of the PTP1B-tungstate complex provides information about the interactions between the enzyme and its substrate.

The structure of PTP1B has been determined to 2.8 Å resolution. It is composed of a single domain with the polypeptide chain organized into 8 α helices and 12 β strands with a 10-stranded mixed β sheet, which adopts a highly twisted conformation, spanning the entire length of the molecule. The structure illustrates that the sequence motif [I/V]HCXAGXXR[S/T]G, which contains the catalytically essential nucleophilic cysteinyl residue and uniquely defines the PTP family of enzymes, forms the phosphate recognition site and is located at the base of a cleft. The hydrogen bonding interactions that are important for phosphate binding are illustrated in Figure 3. Specificity for phosphotyrosine-containing substrates

![Figure 3](image-url)

FIGURE 3 View of the catalytic site of PTP1B to illustrate binding of tungstate (phosphate). (Open lines) Main chain bonds; (closed lines) side chain bonds; (dotted lines) hydrogen bonding interactions.
probably results from the depth of the cleft since the
smaller phosphoserine and phosphothreonine resi-
dues would not reach down to the phosphate-bind-
ing site. In addition, the surface of the protein sur-
rounding the cleft is relatively open and contains a
high density of basic residues. This would permit
numerous modes of peptide binding, consistent with
the poor discrimination between P'Tyr-proteins, and
would explain the apparent preference for acidic
residues adjacent to the phosphotyrosine in target
substrates. We are currently generating mutants of
PTP1B for structural determination to test the role of
particular amino acid residues in the catalytic me-
chanism and also looking at the binding of substrate
detail through cocrystallization of PTP1B with ei-
ther phosphotyrosine or phosphopeptide substrates.
In addition, we are expanding our studies to en-
compass other members of the family, in particular
MKP-1. We hope to elucidate the structural basis for
both the selectivity of MKP-1 and its dual specificity.

PTP1B AND CHRONIC MYELOGENOUS LEUKEMIA

Chronic myelogenous leukemia (CML) is a clonal
disorder of the hematopoietic stem cell characterized
by the Philadelphia chromosome, in which the c-abl
proto-oncogene on chromosome 9, encoding a PTK,
becomes linked to the bcr gene on chromosome 22.
This results in the production of a fusion protein
tered p210 bcr:abl, the PTK activity of which is en-
chanced relative to c-Ab1 generating abnormal patterns
of tyrosine phosphorylation in CML cells. Although
much effort was focused on the p210 bcr:abl PTK
and the role of tyrosine phosphorylation in the dis-
ease, we are looking from the perspective of the PTPs
as potential antagonists of p210 bcr:abl function. We
have found that the levels of PTP1B, both mRNA
and protein, are increased as a result of constitutive
expression of p210 bcr:abl in Mo7 cells. An increase
in the half-life of PTP1B also contributes to the en-
hanced levels of this enzyme. Furthermore, PTP1B
appears to be phosphorylated on tyrosyl residues in
the p210 bcr:abl-expressing cells. What makes these
results exciting is that TCPTP, which is a very close-
ly related homolog of PTP1B (75% identity in the
catalytic domain), is also expressed in Mo7 cells but
is apparently not altered in its expression level, half-
life, or tyrosine phosphorylation state as a con-
sequence of p210 bcr:abl expression. We are current-
ly trying to establish whether PTP1B is a specific
antagonist of p210 bcr:abl function, i.e., does the cell
respond to this aberrantly activated PTK by trying to
produce a natural inhibitor? Through the identifica-
tion of PTPs with the ability to antagonize p210
bcr:abl, we hope to be able to characterize the signal-
ing events that underlie the role of this kinase in
CML. In collaboration with Dr. Bayard Clarkson
(Memorial Sloan-Kettering Cancer Center, New
York), we are also investigating whether the changes
we observe in PTPs in model cell systems for CML
also apply to cells from patients afflicted with the dis-
eease.

Regulation of PTP Activity

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The activity of members of the PTP family may be
regulated at several levels. In the case of the receptor-
like enzymes, there is obviously the potential for
modulation of activity by ligand binding to the ex-
tracellular segment. In addition, it appears that many
of the members of this family will be responsive to
regulation by reversible phosphorylation, intracellular
targeting, and association with other proteins.

PTP-PEST

The protein tyrosine phosphatase PTP-PEST is an 88-
kD cytosolic enzyme that is ubiquitously expressed in
mammalian tissues. We have expressed PTP-PEST
using recombinant baculoviruses and purified the
protein essentially to homogeneity in order to investi-
gate phosphorylation as a potential mechanism of
regulation of the enzyme. PTP-PEST is phosphory-
lated in vitro by both PKA and PKC at two major
sites, which we have identified as Ser-39 and Ser-
435. PTP-PEST is also phosphorylated on both Ser-
39 and Ser-435 following treatment of intact He La
cells with TPA, forskolin, or IBMX. Phosphorylation
of Ser-39 in vitro decreases the activity of PTP-PEST
compared to enzyme obtained from untreated cells.
Our results suggest that both PKC and PKA are
able to characterize the signal-
ing events that underlie the role of this kinase in
CML. In collaboration with Dr. Bayard Clarkson
(Memorial Sloan-Kettering Cancer Center, New
York), we are also investigating whether the changes
we observe in PTPs in model cell systems for CML
also apply to cells from patients afflicted with the dis-
eease.
cesses involving reversible tyrosine phosphorylation. These data again emphasize the interplay between serine/threonine and tyrosine phosphorylation events in signal transduction in vivo.

**PTPX1 AND X10**

We have isolated the complete coding sequences for two distinct PTPs, PTPX1 and X10, from a *Xenopus laevis* ovary cDNA library. The X1 cDNA encodes a protein (PTPX1) of 693 amino acids (~79 kD); the X10 cDNA encodes a protein of 597 amino acids (~69 kD). Both PTPX1 and PTPX10 lack potential membrane spanning sequences and therefore can be classified as nontransmembrane/cytoplasmic PTPs. Although the overall structures of these PTPs are similar, sharing segments of 95% amino acid identity, they differ in that PTPX1 contains a unique 97-amino-acid insert between the amino-terminal segment and carboxy-terminal catalytic domain. The absence of complete identity between PTPX1 and PTPX10 suggests that these two sequences are the products of separate genes and not the result of alternative splicing. This conclusion is confirmed by polymerase chain reaction (PCR) analysis of *Xenopus* genomic DNA. Both PTPs share sequence identities in their amino-terminal segments with two lipid binding proteins, cellular retinaldehyde-binding protein and SEC14p, a phospholipid transferase. In addition, the unique insert sequence of PTPX1 shares identity with PSSA, a protein involved in phosphatidylserine biosynthesis. Sequence comparison suggests that PTPX10 is the *Xenopus* homolog of the human PTP Meg-02, whereas PTPX1 is a structurally related yet distinct PTP. Intrinsic PTP activity of PTPX1 and PTPX10 was demonstrated in lysates of Sf9 cells infected with recombinant baculovirus encoding either enzyme. PTPX1 can associate with membranes in *Xenopus* oocytes, and this association is accompanied by a fourfold increase in PTP-specific activity. We are currently trying to establish the mechanism by which PTP activity is enhanced by membrane association and to determine whether these enzymes are targeted to specific membrane fractions.

**PTPH1**

PTPH1 is a nontransmembrane PTP of 913 residues, characterized by the presence of an amino-terminal domain of 320 residues that has homology with the cytoskeleton-associated proteins, band 4.1, ezrin, and talin. This domain defines a family of proteins that is targeted to interfaces between the plasma membrane and the cytoskeleton. For example, band 4.1, which promotes the association of actin and spectrin in erythrocytes, interacts through this domain with the transmembrane protein glycocholin. We propose that PTPH1 may be similarly restricted in its intracellular localization. In the past year, we have expressed PTPH1 in Sf9 cells and characterized its enzymatic activity. It appears that the amino-terminal segment, containing the band-4.1 homology domain, exerts a negative influence on catalytic activity. Using the purified protein in affinity chromatography approaches, we have identified two proteins that bind to PTPH1. We are in the process of characterizing these proteins and their effect on the activity of PTPH1.

**CD45**

CD45 is the prototypical receptor PTP. It is expressed exclusively on cells of the hematopoietic lineage, and its expression is essential for normal signaling through T- and B-cell receptors. Several reports have indicated that CD45 may be recovered from cell lysates as part of a multiprotein complex. In particular, it has been proposed that CD45 functionally interacts with the PTK p56<sup>ck</sup>, an as yet unidentified p32 protein and cell surface proteins such as CD4 or CD8. We also detect association between CD45 and proteins of 50,000 and 30,000 in human spleen and in cell lines including Jurkat. We are currently testing the regulatory significance of these apparent associations. In particular, we are looking at the possibility that these polypeptides may in fact be derived from CD45 by a proteolytic processing event.

**PUBLICATIONS**


In Vivo Analysis of the Stability and Transport of Poly(A)+ RNA

S. Huang, D.L. Spector [in collaboration with Mark H. Ellisman and Thomas J. Deerinck, University of California at San Diego, San Diego Microscopy and Imaging Resource]

Previous studies from our laboratory have shown that a variety of pre-mRNA splicing factors are localized...
to a subnuclear speckled domain when mammalian cells are immunolabeled with antibodies against these pre-mRNA splicing factors. At the electron microscopic level, the speckled pattern is composed of both interchromatin granule clusters and perichromatin fibrils. A large body of evidence has accumulated from both our laboratory and other laboratories which has suggested that the perichromatin fibrils represent nascent transcripts and the interchromatin granule clusters represent storage and/or assembly sites for pre-mRNA splicing factors. The majority of substrates for these splicing factors are pre-mRNAs which contain a poly(A) tail of approximately 200–300 nucleotides. During the past year, we have studied the distribution of poly(A)+ RNA in the mammalian cell nucleus and its transport through nuclear pores by fluorescence and electron microscopic in situ hybridization. Poly(A)+ RNA was

![FIGURE 1](image)

FIGURE 1 Transport of poly(A)+ RNA through the nuclear pore complexes is directly visualized in HeLa cells by electron microscopic pre-embedding in situ hybridization with photoxidation. Poly(A)+ RNA labeling appears to taper into confined regions as it reaches the nuclear pore complexes (arrowheads in A). In addition, a buildup of RNA is observed at the cytoplasmic side of the pore complex (arrows in A). In grazing section, poly(A)+ RNA is observed to be localized to all of the nuclear pore complexes (B), suggesting that poly(A)+ RNA is transported through all of the nuclear pores. The poly(A)+ RNA appears to be concentrated around the periphery of the pore complexes. Bars: (A) 1 µm; (B) 0.5 µm.
detected in the nucleus as a speckled pattern that totally colocalized with pre-mRNA splicing factors to interchromatin granule clusters and perichromatin fibrils. When cells were fractionated by detergent and salt extraction as well as DNase I digestion, the majority of the nuclear poly(A)+ RNA was found to remain associated with the nonchromatin RNP-enriched fraction of the nucleus. After inhibition of RNA polymerase II transcription for 5–10 hours, a stable population of poly(A)+ RNA remained in the nucleus and was reorganized into fewer and larger interchromatin granule clusters along with pre-mRNA splicing factors. This stable population of nuclear RNA may have an important role in nuclear function other than in the formation of pre-mRNA or mRNA. Furthermore, we have observed that, in actively transcribing cells, the regions of poly(A)+ RNA that reached the nuclear pore complexes appeared as narrow concentrations of RNA, suggesting a limited or directed pathway of movement (Fig. 1A). All of the observed nuclear pores contained poly(A)+ RNA labeling (Fig. 1B), suggesting that they are all capable of exporting RNA. In addition, we have directly visualized, for the first time in mammalian cells, stages in the transport of poly(A)+ RNA through the nuclear pore complexes. Ongoing studies are attempting to determine whether transcripts from individual genes are transported through all or a subset of nuclear pores. If only a subset of the pores are involved in transport of certain transcripts, as has been suggested by the gene gating hypothesis (Blobel, Proc. Natl. Acad. Sci. 82: 8527 [1985]), it will be interesting to determine the spatial relationship of the localization of the gene to the nuclear pores involved in the transport of its RNA.

A Possible Role of the Yeast Nucleolus in mRNA Transport

T. Tani, R.J. Derby, D.L. Spector

Transport of mRNA from the nucleus to the cytoplasm plays an important role in gene expression in eukaryotic cells. The process of mRNA transport can be dissected into several stages: release of the mRNA from transcription and processing sites, movement of the mRNA to the nuclear pore complex and docking at the pore complex, translocation through the nuclear pore complex, binding of the mRNA to cytoskeletal elements, and movement to the proper cytoplasmic location for translation. However, knowledge of the mechanisms involved in each of these processes remains limited.

We have examined the effects of heat shock on mRNA transport in the fission yeast Schizosaccharomyces pombe. As an assay system, we have utilized in situ hybridization with an oligo(dT) probe that hybridizes to the poly(A) tail of mRNA. In normal cells grown at 30°C, poly(A)+ RNA is uniformly distributed in the cytoplasm and nuclei (Fig. 2A). We found that a severe heat shock at 42°C results in accumulation of bulk poly(A)+ RNA in the nuclei with little to no cytoplasmic labeling, suggesting that the mRNA transport pathway is blocked in the heat-shocked cells (Fig. 2B). Pretreatment of cells with a mild heat shock prior to a severe heat shock protects the mRNA transport machinery and allows mRNA transport to proceed unimpeded. The protective effect of the pretreatment is eliminated when cycloheximide is administered before the pretreatment. However, cycloheximide does not block protection of mRNA transport if it is added after pretreatment, suggesting that the induction of heat shock proteins is involved in the protection of the mRNA transport pathway. In situ hybridization using a probe to the intronless alcohol dehydrogenase RNA showed that alcohol dehydrogenase transcripts are also accumulated in the nucleus after a severe heat shock. This result suggests that the accumulation of poly(A)+ RNA in nuclei is not simply due to the retention of unspliced precursor mRNAs after heat shock due to the inhibition of pre-mRNA splicing. Upon heat shock, the nucleolar crescent-shaped region of the nucleus condensed into a few compact structures. Triple-label staining with the oligo(dT) probe, an antibody to the nucleolar protein fibrillarin, and the DNA fluorochrome DAPI, revealed that poly(A)+ RNA accumulates predominantly in the condensed nucleolar regions of the heat-shocked cells (Fig. 2B). High-resolution electron microscopic in situ hybridization confirmed that poly(A)+ RNA is distributed in both the nucleolar region and the DNA-enriched region of wild-type cells and that upon heat shock, RNA accumulates in the condensed nucleolar components of the S. pombe nucleus. On the basis of these results, we propose that the crescent-shaped "nucleolar" region of the yeast nucleus may contain functional domains that are involved in mRNA transport in addition to those domains involved in ribosomal RNA synthesis and packaging.
FIGURE 2 Localization of poly(A)$^+$ RNA in control and heat-shocked S. pombe cells. Wild-type S. pombe cells grown at normal temperature (A,C,E) and cells exposed to heat shock for 60 min (B,D,F) were subjected to triple labeling with an oligo(dT) probe (A,B), an antibody to the nucleolar protein fibrillarin (C,D), and DAPI (E,F). In control cells, poly(A)$^+$ RNA is diffusely distributed throughout the cytoplasm and nucleoplasm (A). In heat-shocked cells, poly(A)$^+$ RNA concentrates in the nucleolar portion of the S. pombe nucleus (B). Magnification, 1050x.

Organization of the Double-stranded RNA-activated Protein Kinase DAI and Virus-associated VA RNA$_1$ in Adenovirus-infected Cells


In a previous study, we examined the localization of total adenovirus-2 (Ad2) RNAs in infected HeLa cells. We found that these RNAs are concentrated in a series of ring-shaped inclusions and dots in infected cell nuclei and are diffusely distributed in the cytoplasm. Since translational control in adenovirus-infected cells involves the participation of the cellular kinase DAI (double-stranded RNA-activated inhibitor of translation) and the small viral VA RNA$_1$, we have examined the cellular distribution of DAI in Ad2-infected and uninfected HeLa cells. In uninfected HeLa cells, DAI was found to be concentrated in the cytoplasm. In addition, DAI was localized in the nucleoli and diffusely distributed throughout the nucleoplasm (Fig. 3a). Cells treated with α-interferon displayed a pattern of distribution similar to that for
FIGURE 3 Immunofluorescent localization of DAI in uninfected control and α-interferon-treated HeLa cells. Cells untreated (a) or treated with α-interferon (b) both display cytoplasmic and nucleolar localization of DAI.

DAI (Fig. 3b). When RNA polymerase I activity was inhibited by the drug actinomycin D, nucleoli segregated and DAI was found to colocalize with the dense fibrillar region of the nucleoli. During mitosis, the distribution of DAI paralleled that of rRNA. In adenovirus-infected cells, the localization of DAI was similar to that in uninfected interphase cells. VA RNA₁ was detected in Ad2-infected cells by 10–14 hours postinfection as fine dots in the nucleoplasm. By 18–24 hours postinfection, VA RNA₁ appeared in bigger and more abundant dots in the nucleoplasm, and the cytoplasm was intensely labeled (Fig. 4a). Transient expression of the VA RNA₁ gene in uninfected cells resulted in a similar localization of the RNA. Our results are consistent with a role for DAI and VA RNA₁ in protein synthesis and suggest

FIGURE 4 Localization of VA RNA₁ in HeLa cells infected with Ad2 for 24 hr, using in situ hybridization. (a) VA RNA₁ appears to be localized in abundant small round dots in the nucleus (arrowheads) as well as being diffusely distributed in the cytoplasm. (b) Corresponding differential interference contrast image. The hybridization signal (dots) are still observed after DNase I pretreatment (c) but are not observed after RNase A pretreatment (d).
that DAI may have an early role in ribosome biogenesis in the nucleolus in addition to its cytoplasmic role in translation.

PUBLICATIONS


In Press


QUANTITATIVE REGULATORY BIOLOGY

B.R. Franza

Y. Li

M. Neumann

B.-C. Ma

J. Schepppler

G. Mak

A. Wilisch

During the past year, we continued to study specific interactions of cellular macromolecules that occur in temporally constrained responses of the cell to its environment.

Drs. M. Neumann and A. Wilisch joined the lab to demonstrate highly specific alterations in the regulatory protein, Rel, as cells are stimulated by the cytokines, interleukin 1, interleukin 3, tumor necrosis factor α, and the retinoid, all-trans retinoic acid. The cell system they were investigating permitted the comparison of the effect of constitutive expression of an altered tyrosine kinase on the response to addition of each cytokine or/and retinoid to the growth environment. The results indicated that Rel can be used as a meter of the differential effects of each cytokine and all-trans retinoic acid, that the meter is fundamentally re-set when a constitutive, disregulated tyrosine kinase is present in the cell, and that interactions between Rel and other macromolecules represent additional meters of changes in the growth environment of the cell, as well as expression of intracellular regulatory components like tyrosine kinases (manuscript submitted).

Dr. J. Schepppler demonstrated conclusively that specific cellular and viral (in this case, human immunodeficiency virus type 1, HIV-1) proteins do interact with single-strand, specific sequence oligonucleotides. In doing so, she uncovered a novel approach to the disruption of a critical HIV-1 regulatory protein, p32 integrase, and set in place (with our collaborators at ISIS Pharmaceuticals) a strategy for deducing an optimum structure for inhibiting the enzyme. The context of her discovery was substantial because she uncovered certain low-abundance cellular proteins that interacted with the same oligonucleotide. The implications include cellular proteins with integrase-like activity or/and complexes of proteins, one of which happens to recognize the single-strand oligonucleotide in a sequence-specific manner, with the others coming along for the ride.

Dr. Y. Li continued her work on the regulation of expression of HIV-1 by demonstrating contributing interactions between various structures that reside within the HIV-1 transcription control region (manuscript submitted). She also brought a substantial amount of work on the promoter of the human cell
cycle control gene, cdc2, to completion. In the cdc2 study, she elucidated a more comprehensive understanding of promoter structures that apparently contribute to the stringent transcriptional control of cdc2 and a clearer indication of regions within the cdc2 promoter that are responsive to disregulators like the cellular-transforming adenoviral gene product, E1A-12S (manuscript submitted).

We continued our excellent collaboration with Nick Tonks on aspects of regulation and activity of the cellular tyrosine phosphatase, PTP1B. Similarly, in two collaborations, Dr. M. Neumann completed further analysis of the tyrosine phosphorylation of Rel in fully differentiated, normal human myelocytes with Brian Druker and extended our understanding of Rel (and the related cellular protein, p65 NF-κB) interaction with the κB site in response to activation of protein kinase A in normal human T lymphocytes with Edgar Serfling and colleagues (manuscript submitted).

Through all of this work, the contributions of Ms. G. Mak and B.-C. Ma have been extensive.

PUBLICATIONS


MOLECULAR CELL BIOLOGY

We are interested in two fundamental problems in molecular and cell biology: (1) the mechanisms responsible for cell-type-specific and developmentally regulated patterns of gene expression and (2) the functional significance of cell-type-specific protein isoform expression. In particular, we have been studying 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, mitosis, and the generation of cell shape. Of interest to our laboratory is the observation that different forms of tropomyosin are expressed from four separate genes in rat. The α-TM gene encodes nine isoforms, the β-TM gene encodes two isoforms, the TM-4 gene encodes a single isoform, and a newly identified fourth TM gene, referred to as the α skeletal muscle slow-twitch gene, encodes at least four distinct isoforms. We have been studying the expression of these genes with particular attention to understanding the mechanisms of their regulation at the posttranscriptional level, i.e., tissue-specific alternative RNA splicing. 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. In addition, we have also continued to study the molecular basis for neuron-specific alterna-
Cis-Elements Involved in Alternative Splicing of the Rat β-Tropomyosin Gene: The 3’ Splice Site of the Skeletal Muscle Exon 7 Is the Major Site of Blockage in Nonmuscle Cells

W. Guo

We have been using the rat β-tropomyosin (β-TM) gene as a model system to study the mechanism of alternative splicing. The β-TM gene spans 10 kb with 11 exons and encodes two distinct isoforms, namely, skeletal muscle β-TM and fibroblast TM-1. Exons 1-5, 8, and 9 are common to all mRNAs expressed from this gene. Exons 6 and 11 are used in fibroblasts, as well as in smooth muscle cells, whereas exons 7 and 10 are used exclusively in skeletal muscle cells. Our previous studies localized the critical elements for regulated alternative splicing to sequences within exon 7 and the adjacent upstream intron. We also demonstrated that these sequences function, in part, to regulate splice site selection in vivo by interacting with cellular factors that block the use of the skeletal muscle exon in nonmuscle cells. We have further characterized the critical cis-acting elements involved in alternative splice site selection. Our data demonstrate that exon 7 and its flanking intron sequences are sufficient to regulate the suppression of exon 7 in nonmuscle cells. We have shown that in both in vivo and in vitro assays that the blockage of exon 7 in nonmuscle cells is primarily at its 3’ splice site.

Role of the Polypyrimidine Tract Binding Protein in RNA Splicing

G. Mulligan

The β-TM transcript contains a mutually exclusive internal cassette, in which exon 7 is spliced into mRNA in skeletal muscle, whereas in all other tissues, exon 6 is used. Our studies demonstrated that in nonmuscle cells, exons 6 and 7 do not compete for splice site recognition, but instead there are cellular factors that block the use of exon 7. We recently found that the polypyrimidine tract binding protein (PTB) specifically interacts with sequences involved in alternative splicing of β-TM pre-mRNA. Having identified that PTB can bind to specific regulatory regions, we are studying how these interactions contribute to splice site selection. One approach to test the role of PTB is to determine if depletion or addition of PTB to nuclear extracts affects the use of the skeletal muscle exon 7 in HeLa cell nuclear extracts. Two methods are being used to deplete nuclear extracts of PTB: (1) passage of nuclear extract over a poly(U) affinity column and (2) immunodepletion. We have prepared both polyclonal and monoclonal antibodies to PTB. We now have 16 monoclonal antibodies and have determined that they bind to epitopes located throughout the length of the protein. We have used the antibodies to inhibit PTB function by adding them to in vitro splicing assays of various pre-mRNAs. Four of the monoclonal antibodies inhibited the in vitro splicing of intron 6 in the β-TM intron yet have no effect on the splicing of introns 5 and 7. It is worth noting that the latter two introns were previously shown not to bind well to PTB in direct binding assays (Mulligan et al., J. Biol. Chem. 267: 25480 [1992]). In addition, we found that splicing of intron 2 in the α-TM gene (which also contains a strong PTB-binding site) was also inhibited by antibodies to PTB, whereas splicing of an intron from β-globin was not. Collectively, these data suggest that PTB is required for splicing of certain introns.

Functional analysis of cellular factors involved in exon 7 blockage had previously been hampered by the lack of an extract that uses the skeletal muscle exon to significant levels. We have now developed a modified procedure for nuclei isolation that allows us to obtain nuclear extracts from both HeLa cells and 293 cells which utilize the 3’ splice site of exon 7. This modification alters the distribution of cellular factors between the nuclear and cytoplasmic fractions. These extracts contain significant levels of PTB, and addition of more PTB did not reduce exon 7 splicing. Affinity chromatography and immunodepletion with PTB antibodies are both being used in attempts to reduce PTB levels such that possible effects on exon 7 usage can be observed. We are also using this extract as the basis for a complementation assay to detect factors blocking exon 7 usage. Addition of a HeLa cell nuclear extract that inefficiently
uses exon 7 indicates the presence of general and specific inhibitory activities. We have obtained a partially purified fraction that specifically reduces exon 7 usage without inhibiting splicing of control pre-mRNAs. Work is in progress to purify this specific inhibitory activity in order to understand both the mechanism and specificity of exon 7 blockage.

Multiple Cis-elements Can Contribute to the Use of the 5′ and 3′ Splice Sites of the Nonmuscle/Smooth Muscle Exon 6 in β-TM Pre-mRNA

C. Casciato

We previously found 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 8 (Genes Dev. 2: 1627 [1988]). Pre-mRNAs containing exon 5, intron 5, and exon 6 are not normally spliced in vitro. 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. We found that mutations in two regions of the pre-mRNA led 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 upstream of the 3′ splice site of exon 6 results in 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 led to splicing of exon 5 to exon 6. Thus, three distinct elements can act independently to activate the use of the 3′ splice site of exon 6: (1) the sequences contained within exon 8 when joined to exon 6, (2) a poly(U) tract in intron 5, and (3) a consensus 5′ splice site in exon 6. Using biochemical assays, we have determined that these sequence elements interact with distinct cellular factors for 3′ splice site utilization. Although HeLa cell nuclear extracts were able to splice all three types of pre-mRNAs mentioned above, a cytoplasmic S100 fraction supplemented with SR proteins was unable to splice efficiently exon 5 to exon 6 using precursors in which exon 6 was joined to exon 8. We also studied how these elements contribute to alternative splice site selection using precursors containing the mutually exclusive, alternatively spliced cassette composed of exons 5 through 8. Introduction of the poly(U) tract upstream of exon 6, and changing the

5′ splice site of exon 6 to a consensus sequence, either alone or in combination, facilitated the use of exon 6 in vitro, such that exon 6 was spliced more efficiently to exon 8. These data show that intron sequences upstream of an exon can contribute to the use of the downstream 5′ splice and that sequences surrounding exon 6 can contribute to tissue-specific alternative splice site selection.

Identification of Cis-acting Elements and Cellular Factors Required for the Use of Certain 3′ Splice Sites

M. Selvakumar

As described above, our experiments have revealed a number of features involved in the alternative splicing of β-TM pre-mRNA, including the fact that the splicing of exon 5 to exon 6 requires that exon 6 first be joined to exon 8. These experiments demonstrated that sequences within exon 8 contain a cis-acting element (referred to as exon recognition element or ERE), which is required for the 3′ splice site of exon 8. Within exon 8, there are three copies of a purine-rich motif that has been shown in other systems to function as a site enhancer for certain 3′ splice sites. Interestingly, exon 6 contains two copies of a purine-rich motif and exon 8 contains three copies. Whether all these repeats are functionally required for splicing remains to be determined. For example, we previously found that pre-mRNAs containing only one repeat from exon 8 joined to exon 6 were still able to splice in vitro. We are currently undertaking a systematic analysis of the different purine-rich motifs to determine which are required for pre-mRNA splicing using both in vitro and in vivo systems. In addition, using a biochemical complementation assay, we are in the process of purifying the factor(s) involved in pre-mRNA splicing.

Alternative Splicing of β-Tropomyosin Pre-mRNA in a Myogenic Cell System

Y.-C. Wang

Studies in our laboratory on alternative splicing of β-TM pre-mRNA have indicated that use of the
skeletal-muscle-specific exon (exon 7) in nonmuscle cells (such as HeLa cells) is subject to negative regulation, due to the presence of an inhibitory activity or factor in nonmuscle cells. It remains to be determined whether skeletal muscle cells lack this negative activity or express a muscle-specific factor that overcomes the negative activity, thereby resulting in the use of exon 7. To address this question, we are developing an in vitro splicing system from the myogenic cell line BC3H1 cells. To test whether the nuclear extracts made from BC3H1 cells have an effect on splicing of exon 5 to exon 7 (skeletal-muscle-type splice), fractions from BC3H1 extracts were added to nonmuscle cell nuclear extracts (made from HeLa or 293 cells) and splicing reactions were carried out. The results revealed that BC3H1 cells contain an activity that can stimulate the inclusion of exon 7 when added to nonmuscle cell extracts. Experiments are under way to determine whether this activity is due to a general splicing factor or a muscle-specific factor.

Since it has been reported that alternative RNA splicing might be achieved by changes in the levels or activities of general splicing factors, we have begun to analyze the protein levels of a variety of factors that are known to have effects on alternative splicing in other gene systems. Previous studies in our laboratory have shown a correlation between the activation of the splicing of exons 5 to 7 in nonmuscle cells and reduced binding of the poly-pyrimidine tract binding protein (PTB) to an intron regulatory element immediately upstream of exon 7 (Mulligan et al., J. Biol. Chem. 267: 25480 [1992]). Studies with other genes have also shown that changes in the relative amounts of certain splicing factors may have a role in regulating alternative splicing of their pre-mRNAs. For example, antagonistic effects on splice site selection were demonstrated in vitro between the essential splicing factor SF2/ASF and hnRNP A1 (Mayeda et al. 1993), as well as interactions between U2 auxiliary factor (U2AF) and the Drosophila sex-lethal gene product (Valcarcel et al., Nature 362: 171 [1993]). Therefore, we examined the levels of these factors in BC3H1 cells during differentiation using Western blot analyses. The levels of PTB, U2AF, SF2, and hnRNP A1 all remained constant throughout the differentiation process. Therefore, changes in the levels of these factors per se are not likely involved in the regulation of alternative splicing of exon 7 in the β-TM pre-mRNA.

**RNA-Protein Interactions Involved in Alternative Splicing of β-Tropomyosin Pre-mRNA**

J. Horwitz, Y.-C. Wang

We have begun to identify proteins that interact with the pre-mRNA during spliceosome assembly. One approach is using biotin-RNA probes to detect proteins that interact with the different segments of the pre-mRNA. This method has been used to identify proteins that are associated with pre-spliceosome and spliceosome complexes, as well as snRNPs associated with the pre-mRNA. Using both wild-type and mutant RNAs, we were able to detect a number of specific interactions with proteins in HeLa cell nuclear extracts. In agreement with the results described above, among the associated proteins, we detect binding of PTB to sequences within intron 6. In addition, we detect a number of other polypeptides ranging in size from 30 to 100 kD. We are currently studying the interaction of proteins obtained from myoblasts and myocytes to determine if there are cell-type-specific factors that interact with distinct regions in the pre-mRNA. Such factors would be candidates for tissue-specific splicing factors.

**Role of Brain-specific Tropomyosin Isoforms TMB-1 and TMB-3**

A. Watakabe

The rat α-TM gene expresses at least nine distinct isoforms, including a unique set of isoforms found in the rat brain, named TMBr-1, TMBr-2, and TMBr-3, and are 281, 251, and 245 amino acids in length with apparent molecular weights on SDS-PAGE of 36,000, 31,000, and 31,000, respectively. Last year, we reported on the expression of TMBr-1 and TMBr-3 using an isoform-specific antibody specific for sequences contained within exon 9c of TMBr-1 and TMBr-3 (Stamm et al. 1993). Western blot analysis reveals that the two isoforms are differentially expressed in development, with TMBr-3 appearing in the embryonic brain at 16 days of gestation, followed by the expression of TMBr-1 at 20 days after birth. In addition, although TMBr-3 was detected by Western blot in all brain regions examined (brainstem, cerebellum, cortex, hippocampus, midbrain, olfactory
bulb, striatum, and thalamus), TMBr-1 was detected predominantly in brain areas that derive from the telencephalon (cortex, hippocampus, olfactory bulb, striatum, and thalamus). Immunocytochemical studies on mixed primary cultures made from rat embryonic midbrain indicate that expression of the brain-specific epitope is restricted to neurons, and this was confirmed by the lack of immunoreactivity when immunocytochemistry was performed on glial cultures of the same origin.

The functions of TMBr-1 and TMBr-3 in neurons are not known. What role TMBr-2 has in brain also remains to be investigated. We have prepared recombinant TMBr-1, TMBr-2, and TMBr-3 using Escherichia coli and insect cell expression systems. The recombinant proteins are being used to study the biochemical properties of the individual isoforms, including the ability to bind to F-actin and interactions with other proteins. In addition, we are currently labeling these proteins with various fluorescent compounds or chromophores. The labeled proteins will be introduced into living cells by microinjection. By so doing, we will determine whether the different isoforms exhibit a differential localization or incorporate into different microfilament structures associated with the neuronal cytoskeleton.

**Functional Differences among Tropomyosin Isoforms**

M. Pittenger

Although much is known about the function of TMs from striated muscle, relatively little is known about the isoforms from nonmuscle cells. This is in large part due to the difficulty in isolating the individual isoforms from cells and tissues. By expressing cDNA clones in bacteria and insect cells, we have been able to obtain the individual TMs for in vitro and in vivo studies. During this past year, we have made significant progress in understanding the products of the β-TM gene. In particular, we have found that TM-1 is a major target for the actions of caldesmon. The latter protein promotes the binding of TM to F-actin, which has a key role in stabilizing microfilaments. Our studies to date have been limited to fixed interphase cells. Experiments are in progress to study the dynamic distribution of the different isoforms in living cells during cell spreading, mitosis, and cell movement. In related experiments using polarized epithelial cells, we have found differences in the localization of some TM isoforms (see below).

**Partial Reversion of the Transformed Phenotype by Stable Expression of Specific Tropomyosin Isoforms in Transformed Cells**

J. Kazzaz, M. Pittenger

One of the classic features of transformed cells in culture is their morphologic appearance. Transformed cells often exhibit a rounded morphology with few
identifiable microfilament bundles or stress fibers. A number of studies have documented a decrease in the expression of specific tropomyosin (TM) isoforms in transformed cells, although it remains to be determined if the suppression of TM synthesis is essential in the establishment and maintenance of the transformed phenotype. As a model system, we are using a Kirsten-virus-transformed cell line (ATCC NRK 1569) to investigate the role of TM expression in transformation. In contrast to normal fibroblasts, the Kirsten transformants contain reduced levels of TM-1 and undetectable levels of TM-2 and TM-3. These cells have a rounded morphology and are devoid of microfilaments. We prepared cDNA expression vectors for TM-2 and TM-3, and stable cell lines were established from the NRK 1569 cells that constitutively express TM-2 or TM-3. We have found that increased expression of these isoforms led to cell spreading and flattening and formation of identifiable microfilaments but did not effect density-independent or anchorage-independent growth. However, expression of TM-2 in these transformed cells altered the serum requirements of these cells. Both the parental cell line and cells stably expressing TM-3 can grow in both high serum and low serum, whereas the cells expressing TM-2 can grow only in high serum. These data demonstrate that different TM isoforms can have distinct effects on cell growth and suggest that the suppression of TM expression in transformed cells has a role in the transformed phenotype.

Characterization of Microfilament Proteins in Epithelial Cells

C. Temm-Grove

Among nonmuscle cells, brush-border-type cells have one of the most structured cytoskeletons. Typical brush-border-type cells are found lining the intestine and the proximal convoluted tubule of the mammalian kidney. Although epithelial cells and fibroblasts both exhibit stable arrays of microfilaments, epithelial cells also contain two additional actin-filament structures: an adhesion belt and on their apical surface microvilli. In collaboration with Brigitte Jockusch (Bielefeld, Germany), we have begun to study the components of the epithelial cytoskeleton in order to understand the basis for these different cytoskeletal structures. As a model system, we have been using the porcine renal cell line LLC-PK1. These cells have been reported to retain many functional properties characteristic of renal proximal tubular cells. Using a combination of molecular, biochemical, and immunological approaches, we have begun to study the LLC-PK1 cells, as well as primary intestinal brush border cells and a human cell line CaCO2 (BBE). Our studies demonstrate that LLC-PK1 cells synthesize villin, fimbrin, ezrin, and a 110-kD protein (myosin I), all of which are incorporated into the microvilli, as is characteristic for typical brush border cells. We also observe not only the presence of spectrin and myosin in a terminal web-like structure, but a differential localization of high- and low-molecular-weight TM isoforms in different microfilament structures. Collectively, these studies demonstrate that LLC-PK1 cells, although an immortalized cell line, retain much of the normal brush border cytoskeletal structure. Work is currently under way to determine which isoforms of tropomyosin are located in the adhesion belt, terminal web, and stress fibers of the brush-border-type epithelial cells. In addition, preliminary experiments indicate that specific forms of TM might be expressed in differentiated epithelial cells whose expression appears to be sensitive to transformation.

Isolation and Characterization of cDNA Clones Encoding a Low-molecular-weight Fibroblast Tropomyosin TM-5

W. Guo

Previous work from our laboratory revealed that rat contains at least three tropomyosin genes: α, β, and the TM-4 gene. The α gene is known to encode at least nine distinct isoforms, including skeletal muscle fast-twitch α-TM, smooth muscle α-TM, three isoforms found in brain (TMBR-1, TMBR-2, and TMBR-3), and four isoforms found in fibroblasts and other nonmuscle cells (TM-2, TM-3, TM-5a, and TM-5b). The β gene encodes two isoforms, namely, skeletal muscle β-TM and fibroblast TM-1. The latter form is also expressed in smooth muscle where it corresponds to smooth muscle β-TM. The TM-4 gene encodes a single isoform that corresponds to fibroblast TM-4 and is expressed in a variety of cells and tissues. A fourth TM gene has been reported in a
number of vertebrates including human, mouse, and chicken. Utilizing cDNA clones to mouse fibroblast TM-5 (kindly provided by Peter Gunning), we have isolated and characterized the rat homolog. The rat fibroblast TM-5 contains 248 amino acids and is encoded by a 1.7-kb mRNA. Interestingly, TM-5 comigrates on one- and two-dimensional gels with fibroblasts TM-5a and TM-5b. Northern blot and RNA protection analyses revealed that the gene encoding fibroblast TM-5 encodes at least three other isoforms, including a skeletal muscle isoform that corresponds to the skeletal muscle slow-twitch α isoform. The full-length cDNA has been used to produce recombinant TM-5 to study its actin-binding properties in vitro. In addition, experiments are under way to fluorescently tag the protein for microinjection studies in order to determine the localization of TM-5 in living cells.

Regulation of Neuron-specific Alternative RNA Splicing
S. Stamm

We are using two approaches to understand the molecular basis for neuron-specific alternative RNA splicing: (1) analysis of model pre-mRNAs derived from the clathrin light-chain B gene to identify cis-acting elements and cellular factors involved in alternative splice site selection and (2) computational analysis of genes that are alternatively spliced in neurons. Clathrin light chains are components of clathrin-coated vesicles which are involved in endocytosis and membrane recycling. The light-chain B (LCB) gene encodes two isoforms, termed LCB2 and LCB3, via alternative RNA splicing. The neuron-specific LCB2 isoform is generated by the inclusion of a single exon, termed EN. We analyzed the cis elements involved in splice site selection by transfecting minigene constructs in primary neuronal and HeLa cell cultures. Both the 5' and 3' splice sites of exon EN deviate from the vertebrate consensus sequences. Changing the 5' or 3' splice site of exon EN into the consensus sequence leads to the use of exon EN in HeLa cells. If both splice sites are converted into the consensus sequence, exon EN is constitutively used in HeLa cells. To investigate general mechanisms involved in neuron-specific splicing, we generated in collaboration with Mike Zhang and Tom Marr (CSHL) a database containing all known neuron-specific alternatively spliced exons (Stamm et al. 1994). Splice site analysis of this database shows that about 20% of alternatively spliced exons in neurons have a suboptimal 3' splice site similar to that of exon EN, revealing that an A at the -3 position is an important feature of these 3' splice sites. In addition, we found that neuron-specific exons can be divided into two subgroups according to their splice site quality. One subgroup has relatively suboptimal splice sites, whereas the second subgroup has the same quality as constitutively spliced exons. Using sequence analysis of this database, we noticed sequence identity between exon EN and other neuron-specific exons. We found that exon 9c of the α-TM gene also contains the same sequence motif as in exon EN of clathrin light-chain B. We demonstrated that use of exon 9c is neuron-specific and follows a pattern of developmental regulation identical to that of exon EN (Stamm et al. 1993). Changing this sequence element eliminates the use of exon EN in neurons. RNA gel-shift experiments detect specific RNA-binding proteins in brain nuclear extracts that bind specifically to exon EN. To determine if SR-type proteins might have a role in alternative splicing of exon EN, in collaboration with J. Caceres and A. Krainer (CSHL), we co-transfected the LCB minigene with SF2 in HeLa cells and observed an increase in EN usage. This effect suggests that exon EN inclusion is regulated in neurons by factors similar to SF2. We propose that the use of exon EN is regulated as follows: exon EN is not spliced in nonneuronal cells because its 5' and 3' splice sites differ from the consensus. In neurons, a protein factor binds to sequences within the exon and promotes its use. We postulate that a developmentally regulated neuron-specific exon-binding factor is responsible, in part, for the regulation of a variety neuron-specific exons, such as clathrin light-chain exon EN and α-TM exon 9c.

PUBLICATIONS

In Press


QUEST PROTEIN DATABASE CENTER

J.I. Garrels
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The QUEST Protein Database Center moved into new areas in 1993. The software development effort reached its goal of producing a version of the QUEST software for 2D gel analysis and database construction that is suitable for distribution to other sites. A course was held in April to introduce the new software package to prospective users. While the effort to support and improve the software system continues, a new effort to distribute databases via Internet was begun. The completed rat database has been made available in graphic form via Internet, and our yeast database is next to be made available on the Internet.

A new focus on the yeast database was initiated in 1993. With the genome sequencing effort for the yeast Saccharomyces cerevisiae going on throughout the world, we have concentrated our efforts on building a 2D gel yeast protein database. The current thrust of this effort is protein identifications. With so much knowledge available from yeast genetics, our 2D gels of normal and mutant yeast strains under various growth conditions (as reported in prior years) gain in significance in proportion to the number of proteins identified. Two CSHL researchers, Dr. Bruce Futcher and Dr. Ryuji Kobayashi, have joined the QUEST program for this effort, and as detailed below, we are engaged in three complementary programs for protein identification.

While Dr. Futcher and Dr. Kobayashi joined efforts with the QUEST center in 1993, two of our colleagues, Dr. Scott Patterson and Dr. Robert Franza, ended their affiliation for other pursuits. Dr. Patterson moved to California in May to take a new position at Amgen, and Dr. Franza is in transition to a new biotechnology venture. Both Scott and Bob were stimulating influences at QUEST, and both will continue their interest in protein databases in their new positions.

A Yeast Protein Database of Known Protein Properties

J.I. Garrels

A good starting point for a 2D gel database of the yeast proteins is to gather as much information as possible from the literature and from the protein sequence databases about the known properties of the yeast proteins. More than 2000 protein sequences
from yeast are now known. Using the sequence data to calculate molecular weight, isoelectric point, and codon bias, we can greatly narrow the number of possible identifications for each spot on a 2D gel. Other relevant information from the literature includes amino- and carboxy-terminal modifications, information on protein phosphorylation and glycosylation, and the identification of precursor peptides for secretory pathway or mitochondrial proteins. Furthermore, knowing the expected subcellular localization and regulators of expression of each protein can help greatly for identification and for understanding of function.

A yeast protein database, called YPD, has been compiled that contains one entry for each known yeast protein. For each protein, the gene name and its synonyms have been determined, and when multiple entries are available in the sequence databases, the sequence most likely to be correct for strain S288C is used. The YPD database is available as a table suitable for spreadsheet analysis. The fields provided for each protein include calculated molecular weight, calculated isoelectric point, codon bias, subcellular localization, molecular environment (association with membrane, DNA, RNA, cytoskeleton, or soluble), and functional categories (protein kinases, phosphatases, heat shock, proteases, tRNA synthetases, GTP-binding proteins). Other fields report known amino-terminal modifications (acetylation or myristylation), carboxy-terminal modifications (prenylation or addition of GPI anchors), and the lengths of any precursor peptides. Finally, the amino acid composition of the mature protein and a brief description are given. All of this information has great relevance to the characterization of proteins on 2D gels. The database will be made available on Internet as part of our yeast database project.

An image of the entire yeast protein map is shown in Figure 1. This map represents the expected size and charge of the mature form of each protein, after

![All Known Proteins of Saccharomyces cerevisiae](image-url)

**FIGURE 1** A "theoretical 2D gel plot" for all known yeast proteins. Each spot is positioned by its calculated molecular weight and isoelectric point, taking into account removal of precursor peptides and amino- and carboxy-terminal modifications. The spot size is determined by codon bias.
compensating for the effects of precursor peptide cleavage and amino-terminal modification. The plotted spot size is a function of codon bias, which is a good predictor of the level of maximal gene expression. The enlargement in Figure 2 shows a section of the map with gene names for the major proteins. Those with known or probable identification on our 2D gel maps are underlined. Each of the identified spots has been checked for consistency with previously run 2D gels, which show the location of heat shock proteins, ribosomal proteins, acetylated proteins, and proteins coded from spliced genes (see work in collaboration with C. McLaughlin and J. Warner reported in prior years).

**Protein Identification by Double-labeled Amino Acid Analysis**

J.I. Garrels, G.I. Latter

To identify proteins that are products of sequenced genes, one method is to compare amino acid compositions. The relative amino acid compositions of proteins on 2D gels can be obtained by labeling cells with different $^{35}$S and $^{14}$C amino acid precursors. This information, coupled with expected size, charge, codon bias, and other information provided by the YPD (see above), can allow many of the spots on 2D
gels to be identified. We have been employing this approach, using yeast cells double-labeled with one $^{35}$S amino acid and one $^{14}$C amino acid.

The relative incorporation of two amino acids can be determined for each spot on 2D gels by the rate of isotope decay. We have exposed our gels every 2 weeks for up to 4 months, and the images have been analyzed by a special version of our image analysis software. After detailed alignment of the multiple images, the rate of decay for each pixel of the image can be calculated, and the average rate of decay for each spot can be calculated. Spots that contain more of the $^{14}$C amino acid will decay slowly, and spots that contain more of the $^{35}$S amino acid will decay more rapidly, approaching the half-life of $^{35}$S which is 3 months.

Using the rate of decay for known proteins, we create a standard curve for each gel. A standard curve for a gel containing $[^{35}]$S methionine and $[^{14}]$C histidine is shown in Figure 3. About 24 known proteins follow closely on the line, which relates isotopic ratio to amino acid ratio. We have analyzed gels so far from about ten different double-labeled amino acid combinations.

The analysis of the data for several hundred spots of each gel is carried out on a spreadsheet. For each spot from a 2D gel, the program selects the proteins from the YPD within the expected range of molecular weight, isoelectric point, and codon bias and then gives the candidate a score from each gel, based on the number of standard deviations from the expected amino acid ratio. When a spot emerges with a score much better than all the others, it is in most cases the correct identification. The method has identified more than 30 spots so far, and more identifications are coming clear as more data are analyzed. Each new identification, from any one of our three methods (protein sequencing, expression from multicopy plasmids, and amino acid composition), improves the quality of the standard curves and aids in making future identifications. Addition of new protein entries to the YPD database, as new gene sequences appear in the sequence databases, also improves the number of identifications that can be made.

Yeast 2D Gel Protein Database: Protein Identification by Overexpression

B. Futcher, T. Volpe

One method for identifying yeast proteins on 2D gels is to overexpress a known protein from a high-copy-number plasmid and see which spot on the gel becomes darker. This method has been tried with several dozen proteins. In cases where the codon bias was less than 0.2 (suggesting a relatively rare pro-
tein), the method did not work, presumably because even the overexpressed protein was too rare to be seen. However, in all cases so far when the codon bias was greater than 0.4 (and in some cases between 0.3 and 0.4), an extra-dark spot was seen at about the expected mobility. In most cases, there were also one or two extra nearby spots that may represent posttranslationally modified forms. We are now collecting high-copy plasmids so that we can use the method to identify all of the abundant proteins. Our initial goal is 200 identifications.

We have also begun to study the phosphorylated proteins. In vivo labeling with $^{32}$P or $^{33}$P gives a pattern containing several hundred spots. Many proteins appeared to have multiple phosphates. Perhaps surprisingly, cell cycle position has very little effect on the pattern.

Finally, we have done pulse-chase experiments with $[^{35}S]$methionine to examine half-lives. Most of the proteins in the 2D gel are stable, but a few turn over rapidly.

Identification of S. cerevisiae Proteins after 2D Gel Electrophoresis by Protein Sequencing

R. Kobayashi, B.J. Schwender

To construct a protein database of S. cerevisiae on 2D gels, we have been identifying proteins in each spot by protein sequencing. The 2D gel electrophoresis was performed at the Cold Spring Harbor Laboratory 2D gel laboratory core facility. Each gel was loaded with several hundred micrograms of protein. We applied the technique for internal amino acid sequence analysis of protein after SDS-PAGE, which is currently used lab-wide at Cold Spring Harbor Laboratory. The method is carried out as follows: After the gel electrophoresis, proteins are stained by Coomassie brilliant blue G and the excised protein bands are transferred to a microfuge tube. The gel is soaked in 1 ml of 50% methanol twice for 20 minutes at room temperature to remove SDS. *Achromobacter* protease I (lysylendopeptidase) is then added to digest the protein in the gel. After digestion for 24 hours, the peptide fragments are extracted by 0.1% TFA in 50% acetonitrile. The digest is then separated by reverse-phase HPLC using a C18 microbore column, and the purified peptides are sequenced by using an automated protein sequencer. This technique usually allows us to obtain sequence information for proteins in the amount of 1–2 µg for 50 kD. After obtaining sequence information, each sequence is searched for homology in the protein database through the computer network. So far, we have obtained good sequencing data to identify nine proteins and two unknown proteins. The nine proteins and their gene names are aldolase (FBA1), heat shock protein 70K (SSB1), triose-phosphate isomerase (TPPI), posttranslationally modified form triose-phosphate isomerase (TPPI1), alcholoh dehydrogenase (ADH1), enolase 1 (ENO1), enolase 2 (ENO2), pyruvate decarboxylase 1 (PDC1), and inorganic pyrophosphatase (PPA1). Two other proteins had no match from the yeast protein sequence database, although one of these has homology with an initiation factor in *B. subtilis*. We will continue this work to identify as many proteins as possible in the 2D gel map.

**QUEST II Software for 2D Gel Analysis**

P.J. Monardo, T. Boutell, J.I. Garrels, G.I. Latter

The QUEST II software for analysis and construction of protein databases has been released and delivered to several sites. A number of users who attended our course entitled Two-Dimensional Gel Analysis and Protein Database Construction now have the software at their own sites. The architecture has proven especially successful for a networking environment, and at the QUEST center, the client-server architecture is allowing multiple users to interact to build new databases.

This QUEST II software system has many new features that make the construction and analysis of databases quicker and easier. The most time-consuming part of database construction in the previous system was the detailed editing of the spot data. Most of this is now done automatically. The combination of the QUEST II software as a database building tool and our 2D Gel Network Publishing system as a publishing tool is proving to be very powerful.

The QUEST II software was chosen as a nominee for the Computerworld Smithsonian Software Award in the category of Scientific Achievement for 1993 and was then chosen as a finalist.
We have completed the first phase of a project to make 2D gel data available via the Internet using a system called World Wide Web. The gels are displayed over the Internet and allow biologists to point and click on the identified protein spots to obtain information about the protein. This information includes the protein name and other annotations, the molecular weight, the isoelectric point, and the Swiss Protein Database Accession number. The links provided in the database allow direct access to SwissProt and other molecular biology databases simply by clicking on a spot on a 2D gel image. The annotative data from the REF52 database is the first database that we have made available. We are now adding more annotations for protein sets, such as the nuclear, cytoskeletal, mitochondrial, mannose-labeled, or phosphorylated proteins from the rat database.

A system has been constructed that automatically accesses the QUEST II databases and prepares data for publication via World Wide Web if the data have been marked to be published. Additionally, a hypertext tutorial on the QUEST II software and general information about the QUEST Protein Database Center have been made available for Internet users.

The 2D gel laboratory in the Demerec building has provided 2D gel services for 14 different groups here at the Laboratory and for 6 outside researchers. Nearly all of the 2152 gels run in 1993 were detected using phosphorimaging, and the images were archived on tape. The computer storage for 2D gels has been enhanced, and Jerry Latter has helped to automate the image handling aspects. A high-quality laser image of each gel is now routinely printed and sent to the user. Other services include silver and Coomassie staining, including the use of a special high-purity Coomassie stain that has been very successful for protein sequencing from 2D gels.

PUBLICATIONS

In Press
In the last century, Cold Spring Harbor Laboratory began its life as a biological institute as an adjunct to the local whaling fleet. With the collapse of the whaling industry, the laboratory was reinvented, and even from these earliest beginnings, an interest in genetics was apparent. This interest took an unfortunate turn with short-lived leanings toward eugenics. The best debunking of the pursuit of eugenics came not from the outcries of moralists but from the unwitting comment of the playwright George Bernard Shaw. The inveterate bachelor was approached one day by a most attractive woman who propositioned Shaw with the following suggestion. "Just imagine what our child would be like with your brains and my looks." The playwright is reputed to have replied laconically, "but my dear, what if the unfortunate child were to have your brains and my looks." Described below are the results of the genetic excursions undertaken at Cold Spring Harbor during the last year.

EUKARYOTIC CELL CYCLE CONTROL

D. Beach S. Allan M. Caligiuri D. Casso T. Connolly S. Davey D. Demetriick H. Feilotter
K. Galaktionov I. Garkavtsev C. Gawel G. Hannon J. Hofmann J. Hudson
N. Kaplan D. Lombardi K. Lundgren S. Matsumoto T. Matsumoto B. Nefsky
K. Okamoto M. Serrano A. Tesoro N. Walworth Y. Xiong H. Zhang

During the course of the year, Harriet Feilotter left to take a staff position at Cold Spring Harbor (Hershey Building) with the Manic Depression Program, and Igor Garkavtsev took a position at the University of Calgary. Karen Lundgren became a Staff Scientist at Glaxo in North Carolina, Yue Xiong accepted a position as Assistant Professor at the University of North Carolina at Chapel Hill, and Nancy Walworth went to the Netherlands Cancer Center. We were joined by one new postdoc, Doug Conklin, and a lab technician, Gretchen Hannon.

Checkpoint Controls

N. Walworth, D. Beach

Cell cycle progression in eukaryotic cells is controlled by the activity of complexes of proteins consisting of a protein kinase catalytic subunit and a regulatory subunit called a cyclin. In fission yeast, a complex between p34\textit{cdc2} and p56\textit{cdct3} regulates the transition from G2 to mitosis. The p34\textit{cdc2} subunit itself is regulated through phosphorylation. The kinase tyrosine residue is maintained by the products of the \textit{weel} and \textit{mikl} genes. Phosphorylation of p34\textit{cdc2} on tyrosine is required to prevent cells from entering mitosis prematurely. Simultaneous loss of function of \textit{weel} and \textit{mikl} results in advanced progression into mitosis and leads to cell death. Mutations in the \textit{cdc2} gene itself can compensate for the loss of function of \textit{weel} and \textit{mikl}. These particular alleles of \textit{cdc2} are cold-sensitive and cause G2 arrest at the restrictive temperature. The proteins encoded by the mutant alleles have low kinase activity at any temperature.

A number of genes were isolated that allow the cold-sensitive alleles of \textit{cdc2} to grow at the restrictive temperature. One of these genes encodes a novel protein kinase which has been called \textit{chk1}. The \textit{chk1}
gene product is not required for cell viability during normal vegetative growth. However, under certain conditions, the chkl gene product is required for cell survival. Characterization of the chkl protein kinase suggests that it is required for the checkpoint in G2 that causes temporary cell cycle arrest when DNA is damaged; chkl encodes a checkpoint kinase.

Cells that have been exposed to DNA-damaging agents normally arrest the cell cycle to repair the damage in order to avoid the accumulation of mutations or errors in the segregation of chromosomes. Cells lacking chkl function fail to arrest the cell cycle in G2 when DNA is damaged by irradiation or when unligated DNA is generated upon inactivation of DNA ligase. The chkl protein kinase is not required for the physical repair of damaged DNA nor is it required to prevent entry into mitosis when DNA replication is blocked. It is likely that the kinase is involved in arresting the cell cycle in response to signals generated by damaged DNA. Since chkl was isolated as a multicopy suppressor of a cdc2 mutant, it is likely that chkl has the ability to influence the activity of p34cdc2. Indeed, overexpression of chkl inhibits cell cycle progression. Identification of the targets of the chkl protein kinase and of the signals that activate it will aid in the understanding of the mechanism of cell cycle arrest in response to DNA-damaging agents.

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**p16INK4: A Specific Inhibitor of the CDK4/Cyclin D Kinase**

M. Serrano, D. Beach

The complexes formed by CDK4 and the D-type cyclins have been implicated in the control of cell proliferation during the G1 phase of the cell cycle. In normal proliferating cells, CDK4 associates with D-type cyclins and with a protein with a molecular mass of 16 kD, p16INK4 (inhibitor of CDK4). In human cells transformed with viral oncoproteins that inactivate the Rb tumor suppressor protein, p16INK4 is overexpressed, being the main, if not exclusive, partner of CDK4. We have isolated a human cDNA clone encoding p16INK4 by using the two-hybrid screening system in yeast with CDK4 as the target protein. p16INK4 specifically associates with CDK4 in vitro and in vivo but not with other CDKs. We have reconstituted active CDK4–cyclin D kinases in insect cells and have found that p16INK4 specifically inhibits the kinase activity of CDK4. In normal cells, the balance between p16INK4 and D-type cyclins could determine the level of CDK4 kinase activity. Inactivation of Rb during the mid-G1 phase is thought to be fulfilled by CDK4 associated with D-type cyclins. In transformed cells expressing viral oncoproteins, such as T antigen, E1A, or E7, Rb is constitutively inactivated and the G1/S control is, at least, partially disrupted. We think that the disruption of the G1/S control is sensed by a signaling pathway that up-regulates the expression of p16INK4, resulting in the inhibition of CDK4 activity; however, this negative loop would be unable to restore the Rb-dependent G1/S control because Rb function in these cells is constitutively abrogated.

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**Target Genes of the Cell-cycle-regulated Transcription Factor cdc10**

J.F.X. Hofmann, D. Beach

The fission yeast cdc10 gene product is required for cell cycle progression at the G1/S boundary. Recently, we isolated two novel target genes of the cdc10 transcription factor, cdt1 and cdt2. Gene disruption experiments have shown that cdt1 is an essential gene whose function is required for the entry into S phase. Surprisingly, although cdt1 null mutants do not synthesize DNA, they enter an abnormal mitosis. cdt1 is therefore required for the normal dependency of mitosis on the previous S phase.

Mutations in two other cell cycle genes, cdc18 and cut5, express essentially identical phenotypes like cdt1. We have thus examined the possibility of genetic interaction between these genes. Indeed, elevated gene dosage of either cdt1 or cdc18 can suppress the lethal phenotypes of cut5 mutations. This and other observations suggest that cdt1, cdc18, and cut5, in addition to their requirement for DNA synthesis, act in an essential checkpoint control pathway to inhibit the premature entry into mitosis. We also conclude that the activation of mitotic pathways is intrinsically completely independent from S phase.

The cell-cycle-regulated expression of both cdc18 and cdt1, which is dependent on cdc10, appears to be essential for cell cycle progression under certain conditions. Ectopic expression of both cdt1 and cdc18 is lethal under conditions where the entry into S phase is rate-limiting for cell cycle progression. This suggests that the expression of cdt1 and
cdc18 must be properly controlled within the cell cycle.

The other novel target gene of cdc10, the cdt2 gene, also is an essential gene. The construction of a null allele has shown that cdt2 is surprisingly not required until the last landmark event in the cell cycle, namely cytokinesis. Mutant cells arrest as binucleate septated cells with each nucleus containing a 1N complement of DNA. Eventually, these nuclei continue to replicate DNA followed by a normal mitosis and cell septation. However, cytokinesis does not occur, which results in the formation of elongated multinucleate cells. These observations suggest that Start is coupled with cytokinesis in a pathway that involves the function of the cdt2 gene.

The GTPase Signaling Cascade Prevents Premature Mitosis

T. Matsumoto, D. Beach

A checkpoint functions during each cell cycle to ensure that DNA synthesis is complete before mitosis is initiated. In fission yeast, loss of pim1+, a homolog of RCC1, disrupts this checkpoint, resulting in premature entry into mitosis. Mutation in RCC1 in hamster BHK cells results in similar phenotype, suggesting functional as well as structural conservation of this gene between species.

spl+, a multicopy suppressor of pim1, encodes a ras-like GTPase, which binds to pim1+. The pim1/spl complex dissociates in the presence of GTP and Mg++, suggesting that pim1 acts as a guanine nucleotide exchanger on spl GTPase.

On the basis of the assumption that pim1+ activates spl GTPase, we hypothesize that pim1+ senses the chromatin state during the cell cycle and changes its activity as a guanine nucleotide exchanger. The activity might be higher during G1 and S phases to prevent cells from entering into mitosis prematurely. As a collaboration with Dr. Bruce Stillman's laboratory, we have demonstrated that bacterially expressed pim1+ can bind in-vitro-assembled chromatin. We are currently examining whether the activity of pim1+ is altered when bound to chromatin.

We are also studying two additional genes that may have a role at the pim1/spl checkpoint. Mutations in esp1+, which encodes a protein phosphatase, can suppress the pim1 mutation. Another mutant, pim2, shows a phenotype similar to that of pim1, and the double mutant pim1 pim2 is a synthetic lethal, suggesting a close relationship between the two gene products. pim2+ could possibly encode a factor acting downstream from pim1+ or spl+, such as an effector of the GTPase.

Reconstitution and Characterization of p21/PCNA/Cyclin/CDK Quaternary Kinase Complexes

H. Zhang, D. Beach

The mammalian cell cycle is controlled by multiple cdc2-like kinases. Differences exist between normal and transformed cells in their ability to respond to various growth conditions and external stimuli during the cell cycle. We have established that these differences appear to be reflected in the changes in the basic composition of the cell cycle kinases. In normal cells, the majority of cell cycle kinases exist in the form of quaternary complexes of cyclin/CDK associated with two universal components, PCNA and p21. In many transformed cells, the majority of the kinases are in the form of cyclin/CDK binary complexes which sometimes associate with other proteins. In the past year, we have focused on the isolation and characterization of these proteins. We have cloned the gene for p21 through its association with cyclin D1. Using a baculovirus expression system, we have reconstituted the cyclin/CDK/PCNA/p21 quaternary complexes in vitro. Biochemical analysis indicated that p21 is a universal inhibitor of cell cycle kinases that exerts its effect by association with the kinases. p21 is also capable of forming an independent complex with PCNA. No effect of PCNA on the kinases has been observed. However, since in normal cells, the majority of cell cycle kinases are in the form of cyclin/CDK/p21/PCNA quaternary complexes, how do these cells divide at normal rate, whereas most kinases are in inactive form? Our study suggested that p21-containing kinases can exist in both active and inactive states. The single p21-containing kinases are catalytically active. The binding of multiple p21 is required for the inhibition of the kinases. The p21 inhibition of the kinases appears to be cooperative. These results suggest that in vivo, the p21-containing kinases can transit between active and inactive forms, depending on the level of p21 protein.
In viral oncogene-transformed cells such as HeLa or 293 cells, p21 and PCNA disappear from the cyclin A/CDK2 kinase. Instead, another protein, p19, associates with cyclin A/CDK2 kinase. p19 has been isolated through its association with cyclin A and its gene has been cloned. The cloned p19 shows no homology with p21 and thus is a new type of protein that interacts with the cyclin/CDK kinase. The characterization of p19 is in progress.

p21, a Potential Effector of the p53 Cell Cycle Checkpoint
G. Hannon, H. Zhang, D. Casso, R. Kobayashi, D. Beach

A wide variety of genetic alterations can contribute to neoplastic transformation. However, one feature common to most transformed cells is their ability to execute the cell division cycle under conditions that would prevent or slow the proliferation of normal cells. This suggested that one of the ultimate effects of alterations in oncogenes or tumor suppressors must lie in at least a partial derangement of cell cycle control.

The pathways that oversee cell cycle progression have been termed "checkpoints." It has recently become clear that the tumor suppressor protein, p53, functions in a cellular checkpoint that responds to damaged DNA. Upon DNA damage (e.g., by ionizing radiation), p53 levels increase, and the cell cycle arrests, allowing the damage to be repaired. p53 is a transcriptional activator, and this property is critical to its function both as a checkpoint protein and as a tumor suppressor. We have recently demonstrated that one of the targets of the p53 transcription factor is the 21-kD subunit of cyclin kinase complexes (p21).

The passage of a mammalian cell through the cell cycle is controlled by a family of related protein kinases known as the cyclin-dependent kinases or CDKs. The activation of specific members of this family is required for the execution of each cell cycle phase. CDKs are regulated posttranslationally by phosphorylation and by complex formation with regulatory subunits. In particular, CDKs require the association of a positive regulatory subunit known as a cyclin for activity. Studies from a number of laboratories suggested that the binary complex of a cyclin and a CDK formed the core enzyme that controls cell cycle progression. This view persisted until these enzymes were examined in normal fibroblasts, rather than in the many oncogenically transformed cell types that had been previously investigated (Xiong et al. 1993).

In normal fibroblasts, the major population of cyclin kinases exists in quaternary complexes consisting of cyclin, CDK, PCNA, and a protein of 21 kD. In vitro, reconstitution using cyclin kinase expressed in baculovirus-infected insect cells revealed that p21 is a universal inhibitor of cyclin kinases, although the affinity of p21 varied for each cyclin/CDK pair.

Previous analysis of cyclin kinase complexes in a large number of cell lines indicated that the presence of p21 correlated with the presence of functional p53. For example, p21 was absent from cells expressing p53-inactivating viral oncoproteins (e.g., HPV-16 E6) or from cells containing inactive, mutant p53 (e.g., Li-Fraumeni fibroblasts). These observations prompted us to test the possibility that p21 might be a target for the p53 transcription factor. Both p21 mRNA and p21 protein are rapidly induced upon activation of the p53 checkpoint pathway. p21 message levels increase approximately 10-fold upon irradiation of ML-1 cells which contain wild-type p53. Furthermore, primary fibroblasts derived from p53 "knockout" mice (kindly supplied by Tyler Jack, Massachusetts Institute of Technology) contain approximately 50-fold reduced levels of p21 as compared to fibroblasts from their wild-type siblings. Finally, genomic sequences upstream of the human p21 gene contain sites capable of binding to p53.

Considered together, our observations suggest that p53 imposes cell cycle arrest through its regulation of the cyclin kinase inhibitor, p21. Thus, our results form one of the few concrete links between the basic machinery of cell cycle control and a genetically identified tumor suppressor protein.

Role of sct1 in the Regulation of Start in the Fission Yeast Cell Cycle
M. Caligiuri, D. Beach

In the fission yeast Schizosaccharomyces pombe, passage through Start and subsequent commitment to the mitotic cell cycle requires the function of the Cdc2 protein kinase (p34cdc2) and the Sct1 and Cdc10 tran-
scription factors (p72sct1 and p85cdc10, respectively). Strains defective in any one of these genes will conjugate from the point of arrest. The Sct1 protein is unique, however, in that it functions in a dual capacity to negotiate passage through the G1/S-phase transition. Cells that carry the sctl null allele arrest in G1 as highly elongated cells that are capable of mating under conditions normally inhibitory to this process. This suggests that p72sct1 functions as a positive regulator of the mitotic cell cycle and as a negative regulator of the cells' alternate developmental fate leading to sexual differentiation.

p72sct1 and p85cdc10 share amino acid sequence similarity with the budding yeast Swi4 and Swi6 transcription factors. The p72sct1 and p85cdc10 proteins associate to form a complex that is involved in the regulation of the cdc18, cdc22, and cdt1 genes whose transcripts are periodically expressed, peaking in late G1/early S phase. Genetic evidence suggests that p72sct1 and p85cdc10 do not, however, perform equivalent functions and that perhaps p72sct1 is required for an additional essential function(s). We have initiated a genetic analysis of the sctl gene in order to elucidate these activities and to identify additional proteins which interact with and perhaps regulate sctl. The basis of this genetic screen exploits the observation that overexpression of the wild-type sctl gene can rescue mutants carrying temperature-sensitive alleles of cdc10 (cdc10ts-). We therefore envisioned that conditional alleles of sctl could be obtained by transforming a cdc10ts- strain with a library of mutagenized sctl plasmids and screening these transformants for temperature-sensitive growth. Plasmids from strains incapable of growth at the restrictive temperature were recovered and transformed into a strain heterozygous for the sctl disruption. With this screen, we have obtained a collection of sctl mutants that display a variety of cell cycle defects. Most interesting are two classes represented by mutants that display a classic cell-cycle-arrest phenotype, some of which mimic the null phenotype (i.e., cell cycle arrest and de-repression of the mating pathway), and those that display defects in the meiotic pathway. Members of this later group initiate the mating pathway and undergo conjugation but apparently fail to execute functions required to activate the meiotic pathway. These strains will be used in a classic suppressor analysis, as well as in a screen to identify downstream targets of the p72sct1 transcription factor by seeking cDNAs capable of rescuing the sctl mutant when expressed ectopically under a heterologous promoter.

**Interaction between the RAN1 Protein Kinase and PUC1 in the Fission Yeast Cell Cycle**

T. Connolly, D. Beach

The mechanism by which *S. pombe* cells recognize nutritional conditions and respond either by arresting in G1 at Start to initiate mating and subsequent meiotic functions or by maintaining the haploid mitotic cycle is not well understood. We have cloned a CLN-type cyclin in fission yeast, puc1, by its ability to complement the loss of the CLN-type cyclins in *Saccharomyces cerevisiae*. We have shown that p40puc1 associates with p34cdc2 to activate its histone H1 kinase activity and defines a G1 form of the p34cdc2 protein kinase in fission yeast. We have begun to investigate the function of the p40puc1 protein complex in the cell cycle by attempting to identify proteins that associate with the p40puc1/p34cdc2 protein complex.

We have identified the p52ran1 protein kinase as a substrate of the p40puc1/p34cdc2 protein kinase. The transition from the vegetative cell cycle to meiosis and sporulation in the G1 phase of the cell cycle is controlled in part by the ran1+ gene product. The ran1 gene encodes a 52-kD protein kinase that is essential for the vegetative cell cycle and functions as a negative regulator of sexual conjugation and meiosis. Immunoprecipitation and Western blot analysis have revealed that p52ran1 and p40puc1 are associated in vivo. A 52-kD protein that comigrates with autophosphorylated p52ran1 is coimmunoprecipitated with p40puc1 and becomes phosphorylated in vitro. Immunoprecipitates of p40puc1/p34cdc2 kinase reactions. We have determined that the phosphorylated product identified in the p40puc1 immunoprecipitates is in fact p52ran1 by partial protease mapping of this 52-kD phosphorylated product against autophosphorylated p52ran1. In addition, the overproduction of puc1 rescues a temperature-sensitive ran1 mutant. These observations support a model in which these two proteins interact to promote G1/S-phase progression. The biochemical consequences of the interaction between the p52ran1 and p40puc1 proteins are under current in-
vestigation. Specifically, we are interested in determining if the phosphorylation of p52ranl by p4opuc/p34cdc2 affects its catalytic activity. To date, no substrates of the p52ranl kinase have been identified; however, we have detected an allele-specific interaction between the p72sctl transcription factor and p52ranl. This suggests that p72sctl may be a substrate of the p52ranl kinase, a possibility that is under current investigation.

**PUBLICATIONS**


**PLANT GENETICS**

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**Enhancer-trap and Gene-trap Transposon Mutagenesis in Arabidopsis**


Last year, we reported on the development of an enhancer-trap/gene-trap mutagenesis system, using maize transposons in Arabidopsis. This approach to insertional mutagenesis, which has been used with spectacular success in *Drosophila*, allows the identification and cloning of genes by their expression patterns. It is particularly advantageous in identifying genes whose mutant phenotypes can mask an essential function or are unrevealing, e.g., genes with multiple essential functions (early as well as late) or genes that are partially or completely redundant. We have utilized the Ac-Ds transposon system discovered by McClintock, engineered for our purpose as follows. The Ds elements carry a GUS reporter gene with either a minimal promoter (enhancer-trap) or a multiple splice acceptor sequence (gene-trap) fused
to the 5' end of the gene. Expression of the reporter gene depends on the element inserting within or close to a chromosomal gene. The gene-trap element (DsG) must insert within the transcribed region of a gene in the correct orientation in order to get GUS expression, whereas insertions of the enhancer-trap element (DsE) in either orientation, or into the nontranscribed region, can also result in GUS expression. Enhancer-trap insertions are more likely to result in reporter gene expression, but gene-trap insertions that generate expression patterns are more likely to result in mutant phenotypes. Furthermore, cDNA cloning of the gene by RACE-polymerase chain reaction (PCR) is feasible for gene-trap insertions (as described in the following section; Springer et al.), and gene trap insertions can result in translational fusions that provide information about the intracellular localization of the gene product. Details of the construction of these elements are given in the 1991 and 1992 Annual Reports.

A major limitation with the Ac/Ds transposon system is the preferential transposition of the elements to closely linked sites. To overcome this limitation, we have designed a novel selection scheme that selects against closely linked transpositions. This selection scheme has been described in detail in the 1992 Annual Report and is summarized briefly here. The donor Ds element is linked to the IAAH (indole acetic acid hydrolase) gene which confers sensitivity to the auxin analog NAM (naphthalene acetamide). The Ds element also carries an NPTII gene that confers kanamycin resistance. The Ac element that we use is a fusion of the 35S promoter to the Ac transposase gene; this element cannot transpose since one end has been deleted. The Ac element is linked to both an NPTII gene and an IAAH gene. The Ds element is mobilized by crosses of Ds homozygous plants to Ac homozygous plants. The transpositions occur in the F1 plants, and the progeny of these plants are selected for resistance to both kanamycin and NAM. Therefore, we select against the donor site of the Ds element, as well as against the Ac element, and transposed Ds elements that are not linked to the donor site will be preferentially recovered. Last year, we described results showing that the selection scheme could be successfully implemented and that plants containing new independent transposed Ds elements (transposants) can be generated using this scheme. By crossing transposants back to plants that carry the donor Ds, it is possible to estimate their linkage to the donor site. In three cases examined so far, we find that two of the transposed Ds elements are unlinked to the donor site and the third showed loose linkage (about 25 cM). A more detailed analysis of the linkage of the transposed Ds elements is underway, but these preliminary data are further evidence that the selection scheme is working as planned. We have initiated large-scale mutagenesis of Arabidopsis by crossing Ac lines to the DsG and DsE lines. From about 100 crosses, approximately 7500 F1 progeny have been generated, of which about 3000 have been selfed. The selection for transposed Ds elements has been carried out on 1282 of these F2 families, and 496 independent transpositions have been recovered. Therefore, approximately 39% of the F1 progeny from the crosses of Ac to Ds give rise to transpositions that are selected for in our scheme (i.e., transpositions that are unlinked or loosely linked to the donor site). This encouragingly high frequency suggests that generation of large numbers of independent transposition events is feasible starting with a relatively small number of crosses. Currently, we are scaling up our efforts with the aim of generating 4000 transposon insertions in the coming year.

The plants carrying transposon insertions (transposants) are maintained as individual lines by selfing. These lines will then be screened for patterns of reporter gene expression and for mutant phenotypes due to the insertion. In a preliminary screen, 151 of the gene-trap insertion lines and 23 of the enhancer-trap insertion lines were examined for GUS expression in seedlings, leaves, and flowers. About 18% of the gene-trap insertions and 40% of the enhancer-trap insertions were found to result in GUS expression in this screen. The lower frequency observed with gene-trap insertions is expected from the requirement that the gene-trap DsG element must insert within the transcribed region of a gene and in the correct orientation in order to get GUS expression, whereas insertions of the enhancer-trap DsE element outside the transcribed region can result in GUS expression. Many different GUS expression patterns were observed in transposant lines using both DsG and DsE. The seedling patterns included expression restricted to the cotyledons, expression in the shoot and root meristematic zones, and expression in the hypocotyl (see below). The leaf patterns included expression in leaf primordia and expression restricted to the vasculature and hydathodes. The floral patterns included expression in the whole flower, expression restricted to the outer two whorls of the flower, expression restricted to the filaments of
the stamens, expression only in the anthers, expression in the carpels and the vasculature of the sepals, expression only in the carpels, and expression restricted to the zone of fusion of the carpels. The number of diverse patterns observed is extremely encouraging and suggests that many different genes can be identified even in the relatively small number of lines examined. In addition, a preliminary screen for new mutations was carried out using about 100 insertion lines, and two visible mutations were identified. One of these mutations affects the vasculature of the hypocotyl in the seedlings, and specific GUS staining of the hypocotyl is observed in this line, consistent with the mutant phenotype. This mutation is currently being characterized further. The other mutation was identified as a gametophyte lethal and is discussed in detail below (Springer et al.).

Analysis of indeterminate Gene Function in Maize

J. Colasanti, V. Sundaresan

A maize plant is normally programmed to make a particular number of vegetative structures (e.g., leaves), followed by the reproductive structures (flowers), and eventual senescence of the plant. Plants that are homozygous for the indeterminate (id) mutation are defective in the execution of this program and exhibit several developmental phenotypes: (1) The vegetative to reproductive transition is altered such that the vegetative phase is prolonged, resulting in plants with an extensive (or indeterminate) life span; i.e., they flower much later than normal plants, or not at all. (2) The vegetative phase expands into the reproductive phase of development and causes abnormal flower development; i.e., the female flower (ear) exhibits vegetative characteristics and is usually sterile, and the male flower (tassel) often undergoes a complete developmental reversion such that new vegetative shoots emerge from tissues that begin to develop as floral tissue. In the latter case, terminally differentiated cells that comprise floral tissue redifferentiate into vegetative tissue and resume proliferative growth. These phenotypes suggest that the function of the normal Id gene is to suppress vegetative growth and signal the beginning of reproductive growth at a specific time during the life cycle of the plant. Loss of Id function results in the failure to demarcate this transition and causes prolonged vegetative development.

In an attempt to understand better the vegetative to reproductive transition in plants, we are using transposon tagging to isolate this gene from maize. The Id gene maps near the kernel pigmentation gene, Bz2, on chromosome 1. A mutable allele of the Bz2 gene, bz2-m2, results from the insertion of a Ds2 transposon at this locus. (Ds2 is a defective derivative of the Ac-Ds family of transposable elements and is able to transpose only in the presence of an Ac element which provides transposase.) Taking advantage of the proximity of Id to bz2-m2, and the fact that Ac/Ds elements often transpose to linked sites, we selected for id mutants from germinal revertants in the bz2-m2 population; i.e., by selecting for completely purple kernels that resulted from germinai excision of the Ds2 element (i.e., bz2-m2 to Bz2), an F1 population with the Ds2 excised and inserted elsewhere was generated. From an F2 population of these revertants, one id mutant was isolated from 600 families examined and designated id*. Crosses of id* to known alleles of id confirmed that it is allelic to the id mutation of chromosome 1.

Through several generations of out-crosses and back-crosses, the id* allele was introduced into genetic backgrounds with or without active Ac elements. Preliminary data suggest that id* plants with active Ac elements have a less severe phenotype than those with no Ac; i.e., they exhibit fewer vegetative nodes and have near normal flower development. This result is expected if the Ac element mediates somatic excision of the Ds2 element from the id* gene during growth. Excision would restore Id function and result in partial restoration of normal development. Furthermore, the observation that these plants do not show patterns of defined sectoring (i.e., a sharp demarcation of normal tissue juxtaposed next to mutant tissue) suggests that Id is non-cell-autonomous. This result implies that the Id gene product is a diffusible factor or that it is involved in the regulation of a diffusible factor.

Southern blot analysis using the Ds2 element as a probe shows that a 4.5-kb SacI fragment cosegregates with the id* allele in more than 50 outcross progeny tested so far. This fragment is absent in plants that do not carry the id* allele. The cosegregation of this fragment with the id* allele is evidence that this gene is tagged with the Ds2 transposable element. In future work, the Ds2-containing fragment cosegregating with the mutation will be cloned in or-
der to isolate the sequence flanking the \(D_{s2}\) element. Confirmation of the identity of this flanking sequence as part of the \(Id\) gene will be possible through the comparison of this region in normal plants with that of plants in which the \(D_{s2}\) element has excised and restored the phenotype to normal (germinal reversion). Isolation of the \(Id\) gene from maize will allow us to characterize an important developmental function that mediates the transition from vegetative to reproductive growth in plants.

**Cell Division in Plants: Higher Plants Have Three Structurally Distinct Groups of Mitotic Cyclins**


There are many significant differences between plants and animals in the control of cell division during development. These include the reversibility of the terminally differentiated state, control by phytohormones, regulation of the planes of cell division, and persistence of meristematic lineages through development. Although there has been considerable focus on the molecular biology of cell division control in animals and yeasts, comparatively little is known at the molecular level about the machinery that regulates cell division in plants. For example, it is not known how the plane of cell division is controlled during division of plant cells. This control is very precise in plant development, since plant cells cannot migrate and are fixed in their relative positions following division. Previously, we have cloned a gene for \(p34^{cdc2}\) from maize, generated antibodies specific to the protein, and studied its intracellular localization. We have shown that it is nuclear in interphase and associates with the division site prior to mitosis, suggesting that it is involved in establishment of the division site. In particular, we found that the \(p34^{cdc2}\) kinase is targeted to the division site by the preprophase band of microtubules, and we proposed that it modifies sites on the cortex by phosphorylation that subsequently attract the expanding cell plate at telophase.

In animal cells, the distribution of \(p34^{cdc2}\) kinase and its substrate specificity depends on the associated cyclin and is markedly different for cyclins A and B. To study the mechanism by which the plant \(p34^{cdc2}\) kinase may be targeted to the division site and to identify the cortical substrates of this kinase, we have conducted an exhaustive search for homologs of mitotic cyclins in maize. We used degenerate primers to conserved regions of mitotic cyclins A and B (provided by I. Fitch and B. Futcher, CSHL) to amplify cDNA from maize meristems. The PCR products were cloned, and 21 clones were sequenced from two maize inbreds. The sequences were found to belong to four distinct types, representative of four different cyclins, all related to the A and B cyclins. One PCR product of each type was used to probe a maize immature ear library (B. Veit and S. Hake, USDA), and several cDNA clones corresponding to each type were isolated and sequenced. The longest clones from each type were called \(I_aZm, I_bZm, I_IIZm,\) and \(I_IIIZm (Zm = \text{Zea mays}).\) The designations are based on their relationships to each other as well as to other plant mitotic cyclins as discussed below. All four cyclins were shown to be functional for driving the \(G_2/M\) transition, since they could induce oocyte maturation, following microinjection of the corresponding RNAs into immature oocytes. The expression pattern of the cyclins was analyzed by Northern blots. All four cyclins showed a similar pattern of expression, with the mRNA levels correlated to cell division activity of the tissue. The expression of the maize cyclins appears to be more tightly associated to mitotic activity than expression of the \(cdc2\) gene. For example, there is \(cdc2\) expression, but no detectable cyclin expression, in fully differentiated, nondividing plant tissue. Since fully differentiated cells in many plant species can be induced to divide (and in some cases to regenerate new plants), our results suggest that the control of proliferation in fully differentiated plant cells is in part effected by turning off transcription of cyclins while maintaining a basal level of \(cdc2\) transcription.

The sequences of the four maize cyclins showed that they were clearly related to those of the A and B cyclins but that none of the maize cyclins can be classified as either A or B cyclins, based on sequence homologies or consensus motifs. While this work was in progress, five additional mitotic cyclin genes have been cloned from other plant species; one each from soybean, \(\text{Arabidopsis},\) and carrot, and two closely related cyclins from alfalfa (Hata et al., *EMBO J.* 10: 2681 [1991]; Hemerly et al., *Proc. Natl. Acad. Sci.* 89: 3295 [1992]; Hirt et al., *Plant Cell* 4: 1531 [1992]). These plant cyclins also could not be assigned as either A- or B-type cyclins. The
partial homologies of the plant cyclins with both A- and B-type consensus motifs suggest the separate evolution of the plant cyclin group. For this reason, we propose, instead of the A and B designations, to use the Roman numeral system to describe the different structural groups of plant cyclins that we have been able to define using data from these studies. The nine plant cyclins for which sequence data are available fall into three groups: I, II, and III. Since at least one cyclin from each group can be found in maize, it is likely that all higher plants have these three types of mitotic cyclins. Although the amino-terminal domains of the cyclins are highly variant, consensus motifs typical to the groups of plant cyclins, and absent in animal and yeast cyclins, can be identified in this region, as shown in Figure 1. Maize has two related homologs of group I cyclins, which we have called cyclin IaZm and cyclin IbZm. They are most homologous to the cyclins from soybean and Arabidopsis. Group I cyclins have a conserved motif KXXXXTLSSVLSARSKAAC located upstream of the cyclin box (Fig. 1). They also share with group III cyclins a motif HRPITRSF located close to the cyclin destruction box (Fig. 1). Group II of plant cyclins encompasses cyclin IIZm in maize and the carrot cyclin, which share some short motifs in the amino-terminal region (Fig. 1), and have an overall high degree of homology with each other (not shown). Group III of plant cyclins includes the maize cyclin IIIZm and the alfalfa cyclins. In addition to the HRPITRSF motif that they share with group I cyclins, the three cyclins of group III have a conserved EMEDI motif in the amino-terminal region (Fig. 1).

To summarize, mitotic cyclins in higher plants do not belong to either A or B types, but we have shown that they form three distinct structural groups that have been conserved in both monocotyledonous and dicotyledonous species. Cyclins from all three groups are present in maize and probably in all other plant species as well, suggesting that they specify different essential functions. We are currently generating antibodies to the three types of cyclins in order to study their intracellular locations in dividing plant cells and to determine which cyclin is involved in the localization of p34cdc2 with the division site. Future work will be aimed toward characterizing cyclin-associated proteins in order to identify factors that are required for division site localization, as well as identification of cortical substrates that are phosphorylated during the establishment of the division site.

**Gene Trap Tagging of a Semisterile Mutation in Arabidopsis**

P. Springer, V. Sundaresan, R. Martienssen
[Spring Harbor Laboratory]

During preliminary screening of enhancer- and gene-trap transposant lines, one insertion line containing a

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**FIGURE 1** Comparison of the amino-terminal sequences of the plant mitotic cyclins. Amino acid sequences of the maize cyclins IaZm, IbZm, IIZm, and IIIZm have been aligned with each other and with cycMsl and cycMs2 from alfalfa, cyc1At from Arabidopsis, S13-6 from soybean, and C13-1 from carrot. Only the amino-terminal region of the alignment is shown here. The putative destruction box is in boldface and italicized. Residues conserved in one or several groups of plant cyclins have been indicated and underlined. Dashes indicate positions of spaces required to align the sequences maximally. cycMs2, cyclin IaZm, cyclin IIIZm, and cyclin C13-1 are incomplete open reading frames from cDNAs truncated at their 5' ends, and the cycMsl sequence is truncated at its carboxy-terminal end.
gene-trap transposon was chosen for further characterization. This transposant (GT148) shows GUS expression throughout embryos and very young seedlings; expression is later localized to lateral root and leaf primordia, young floral buds, and, eventually, ovules (see Fig. 2). A partial cDNA clone was obtained corresponding to the chromosomal gene disrupted by the gene-trap insertion in this line. RACE-polymerase chain reaction (PCR) products were generated using nested primers from the GUS gene (Fig. 3) and cloned into M13. Sequencing revealed that they contained a fusion of GUS sequences and chromosomal exon sequences joined at the second splice acceptor site. The cDNA clone was used to probe Northern blots of RNA from different tissues of wild-type plants, as well as plants heterozygous for the gene-trap insertion. In each case, a 3.0-kb transcript was detected in roots and flowers but not in

![Image of reporter gene expression](A, B, C, D, E)

**FIGURE 2** Reporter gene expression in plants heterozygous for the gene-trap insertion, GT148. Plant tissues were stained with X-gluc and cleared in 70% ethanol. (A) Late heart/early torpedo-stage embryo showing subcellularly localized reporter gene activity; (B) uniform staining in lateral root primordia; (C) staining in proximal domain of immature leaves; (D) inflorescence with staining in flower buds, carpels, and ovules; (E) unfertilized mature flower with staining in ovules.
FIGURE 3 A scheme for generating RACE-PCR products from gene-trap insertions. (A) First-strand cDNA is synthesized from primer 2 within the GUS gene. A poly(dA) tail is added with terminal transferase, and the second strand is synthesized using a hybrid adapter-poly(dT) primer. cDNA products are then amplified using nested primer 1 within the GUS gene and a primer from the adapter sequence. (Stippled boxes) Exons within the target gene. Both spliced and unspliced products are recovered using this approach. (B) The sequence of a RACE-PCR product corresponding to a spliced fusion RNA from the gene-trap line GT148.

mature leaves. In addition, a 5.0-kb transcript was detected in plants heterozygous for the DsG insertion. This larger transcript hybridized to a probe specific for the GUS gene, indicating that it was derived from a transcriptional fusion with the gene-trap reporter gene. A cDNA library made from flower RNA (provided by D. Weigel, Salk Institute) was screened using the partial cDNA clone as a probe, and several additional clones were obtained.

The DNA sequence of these clones was determined by R. McCombie, and database searches revealed strong homology with the MCM3 family of proteins (which includes MCM2, MCM3, and CDC 46/MCM5 in Saccharomyces cerevisiae, CDC21 of Schizosaccharomyces pombe, and the mammalian Pl protein). In the conserved region, the Arabidopsis sequence is 65% identical to that of the mammalian Pl protein. These proteins are involved in site-specific initiation of DNA replication and are encoded by essential genes in yeast (Tye, Trends Cell Biol. 4: 160 [1994]).

The GT148 insertion line carries a mutation affecting fertility: Plants heterozygous for the DsG element are semisterile, having about 50% seed set. The DsG insertion carries a kanamycin resistance gene, and progeny from the self-pollination of a plant
heterozygous for the insertion segregate 1:1 kanamycin-resistant:kanamycin-sensitive. These segregation ratios suggest that the insertion results in gametophytic lethality (in contrast, a 2:1 ratio would be predicted for embryo lethality). Outcrosses to wild-type plants (carrying no insertion) show 75% transmittance through the male and 32% through the female. These ratios were even more exaggerated in individual plants, suggesting that the insertion primarily affects the female gametophyte. Early embryo lethals were also observed in self-pollinated siliques, suggesting that the few homozygotes that escape gametophytic selection abort early during embryogenesis.

The Ds element in GT148 cosegregates with semisterility in heterozygous plants, suggesting that disruption of the MCM3-like gene is responsible for the mutation. In the absence of Ac, the semisterile phenotype is completely stable, but when Ac transposase is re-introduced by crossing, the phenotype becomes unstable: Most siliques from plants carrying the DsG insertion and Ac are semisterile, but occasional siliques are fully fertile, suggesting that the mutation has reverted to wild-type in these flowers. Analysis of these apparent revertant events is under way.

Mutagenesis in plants is complicated by the extended haploid phase of the plant life cycle (the gametophyte generation). Studies using chromosomal deficiencies indicate that many mutations are lethal to the male and/or the female gametophyte, resulting in poor transmission and semisterility. For example, mutants blocked in DNA replication would be expected to fall into this class. Enhancer-trap and gene-trap transposon mutageneses are uniquely suited to identifying these genes: In the absence of expression data, mutations causing early lethality form a non-descript class, but with our approach, we can determine the expression pattern in viable heterozygotes. This allows those genes with additional functions later in development to be identified.

The iojap (Ij) Protein Is Associated with 50S Chloroplast Ribosomal Subunits in Maize

C.-D. Han, R. Martienssen

Plants homozygous for the iojap (ij) mutation have patterned white stripes on their leaves (Jenkins, J. Hered. 15: 467 [1924]) and give rise to albino seedlings in their maternal progeny when pollinated by wild-type plants (Rhoades, Cold Spring Harbor Symp. Quant. Biol. 11: 202 [1946]). The degree and extent of striping, and the maternal transmission of the ij-affected plastids, are largely dependent on genetic background (Jenkins, J. Hered. 15: 467 [1924]; Coe et al., Am. J. Bot. 75: 634 [1988]). These properties have made iojap a genetic paradigm for cytoplasmic inheritance and nuclear-organelar interactions, and many models have been proposed to account for them.

We cloned the Ij gene by transposon tagging, but the sequence of the Ij protein showed no homology with proteins in current databases (Han et al., EMBO J. 11: 4037 [1992]). To characterize further the role of the Ij protein in chloroplast and leaf development, antibodies were raised against Ij fusion proteins and purified by APT membrane affinity purification. By using in situ immunolocalization, we found that most cells in immature leaves expressed the Ij protein, which appeared to be concentrated in the plastids of each cell type.

On the basis of ribosomal RNA profiles of mutant and wild-type plants, Walbot and Coe (Proc. Natl. Acad. Sci. 76: 2760 [1979]) proposed that iojap plants suffered a programmed loss of plastid ribosomes that could account for the maternal transmission of ij-affected plastids. We therefore analyzed plastid subfractions to determine whether Ij was associated with plastid ribosomes or with other macromolecular structures. In chloroplast lysates, most of the protein was found in high-speed pellets, but it was found in the soluble fraction in the presence of 20 mM EDTA. This suggested that the Ij protein was associated with an EDTA-sensitive complex. Whole seedling lysates were further fractionated by pelleting through a sucrose cushion, followed by sucrose gradient sedimentation in the presence of nonionic detergents (polysome gradients). Sedimentation profiles were determined by UV absorption (not shown) and by Western blotting (Fig. 4) using anti-Ij and anti-50S ribosome antibodies. The Ij protein was found in the same fractions as the 50S ribosomal proteins under these conditions (Fig. 4).

Direct association of Ij with 50S subunits was examined by immunoprecipitation with affinity-purified anti-Ij antibodies. Immunoprecipitates were fractionated by SDS-PAGE, and Western blots were probed sequentially with anti-Ij, anti-50S ribosomal proteins and control anti-LHCP2 (light-harvesting
FIGURE 4 Western blots of lJ and 50S ribosomal proteins fractionated by sucrose gradient sedimentation. Plastid lysates were pelleted through 0.75 mm sucrose cushions, resuspended in 0.5 mm NH4Cl and 2% PTE, and loaded onto a 10-50% sucrose gradient. The gradient was centrifuged at 150,000 g for 16 hr and fractions were collected. Proteins from each fraction were analyzed by SDS-PAGE and by Western blotting using either affinity-purified anti-IJ antibody (top panel) or anti-spinach 50S plastid ribosomal protein antibodies (lower panel), respectively.

FIGURE 5 IP-Westerns using anti-lJ antibodies. Samples pelleted through sucrose cushions (Fig. 4) were resuspended in 50 mM Tris (pH 8), 5 mM MgCl2, 20 mM KCl and were used for immunoprecipitation with affinity-purified antibodies. The immunoprecipitates and supernatants were analyzed to detect IJ protein, 50S ribosomal proteins, and LHCP II membrane proteins, respectively, by sequential probing of the same blot. (Lane 1) Sample before immunoprecipitation; (lanes 2, 3, and 4) immunoprecipitation with 0, 5, and 25 μl of anti-IJ antibody; (lanes 5, 6, and 7) supernatants from immunoprecipitates loaded in lanes 2, 3, and 4, diluted fivefold relative to the precipitates. The last lane was loaded with protein A beads alone.
complex membrane protein) antisera (Fig. 5). Ij protein was quantitatively precipitated by increasing amounts of anti-Ij antibody (first panel). 50S ribosomal proteins were also specifically recovered in the anti-Ij immunoprecipitates (second panel, I.P.), although most of the proteins remained in solution (second panel, sup.). LHCPII proteins were not recovered in the precipitates (third panel), demonstrating that the coimmunoprecipitation was specific and did not reflect contamination with residual membrane-bound ribosomes. We are currently determining which ribosomal proteins are specifically bound to Ij.

Association with 50S ribosomal subunits suggests a role for the Ij protein in the regulation of plastid translation and supports the model first proposed by Walbot and Coe for the programmed loss of plastid ribosomes in ij mutants: Some ribosomal proteins are encoded by plastid genes, so that loss of plastid translation due to homozygous nuclear mutation could lead to the loss of plastid-encoded ribosomal proteins. Restoration of the nuclear components of the plastid translational apparatus in heterozygous maternal progeny would not be expected to restore plastid ribosomal function if the plastid-encoded components were no longer present. Heritable ribosome-deficient plastids would result.

Lineage Analysis of iojap Maize Plants

R. Martienssen

Maize plants homozygous for the nuclear mutation iojap have variegated leaves. Typically, the margins of each leaf are albino, whereas the middle of the leaf is variegated, comprising a mixture of white, yellow, and green stripes. The pattern of striping changes from leaf to leaf but is remarkably uniform within a given inbred variety. However, in extreme cases, iojap plants can be solid white, or mottled green in different inbred varieties. The variegated pattern found in most inbreds resembles the patterns found in periclinal chimeras in linear-leaf monocotyledons, such as Chlorophytum comosum (spider plants). This pattern reflects the contribution made by LI cells of the shoot apical meristem to the inner photosynthetic tissues of the leaf. LI cells are restricted to the outermost layer of the shoot apex by stereotypical anticlinal divisions (i.e., divisions in the plane of the cell layer). Cells from this lineage give rise to the epidermal, primarily nonphotosynthetic, tissues of the leaf. However, some cells from the LI lineage undergo periclinal divisions in the leaf and so contribute to the underlying photosynthetic tissues. These contributions occur primarily at the leaf margins, but they also occur in "stripes" elsewhere in the leaf. These stripes are descended from groups of cells that divided out-of-plane early in the initiation of leaf primordia. Coe et al. (Am. J. Bot 75: 634 [1988]) proposed that the pattern of variegation observed in iojap plants might be accounted for if the stripes of albino tissue were derived from cells from the LI lineage of the shoot apex.

To test this model, plants of the genotype jlj; B/b; Pl/pl; Bz/bz were constructed and irradiated with γ-rays to induce chromosome breakage. The irradiation was performed at the Brookhaven National Laboratory with the help of Rich Sautkilis. The genes B (booster), Pl (purple), and Bz (bronze) are required for accumulation of purple anthocyanin pigments in the epidermal and certain subepidermal tissues of the maize leaf. A cell in which the functional allele of any of these genes has been lost by chromosome breakage gives rise to a clone of cells in which the pigments fail to accumulate. When this clone arises in a given lineage in the shoot apex, its contribution to mature tissues of the leaf can be readily followed by anthocyanin pigmentation. Of 761 jlij plants, 125 sectors were examined. Sectors were chosen that passed through at least two leaves, so that they must have arisen in the shoot apex prior to leaf development. Twenty-two sectors were restricted to subepidermal tissues of the leaf: All but one of these were in green leaf tissue and had no effect on chloroplast development. Nine sectors were completely restricted to the epidermal layer, and, as expected, these had no effect on chlorophyll accumulation in the underlying tissue. Ninety-four sectors were found that, at some point in the sector, were present in both the epidermal and the subepidermal layers; 89 of these became white or mottled in appearance in the subepidermal layer. In 36 of these 89 sectors the sector was restricted to one or the other layer for part of its length, so that it was possible to determine whether the sector was derived from the LI of the shoot apex and later contributed to the subepidermal layer, or vice versa. In all the cases in which the sector moved from LI to LII, it was white in appearance. In both of two cases in which the sec-
tor had moved in the opposite direction (LII to LI), it was unchanged in pigmentation. Seventy-nine anthocyaninless sectors were observed on 470 control \(ij/+\) heterozygous plants that were phenotypically wild type. All but one of these sectors were green. Molecular analysis revealed that the one exceptional white sector had lost both the \(ij\) gene and the \(B\) gene in successive cell divisions, so that the white sector was entirely contained within hemizygous \(ij\)-tissue.

These results show that chloroplasts in \(ij\) mutant plants fail to differentiate in cells derived from the LI lineage of the shoot apical meristem when these cells contribute to the subepidermal tissues of the leaf. In contrast, chloroplasts in wild-type plants develop normally regardless of their origin in the meristem. Cells in higher plants typically adopt specific fates relatively late in development, independent of cell lineage. However, in \(ij\) mutant plants, chloroplast differentiation depends on cell lineage, so that LI cells must retain their clonal history. This suggests that the lack of lineage restriction in wild-type plants is mediated by specific gene products and may be amenable to mutational analysis.

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**Isolation of a Derivative Allele in Maize by PCR Screening**

L. Das, R. Martienssen

The \(hcf106\) mutation in maize is caused by the insertion of a \(Mu1\) transposable element into the promoter region of a gene required for chloroplast development. The \(hcf106\) mutant phenotype depends on transposon activity, and as a result, the phenotype is leaky and difficult to maintain in new genetic backgrounds. To obtain stable alleles, we used a PCR screening strategy to select plants that had suffered a deletion flanking the \(Mu1\) element at the \(hcf106\) locus; 1500 seedlings from the cross \(hcf106/+ \times +/+\) were germinated in the greenhouse in \(x\)-\(y\) grids to facilitate pooling. \(Mu\) activity was monitored using a second independent mutation (\(Les28\)), and calculations showed that the 1500 seedlings represented a collection of about 400 \(Mu\)-active \(hcf106\) chromosomes. DNA was prepared from pools of 32 seedlings from each row \(x\) and column \(y\) of the grids. The DNA was digested with an enzyme that cuts uniquely in the \(hcf106\) gene at the initiator ATG codon. DNA from the \(hcf106\) locus was then amplified using a primer from the promoter and a primer from the second exon of the \(hcf106\) gene. Digestion ensured that only alleles that had lost the initiator ATG codon via deletion would be amplified in subsequent PCR. Three seedlings were identified by cross-referencing \(x\) and \(y\) pools that had PCR products of the predicted size. DNA was made from these seedlings individually, and they were found to have suffered a 200-bp deletion, and two more complicated rearrangements, respectively, that had removed or interrupted the translational reading frame as desired. These heterozygous plants have been self-pollinated, and the progeny will be examined for mutant phenotypes this summer.

This method provides a powerful way to identify deletion (null) derivative alleles without phenotypic selection. The plants were recovered as heterozygotes, so that even if the deletion alleles are lethal, they can still be propagated and studied. Sequencing of the PCR products can be used to define precisely the derivative alleles before their phenotype is known, so that this technique is ideal for studying genes with lethal phenotypes or those for which only dominant, gain-of-function alleles are known.

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**DNA Methylation-deficient Mutants in Arabidopsis**

L. Das, R. Martienssen [in collaboration with E. Richards, formerly at CSHL, now at Washington University, St. Louis; and with M. Stammers and C. Dean, John Innes Institute, Norwich UK, and K. Edwards, Zeneca Seeds, UK]

Last year, Eric Richards reported the isolation of a new class of mutants in Arabidopsis that are deficient in cytosine methylation (Vongs et al. 1993). These \(ddm\) mutants (for decrease in DNA methylation) were isolated by direct screening of mutagenized populations for mutants whose repetitive DNA was sensitive to restriction digestion using enzymes normally inhibited by DNA methylation. These mutants have about 25–30% of wild-type 5-methylcytosine levels, but no obvious morphological phenotypes. However, they may have altered genetic properties (Vongs et al. 1993). We have backcrossed three different \(ddm\) mutants into the Landsberg ecotype over six generations, so that we can study recombination between polymorphic DNA markers in crosses with multiply
marked strains of the ecotype Columbia. In the coming year, we will quantify genetic recombination in an interval between phenotypic markers on chromosome 4 that has been cloned in overlapping YAC and cosmid clones by Caroline Dean’s group in the UK. Holliday (Science 238: 163 [1987]) suggested the possible link between methylation and recombination based on studies in prokaryotes, and it will be of interest to determine whether recombination in Arabidopsis is affected in methylation-deficient mutants.

**Differential Transcriptional Regulation Mediated by Two Maize Myb-domain Proteins**

E. Grotewold, T. Peterson [in collaboration with B. Drummond and B. Bowin, Pioneer Hi-Bred International]

The c-myb proto-oncogene is the cellular homolog of the transforming v-myb oncogene of the avian myeloblastosis virus. This virus causes acute myeloblastic leukemia in chickens and transforms myelomonocytic cells in vitro. The product of the transforming v-myb gene is a truncated version of its cellular progenitor, c-myb. Both c-myb and v-myb encode sequence-specific DNA-binding proteins that activate transcription. Most likely, c-Myb controls erythroid cell growth and differentiation through the transcriptional regulation of specific genes. Several proteins have been described in a variety of organisms that share high homology with c-Myb in the amino-terminal DNA-binding domain, the Myb domain. In no case, however, have the mechanisms by which Myb-domain proteins regulate transcription been elucidated.

In maize, two Myb-domain proteins, P and C1, regulate flavonoid biosynthesis: P regulates the accumulation of 3-deoxy flavonoids and derived pigments by activating transcription of three genes of the flavonoid biosynthetic pathway, C2, CH11, and A1. C1 regulates 3-hydroxy flavonoid and anthocyanin biosynthesis through the activation of the P-regulated genes (C2, CH11, and A1), in addition to genes only responsive to C1 (A2, Bz1, and Bz2). C1, however, is not able to achieve this regulation by itself and requires the basic helix-loop-helix (bHLH) protein R. The interaction between C1 and R has been shown by two-hybrid analysis to occur through the highly conserved Myb domain of C1 and the amino-terminal region of R (which does not contain the bHLH domain).

This system provides us with the first opportunity to study the mechanism by which two Myb-domain proteins direct distinct patterns of gene expression, probably not only by different DNA-binding preferences, but also through specific interactions with the co-activator R.

Our studies have shown that a protein expressed from a cloned P cDNA (Grotewold et al., Proc. Natl. Acad. Sci. 88: 4587 [1991]) activates the A1 promoter, but not the Bz1 promoter, in transient expression experiments. Our system for transient expression analysis consists of co-bombarding maize embryogenic callus cells (in which P, C1, and R are not normally expressed) with the activator protein (P, R, or C1) expressed from a plant constitutive promoter (the CaMV 35S promoter) and the reporter luciferase gene controlled by the A1 or Bz1 promoter fragments. The differential activation of A1 and Bz1 by P is independent of R: R is not required for the activation of A1, nor does R enable P to activate Bz1. C1, in the presence of R, is able to activate both the A1 and Bz1 gene promoters.

We have determined that 220 bp of the A1 promoter are sufficient for both P and C1 regulation of A1. Gel mobility-shift experiments using Escherichia coli-expressed P and C1 proteins indicated that these proteins bind to this promoter fragment in vitro, in the absence of other maize proteins. Footprint experiments showed that the P and C1 proteins bind to a region 60 nucleotides upstream of the A1 start of transcription which contains the sequence ACCTAC-CAACC. Gel mobility-shift experiments using oligonucleotides with specific mutations in this sequence confirmed that, indeed, this sequence is recognized by P and that it consists of two overlapping P- and C1-binding sites, ACCTACC and ACCACCC.

To test whether the sequences recognized by both P and C1 in vitro had a role in the regulation of A1 in vivo, mutant and wild-type A1 promoters were tested for trans-activation by P and C1 in our transient expression system. Mutations that prevent the binding of P and C1 in vitro decreased the activation by P or R+C1 four- to fivefold. When the wild-type P and C1 binding sites were placed upstream of a minimal promoter, normally not regulated by P or R+C1, regulation by P and R+C1 was obtained. These observations indicate that the P- and C1-binding sites pres-
ent in the Al promoter are not only necessary, but also sufficient, for regulation by P and Cl (Grotewold et al., Cell 76: 543 [1994]).

Since the P- and Cl-binding sites present in the Al promoter are very different from sequences to which c-Myb and v-Myb bind, we performed parallel binding selection experiments with E. coli-expressed P and v-Myb proteins. Our results showed that the two proteins have different binding preferences in vitro, although P and v-Myb have highly related Myb domains. The binding consensus deduced for P was ACC-A/TAACC-A/C(T), whereas the deduced v-Myb DNA-binding consensus was G-I/C/CAACGG-T/C/A (Grotewold et al., Cell 76: 543 [1994]). The two P-binding sites in Al (CCTACC and CCAACC) match the deduced P consensus binding site, and as expected from the binding selection experiments, v-Myb does not bind to the P-binding sites of the Al gene, nor does P bind to the Myb-binding sites in the mim-I gene, the only gene so far described as directly regulated by Myb (see Fig. 6). These results provide the first evidence that two members of the Myb family of transcriptional activators bind different sequences.

P does not regulate Bzl, and consistent with this pattern of regulation, we did not detect any binding of P to Bzl promoter sequences. Surprisingly, however, there is also no binding of Cl to the Bzl promoter, and yet, Cl is required for Bzl regulation. One possibility is that R is required for binding of Cl to the Bzl promoter. When the Myb domain of Cl (residues 1-121) is fused to activation domains of VP16 or GAL4, activation of the Al or Bzl promoters is only achieved in the presence of R, indicating that R is probably required for binding in vivo to both Al and Bzl. Surprisingly, the Myb domain of P

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**FIGURE 6** Gel mobility-shift experiments using wild-type (APB1) or mutant (APB2-4) oligonucleotides containing the P-binding sites present in the Al gene promoter or the Myb-binding sites present in the mim-I gene promoter. A summary of the sequences preferentially bound by P and v-Myb is shown.
fused to the activation domain of VP16 does not activate the Al promoter. This could indicate that P, like C1, requires an accessory factor. There is no genetic evidence, however, to suggest that such a factor exists.

The study of the mechanisms by which the Myb-domain proteins P and C1 regulate transcription of overlapping sets of genes thus provides us with a unique opportunity to study how Myb-like proteins can differentially regulate gene expression through different binding specificities and combinatorial interactions with accessory factors.

Cloning and Characterization of a P-regulated Maize Chalcone Isomerase

E. Grotewold, T. Peterson

Flavonoid-derived pigments are found almost universally in the plant kingdom, providing color to flowers, fruits, leaves, and storage organs. Their biosynthesis is probably the best described biochemical pathway in plants and therefore presents a very convenient system to study regulation of gene expression. The most commonly found flavonoid-derived pigments, the anthocyanins, are derived from 3-hydroxy flavonoids. However, pigments lacking the 3-hydroxyl group characteristic of the anthocyanins (the 3-deoxy flavonoids) exist in some species, including sorghum and maize. Besides being involved in plant pigmentation, flavonoids have important roles in protection from the damaging effects of UV radiation, in response to attack by phytopathogens, as signal molecules for the induction of nodulation genes, and as stimulants for the development and germination of pollen and for pollen tube growth.

Chalcone, the first flavonoid in the pathway, is synthesized by the enzyme chalcone synthase, which has been characterized in several plants, and in maize, it is encoded by the C2 locus. Chalcones can be converted into the corresponding flavonones by an intramolecular reaction in which the C ring is closed by the enzyme chalcone isomerase (CHI). Although mutations have been found in genes encoding several regulators and structural enzymes of the maize flavonoid biosynthetic pathway, no mutations affecting the activity of CHI have been identified. The existence of a functional CHI gene in maize has been inferred from biochemical analysis of flavonoids and the similarity in the flavonoid biosynthetic pathways of maize and other plants. Because we are interested in understanding the mechanisms by which flavonoid biosynthesis is regulated, we decided to isolate putative maize CHI genes.

Using polymerase chain reaction (PCR) based on conserved regions of the CHI genes of Petunia, Antirrhinum, and bean, we cloned a fragment of a maize CHI cDNA. This fragment was used to obtain cDNA clones from a P-rr pericarp-specific cDNA library and genomic clones from a maize genomic library.

The analysis of the sequence of the cDNA clones and the structure of the genomic clones indicated that two to three CHI genes are present in most maize inbred lines, only one gene (CHI1) being regulated in the pericarp by P, a Myb-domain protein (see above). By RNase A and S1 protection experiments, together with primer extension, we determined the start of transcription of CHI1. The intron/exon structure of CHI1 is identical to the structure of the CHIb gene from Petunia, and CHI1 encodes a 24.3-kD protein, very similar in size and about 56% identical in sequence to that of the Petunia CHI proteins (Grotewold and Peterson 1994).

We analyzed 850 bp of the CHI1 promoter sequence for high-affinity P-binding sites (Grotewold et al., Cell 76: 543 [1994]), and none were found. Furthermore, E. coli-expressed P protein does not bind to these CHI1 promoter sequences. These observations could indicate (1) that CHI1 is not regulated directly by P, (2) that P has a different binding specificity in vivo and in vitro, or (3) that an accessory factor could be responsible for recruiting P to the CHI1 promoter. This situation is similar to what is found in the regulation by C1: The Myb-domain protein C1 regulates A1 and Bz1 only in the presence of the bHLH protein R. However, only the A1 gene promoter has C1-responsive elements, whereas the Bz1 promoter does not. We believe that the analysis of the mechanisms by which P regulates transcription of promoters with P-responsive elements (like A1) and without P-responsive elements (like CHI1) will provide us with important information about the mechanisms involved in gene regulation by the maize Myb-domain proteins P and C1.

PUBLICATIONS


In Press
Grotewold, E. J.B. Drummond, B. Bowen, and T. Peterson.

ARABIDOPSIS SIGNAL TRANSDUCTION AND FLOWER DEVELOPMENT

H. Ma J. Carruthers H. Huang K. Mukai
C. Flanagan D. Kostic P. Rubinelli
Y. Hu Y. Mizukami M. Tudor
K. Mukai C. Weiss

Research in our laboratory addresses two problems of plant biology: (1) the function(s) of G protein(s) in plant signal transduction pathways and (2) the molecular mechanisms controlling flower development, especially the roles of members of a conserved gene family (the MADS-box genes).

Plant cells respond to a variety of external signals, including light, gravity, temperature, moisture, pathogens, and physical touch. In addition, in multicellular plants, cells respond to molecules generated by other plant cells, such as hormones and nutrients. Because plants and individual plant cells are generally immobile, the kinds of reactions that plant cells generate in response to a stimulus are often different from those of animals. It is therefore of fundamental importance to study the molecular mechanisms of the plant signal transduction pathways. It is well established that, in animals and simple eukaryotes, heterotrimeric G proteins have important roles in signal transduction. However, the function of G proteins in plants is not known. To study the function of plant G proteins, and to test their possible involvement in plant signaling processes, we have continued the analysis of a previously isolated Arabidopsis gene (GPA1) encoding a G protein subunit (Ma et al., Proc. Natl. Acad. Sci. 87: 3821 [1990]). In addition, we have isolated a new Arabidopsis gene that encodes a G protein β subunit.

Much progress has been made in recent years toward the understanding of the molecular mechanisms controlling flower development. In particular, a number of Arabidopsis floral homeotic genes have been characterized (Coen and Meyerowitz, Nature 353: 31 [1991]; Ma 1994). Mutations in one of these genes, AGAMOUS (AG), cause double-flower phenotypes, where the stamens are converted to extra petals and the ovary is replaced by a new flower. The AG DNA sequence (Yanofsky et al., Nature 346: 35 [1990]) indicates that it encodes a protein with strong similarity to 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, DEF A from Antirrhinum majus (Sommer et al., EMBO J. 9: 605 [1990]), and has been referred to as the MADS-box, for MCM1-AG-DEFA-SRF (Schwarz-Sommer et al., Science 250: 931 [1990]). Additional MADS-box genes were isolated using AG as a probe, and these were designated AGL1 through AGL6 for AG-Like. We have focused our efforts on AG, AGL1, AGL2, and AGL3. To identify new genes required for flower development, we have collaborated with Drs. V. Sundaresan and R. Martienssen (see this section) to establish an enhancer trap/gene trap transposon insertional mutagenesis
system, and we have continued our efforts to generate lines carrying new insertions. In an alternative approach to isolate new genes that function during flower development, we have begun a new project aimed at identifying genes specifically expressed in carpels.

In 1993, in addition to the people already here, Peter Rubinelli came from SUNY Stony Brook, where he is a graduate student in the Molecular and Cellular Biology Program; Matt Tudor, an undergraduate student at Cornell, returned for a second summer, studying with Hai Huang the in vitro DNA-binding properties of AG and AGLs; Julie Car ruthers, an undergraduate at the University of California, Santa Cruz, spent the summer with us as an URP, working with Cathy Flanagan on the analysis of AG expressed in yeast; Kanae Mukai, who attends Bronx School of Science, worked with Catherine Weiss during the summer to isolate a G protein β subunit gene from Arabidopsis; and Dusan Kostic has recently completed his studies at the University of Utah and started his postdoctoral training in the fall.

Isolation and Characterization of Plant G Protein β Subunit Genes

C. Weiss, K. Mukai, Y. Hu, H. Ma [in collaboration with C. Garnaat, Pioneer Hi-Bred Seeds]

Heterotrimeric G proteins consist of three subunits α, β, and γ. Upon interaction of the inactive GDP-bound G protein with an activated receptor, the GDP is replaced with a GTP and the Py complex dissociates from the GTP-bound α subunit, thus liberating two complexes. Until recently, the GTP-α subunit complex was thought to be the only component interacting with downstream effectors, whereas the role of the βγ complex in signal transduction, if any, was controversial. However, a number of reports indicate that the βγ complex is also able to interact directly with effectors, such as adenylate cyclase, K+ channels, phospholipase A2 and the isoform β2 of phospholipase C, and receptor kinases (Clapham and Neer, Nature 365: 403 [1993]). Furthermore, it has recently been shown that in some cases, the βγ complex is involved in receptor recognition. In plants, biochemical studies have suggested G protein involvement in a number of pathways such as the control of K+ channel opening in guard cells and mesophyll cells or the transmission of signals such as red and blue light, stress, and auxin (Terryn et al., Plant Mol. Biol. 22: 143 [1993]). Furthermore, we have demonstrated the existence of heterotrimeric G proteins in plants by isolating a G protein α subunit gene from Arabidopsis and its homolog from tomato (Ma et al., Gene 107: 189 [1991]). To obtain additional tools to study G protein functions in plants, it is important to isolate and characterize gene(s) encoding G protein β subunits.

Recently, a maize cDNA was isolated from a subtracted tassel library, and it was found to encode a peptide homologous to a portion of known G protein β subunits. Further analysis of longer cDNAs with a complete open reading frame indicates that the predicted protein is indeed a homolog of known G protein β subunits. The gene represented by the cDNA was designated ZGB1. Using ZGB1 cDNAs as probes, we isolated the Arabidopsis homolog, AGB1, from a cDNA library. The predicted amino acid sequences of the maize and Arabidopsis G protein β subunit homologs are 76% identical to each other and more than 42% identical to animal G protein β subunits. In addition to G protein β subunits, a number of other proteins contain WD-40 repeats. Although the function of the WD-40 motif is not known, it is thought to be involved in protein-protein interaction. Two plant genes have been isolated, from Chlamydomonas (Chlp; Schloss, Mol. Gen. Genet. 221: 443 [1990]) and tobacco (arca; Ishida et al., Proc. Natl. Acad. Sci. 90: 11152 [1993]), that encode proteins with several copies of the WD-40 motif; their amino acid sequences are 66% or more identical to each other and to that of a chicken WD-40 protein (C12.3; Guillemot et al., Proc. Natl. Acad. Sci. 86: 4594 [1989]), suggesting that they may have similar functions. However, these proteins share only low levels (about 25%) of sequence identity to animal G protein β subunits; therefore, they are probably functionally distinct from the G protein β subunits. Several other WD-40 proteins have been identified in animals and yeast; these proteins are not functionally related to G proteins and are involved in a number of processes, such as RNA splicing, cell cycle control, cytoskeleton organization, and transcriptional repression.

Hybridizations to genomic DNA indicate that
ZGB1 and AGB1 are single-copy genes and that this G protein β subunit homolog is conserved among other plant species. Northern analysis indicates that ZGB1 mRNA is present in the root, leaf, and tassel and that AGB1 mRNA is expressed in the root, leaf, and flower. The isolation of AGB1 and ZGB1 provides further indication that a heterotrimeric G protein functions in plants, and it will be very important to learn what role these genes may have in plant signaling pathways. In particular, it will be of interest to investigate whether the α and β subunits encoded by the GPA1 and AGB1 genes, respectively, interact with each other directly.

**Expression of the AGL2 Gene in Wild-type and Mutant Arabidopsis Flowers**

C. Flanagan, H. Ma, Y. Hu

To obtain clues about AGL2 function in flower development, we have carried out a detailed analysis of the expression patterns of AGL2 in wild-type and mutant flowers using RNA in situ hybridizations. AGL2 expression begins early in flower development (at stage 2), after the floral meristem has emerged, but before any floral organ primordia appear. AGL2 expression is uniformly high in floral primordia and floral organ primordia between stages 2 and 7. However, the expression of AGL2 becomes organ-specific during later stages of flower development. At stage 8, AGL2 expression begins to wane in the sepals but remains high in the other organs. Later, during stages 9–10, AGL2 expression remains high in the carpel, petals, and (to some extent) in the filaments, whereas expression is reduced in the sepals and anthers. By stage 12, AGL2 expression is localized primarily to the ovules. After fertilization, the expression of AGL2 remains detectable in the developing seed coats, but it diminishes gradually. Interestingly, AGL2 is also expressed in developing embryos. The AGL2 expression pattern suggests that AGL2 functions in the floral meristem, and in all floral organs during early flower development, and that its function is required to a different extent in different organs, with carpels (ovules) requiring AGL2 function for the longest period. The presence of the AGL2 message in embryos suggests that it may also have a role in embryo development.

Since the floral organs that exhibit different late AGL2 expression patterns not only are of distinct identities (e.g., sepals or petals), but also occupy different positions (first or second whorls), it was not known whether late AGL2 expression is regulated by the identity or the position of the floral organs. We therefore tried to distinguish between these possibilities by examining AGL2 expression in floral mutants, ag, ap2, and ap3, which have floral organs occupying positions different from those in wild type (e.g., the ag flower has petals in the third whorl, the ap2 flower has carpels in the first whorl, and the ap3 flower has sepals and carpels in the second and third whorls, respectively). The patterns of AGL2 expression are altered in the mutant flowers in ways that are consistent with the organ identities but not their positions. In ag mutant flowers, the outer two whorls are normal, as is AGL2 expression in these organs. The third whorl of an ag flower has petals instead of anthers, and the AGL2 expression pattern in the third whorl is the same as that in the second whorl petals. The ag flower produces a secondary flower instead of the ovary, and AGL2 expression in the secondary flower follows the pattern in the primary flower, both spatially and temporally. In ap2 and ap3 mutant flowers, AGL2 expression is also altered such that the patterns are consistent with the type of organs, regardless of their positions. Therefore, AGL2 is regulated (directly or indirectly) by the floral homeotic genes such that the pattern of expression is in agreement with wild-type AGL2 expression in terms of organ types but not organ positions.

**Characterization of AGL1, AGL2, and AGL3-binding Sequences**

H. Huang, M. Tudor, Y. Hu, H. Ma

AGL1, AGL2, and AGL3 are sequence-specific DNA-binding proteins

AGL1, AGL2, and AGL3 share a conserved motif, the MADS-domain, with known transcription factors (SRF and MCM1) and floral homeotic gene products (e.g., AG and DEFA); however, it was not known whether the AGL proteins are indeed DNA-binding proteins. To test for DNA binding, we expressed the AGL proteins using the T7 expression system in Escherichia coli and used the proteins for in vitro studies. We have recently shown (Huang et al. 1993)
that the *Arabidopsis* AG protein can bind sequences resembling the target sequences (with a core consensus of CC[A/T]₆GG, or the CArG box) of SRF and MCM1. We therefore tested whether the AGLs can bind to an 80-bp oligonucleotide (oligoA) containing a sequence (CCATTAATGG) to which AG binds (Huang et al. 1993). Our results showed that AGL1, AGL2, and AGL3 all can bind to oligoA but not to another oligo (oligoB) differing only at six nucleotides (GGATGCATCC) within the putative binding sequence. Furthermore, nonradioactive oligoA but not oligoB, competes for binding with AGL1, AGL2, and AGL3, indicating that the binding is sequence-specific.

**AGL1-, AGL2-, AND AGL3-BINDING SEQUENCES ARE SIMILAR BUT NOT IDENTICAL**

With active proteins and both a positive and a negative control at our disposal, we next asked what the spectra of sequences to which AGL1, AGL2, or AGL3 bind are, since SRF, MCM1, and AG are known to bind a number of similar but different sequences. We used the in vitro procedure that we used for AG (Huang et al. 1993) to select for sequences that bind to AGL1 (AGL2 or AGL3) from a pool of random sequences (flanked by known invariant sequences for polymerase chain reaction [PCR]). Sequences bound to AGL1 (AGL2 or AGL3) were separated from free DNA using a polyacrylamide gel mobility-shift assay, amplified by PCR, and used for the next round of selection. We observed an enrichment of sequences that bind AGL1 (AGL2 or AGL3) following each round of selection and amplification. After four rounds of selection, the DNA was ligated to a plasmid vector for cloning and sequence analysis. Several dozens of the selected DNAs were sequenced and the consensus sequences were deduced, as shown in Figure 1.

The AGL1 target consensus is very similar to that of AG, consistent with that fact that the MADS-boxes of these two proteins are nearly identical (3 conservative replacements out of 56 residues; Fig. 1). Since these three differences are within the carboxy-terminal one fourth of the MADS-box, this region may not be involved in determining sequence specificity, in agreement with the finding that the amino-terminal half of the MADS-box is needed for the sequence specificity of SRF and related mammalian proteins. The AGL2- and AGL3-binding consensus sequences are very similar to each other but are somewhat different from those of AG and AGL1, possibly because there are ten (nine) amino acid replacements in the MADS-box between AGL2 (AGL3) and AG or AGL1. Six (five) of the ten differences are in the amino-terminal half of the MADS-box; it is possible that these amino-terminal residues may be important for sequence specificity.

Although AG and AGL1 have a similar binding sequence consensus, their expression patterns suggest that they have different functions. AG is expressed in stamens and carpels, whereas AGL1 is found only in carpels. It is possible that AG and AGL1 regulate different genes by interacting with different factors, since they have quite different carboxy-terminal regions. Such interactions could potentially enhance binding of the proteins to some binding sites but not to other binding sites, as is the case for MCM1. In addition, interactions with some accessory factors may
modulate the transcriptional activity of AG or AGL1 proteins, even though the interactions may not affect binding of these proteins to DNA. AGL2 and AGL3 are also expressed very differently; AGL2 is specific to flowers, but AGL3 is expressed in flowers and vegetative organs. Since some of the plant MADS-domain proteins that share common regions of expression (e.g., AG and AGL2) show different sequence specificities, one of the mechanisms by which they might have different functions is by regulating different genes. Our results on the AG- and AGL-binding consensus sequences should also facilitate the identification of potential target genes for these presumptive transcriptional regulators.

Analysis of AG Function Using Ectopic Expression in Transgenic Arabidopsis Plants

Y. Mizukami, H. Ma

We have previously studied AG function in specifying organ identity by ectopically expressing AG in transgenic plants using the cauliflower mosaic virus 35S promoter and found that such ectopic expression of AG results in phenocopies of the ap2 mutant (Mizukami and Ma, Cell 71: 119 [1992]), which have flowers with floral organ conversions opposite to those in the ag mutant. Using the transgenic plants carrying the 35S-AG construct, we have continued to probe AG function in flower development.

ECTOPIC EXPRESSION OF AG AFFECTS SEED DEVELOPMENT

We have previously observed that some of our 35S-AG transgenic plants are sterile. To learn more about AG function, we have characterized the phenotypes of the AG transgenic plants after pollination and found that the AG transgenic plants have abnormalities in seed development. First, in the transgenic seedpod, many seeds fail to desiccate and continue to develop precociously into seedlings (vivipary), which can develop to maturity if transferred onto a defined culture medium. Second, although some embryos develop normally at the beginning of embryogenesis, they become overgrown mature seeds even though they have desiccated and become dormant. Third, the transgenic seed coat is abnormal. Fourth, mature seeds exhibit reduced sensitivity to the exogenously provided hormone ABA. Finally, the levels of 12S seed storage proteins (cruciferins) in the transgenic seeds are reduced compared to those in normal seeds. We have also examined the seeds of ap2 mutant plants and observed that ap2 mutant plants have seeds with phenotypes similar to those seen in 35S-AG transgenic plants. Since AP2 was shown to regulate AG expression negatively, it is possible that AG is ectopically expressed in seeds of the ap2 mutants, leading to the abnormal phenotypes. Our results show that ectopic expression of AG using the 35S promoter produces a phenocopy of ap2 mutants not only with respect to floral organ identity, but also in terms of seed development.

FIGURE 2 Schematic representation of wild-type, ag mutant, and 35S-AG antisense transgenic flowers. (A) Wild-type; (B) ag mutant, and severe AG antisense; (C [external] and D [internal]) mild AG antisense phenotypes. (Se) Sepal; (Pe) petal; (St) stamen; (Ca) carpel; (PS) primary stamen; (PC) primary carpel; (PO) primary ovule; (SS) secondary stamen; (SO) secondary carpel; (SC) secondary carpel; (SS) secondary ovule.

AG FUNCTIONS IN THE MAINTENANCE OF FLORAL MERISTEM IDENTITY

The ag mutant flowers are indeterminate, with a repeating pattern of a flower within a flower (Fig. 2B), suggesting that normal AG function is needed to maintain the floral meristem. This is supported by the observation that when the plants are grown under...
short-day conditions, the ag floral meristems are converted to indeterminate inflorescent meristems, whereas wild-type floral meristems are determinate. To test the possible AG function in floral meristem further, we examined the phenotypes of the 35S-AG transgenic plants in several ways. We found that the transgenic plants flower much earlier than wild-type plants and that the apical (inflorescence) meristem terminates growth with a cluster of sessile ap2-like flowers at its apex, resulting in a determinate inflorescence with a much lower number of flowers compared to that of wild type. On the other hand, ectopic expression of a defective AG transgene does not cause either floral homeotic changes or inflorescent phenotypes. The defective AG transgene encodes a truncated AG protein lacking regions carboxy-terminal of the MADS-domain. Therefore, ectopic expression of a functional AG protein is needed for the early flower and inflorescent phenotypes. It is intriguing that the early flowering and terminal flower phenotypes are very similar to those of terminal flower (tfll) mutants, in which the inflorescent meristems are converted to floral meristems. It will be interesting to determine whether AG is ectopically expressed in the tfll mutant. Our results suggest that, in addition to controlling floral organ identity, AG also regulates floral meristem identity. It is possible that the ectopic expression of AG causes premature formation of the determinate floral meristem, resulting in early flowering, and the ectopic AG expression at the apical meristem leads to the terminal flowers.

Dose-dependent Functions of AG in Transgenic Arabidopsis Plants Expressing Antisense RNA

Y. Mizukami, H. Ma

AG functions in two aspects of flower development after the floral meristem is established: (1) specifying stamen and carpel identity and (2) regulating determinate growth of floral meristems. However, little is known about how AG functions in these two floral developmental processes. To begin to dissect the AG functions, we have generated transgenic Arabidopsis plants expressing a constitutive antisense AG RNA under the control of the 35S promoter and analyzed how different AG mRNA levels affect the floral phenotype. All independent transformants display a range of abnormal floral phenotypes; they all exhibit meristem indeterminacy but not always floral organ homeotic conversion, suggesting that one of the AG functions is separable from the other. Further analysis of AG mRNA levels in the transgenic plants reveals that partial reduction of AG expression results in flowers that exhibit only meristem indeterminacy; these flowers contain within their gynoecia another flower (Fig. 2C,D). Further reduction of AG expression is necessary to produce an ag-l phenocopy (Fig. 2B). These results suggest that the two AG functions are modulated in a dose-dependent fashion during flower development. Because AG likely encodes a transcription factor, the proposed concentration-dependent AG functions could be explained if the critical amount of AG activity required to regulate those AG target genes specifying organ identity is different from the amount of AG needed to regulate genes that are necessary for regulating a determinate floral meristem.

Isolation and Initial Characterization of Two Mutants with Altered Floral Organ Number

H. Huang, H. Ma

ISOLATION AND PHENOTYPES OF fonl AND fon2 MUTANTS

Among the more than 300 Arabidopsis transformants carrying various GPA1 constructs, two were observed to segregate for plants that have abnormal number(s) of floral organ(s). They were designated fonl and fon2, respectively, for floral organ number. Both of the mutations were heritable, because the progeny of the mutants continued to exhibit the same phenotypes as the parents. A fonl mutant plant was crossed to the wild type, and the F1 plants have normal phenotypes, indicating that the fonl mutation is recessive. Furthermore, the progeny of the F1 heterozygotes segregated 3 to 1 (74 to 20 plants) for normal to mutant flowers, indicating that the phenotype was due to a single gene defect. For fon2, the progeny of the original transformant (normal phenotype) segregated 3 to 1 (38 to 13 plants) for normal to mutant plants, suggesting that the mutation is also in a single gene and recessive to wild type. We have crossed fon2 to wild type and the progeny will be examined to confirm this in the near future.

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The overall floral phenotypes of these mutants are quite normal, with all four types of floral organs present: sepals, petals, stamens, and carpels. Although the number of sepals and petals are normal (four each) in both mutants, fonl plants have flowers with an increased number of stamens and carpels, and fon2 plants produce flowers with a reduced number of stamens. Compared to the wild-type flowers, which have six stamens and two carpels (n = 20), the fon1 flowers have six to ten stamens (n = 52, the average is 7.08) and three to four carpels (average 3.44). In addition, fon1 flowers that appear early tend to have more stamens (for 11 #1-3 flowers, the average is 8.09), and fon1 flowers that appear late tend to have slightly fewer stamens (for 25 #7-12 flowers, the average is 6.31). The stamen number in fon2 flowers ranges from four to six (n = 57, average is 4.86), whereas the carpel number in most fon2 flowers is normal (5 of the 57 flowers counted have three carpels, the rest have two), and the shape of the fon2 seedpod is normal. Phenotype variations among flowers of the same mutant plants have been observed for many other floral mutants, including ap2, ap3, ag, and ify.

**COMPLEMENTATION TEST BETWEEN fon1 AND clvl**

Although the increase in stamen and carpel numbers of the fon1 mutant is similar to that of a previously identified floral mutant, clvl (Leyser and Furner, Development 116: 397 [1993]; Clark et al., Development 119: 397 [1993]), there are several significant differences between fon1 and clvl mutants. First, clvl mutants have fasciated (flat but not round) stems, whereas the stems of fon1 plants appear to be normal. Second, clvl mutants have an increased number of organs in all four whorls. Third, clvl mutants have club-shaped ovaries and seedpods, whereas fon1 ovaries and seedpods have about the same diameter from one end to the other. Nevertheless, because fon1 and clvl both have an increased number of stamens and carpels, we tested for complementation between them. We observed that the progeny of crosses between homozygous fon1 and clvl plants produce normal flowers with normal numbers of organs. Therefore, fon1 and clvl are in different genes.

**THE fon1 MUTATION IS TIGHTLY LINKED TO A T-DNA INSERTION**

Cosegregation studies indicate that fon2 is a mutation that probably arose during T-DNA transformation, but it is not due to a T-DNA insertion. Similar studies, however, found that the fon1 mutation is tightly linked to a T-DNA insertion. This insertion apparently has more than two copies of the T-DNA. We isolated DNAs from 94 F2 progeny from the cross between fon1 and wild type and found that all 20 mutant plants contain the same two expected T-DNA bands; 53 of the normal plants also have these bands, indicating that they are heterozygotes. Indeed, when the progeny from one of these heterozygotes was grown up, they segregated 39 to 10 for normal to mutant plants, very close to the expected 3 to 1 ratio. Although the T-DNAs contain a copy of the GPa1 cDNA fused to the 35S promoter, several lines of evidence indicate that GPa1 is not responsible for the phenotype. First, fon1 is the only transformant with this phenotype among 53 transformants with the same T-DNA. Second, fon1 is recessive, whereas transgenes are generally dominant. Third, Western analysis indicates that the level of GPa1 protein in flowers and leaves of the fon1 mutant is the same as that in several other transformants, which have no floral abnormalities.

The isolation of fon1 and fon2 mutants provides new genetic materials to study a different aspect of flower development, the control of organ number about which we know very little. The analysis of the fon1 and fon2 mutant phenotypes, and the isolation and characterization of the FON1 gene, should uncover some of the mechanisms regulating floral organ number and contribute to our understanding of flower development.

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**Subtractive Hybridization between Wild-type and ag cDNAs**

P. Rubinelli, H. Ma

Considerable progress has been achieved in the understanding of factors controlling floral organ identities. The ag-1 mutant flowers exhibit homeotic conversion of stamens to petals and conversion of the carpels to another ag flower, which lacks stamens and carpels. Thus, the ag mutant has neither stamens nor carpels. The sequence of AG suggests that it is a transcription factor that specifies the identity of stamens and carpels. However, little is known about the genes whose functions are responsible for the
proper differentiation of each organ type. In an attempt to identify genes that are involved in the stamen and carpel developmental "pathways," we have performed subtractive hybridizations between cDNAs derived from the mRNAs of wild-type and ag mutant inflorescences. Through several subtraction steps, it should be possible to eliminate most of the common cDNAs and greatly enrich for cDNAs representing genes expressed in wild-type but not in the ag mutant flowers. These cDNAs should be stamen- and/or carpel-specific and should have a role in the development of these organs.

In this method, cDNAs from wild-type inflorescences are hybridized with a 20-fold excess of biotinylated cDNAs from ag mutant inflorescences. The hybrid duplexes are then removed by addition of streptavidin, which binds very tightly to the biotin groups (K_D = 10^-15 M), followed by phenol/chloroform extractions to remove the streptavidin/DNA complexes. The remaining cDNAs are amplified by PCR and two additional rounds of subtraction are performed. The resulting subtracted cDNAs are then cloned into a plasmid vector and clones are screened with both wild-type and ag cDNAs as probes to identify clones that are expressed in wild-type but not in the ag mutant. These clones can be further screened using cDNA probes from the ap3 mutant, which lacks stamens but has carpels. Thus, the stamen- and/or carpel-specific clones can be further distinguished as either stamen- or carpel-specific. With this accomplished, selected clones can be further analyzed by DNA sequencing and other methods.

So far, the subtraction has been performed and verified using marker cDNAs as probes of Southern blots containing the amplified, subtracted cDNAs at each step during the subtraction. One marker for subtraction, GPA1, is ubiquitously expressed throughout the flower. We have observed a gradual loss of GPA1 sequences over the course of the subtraction steps, culminating in a loss of the GPA1 signal to below detection levels in the final subtracted cDNA. A similar result was found using AGL2, which is also expressed throughout the flower, as a probe. Another marker cDNA, AGL1, is normally expressed only in the carpels; in ag mutant flowers, AGL1 expression is drastically reduced. We expected that AGL1 cDNA should be preferentially retained, compared to GPA1. We found that the amount of AGL1 cDNA was maintained after early subtractive hybridizations but reduced slightly after the third round of hybridization. These results suggest that the subtraction method eliminates much of common cDNAs (e.g., GPA1), reduces the amount of cDNAs (e.g., AGL1) expressed at low levels in ag mutant, and presumably enriches inner-whorl-specific cDNAs.

A Northern blot containing both wild-type and ag poly(A)^+ RNAs was hybridized with the final subtracted cDNA as probe. This Northern blot showed that there are still some common cDNAs remaining in the subtracted cDNA, indicating that the subtraction was not complete. We are presently analyzing individual plasmid clones containing cDNA inserts from the subtracted cDNAs by Southern blot analysis of plasmid DNA, probing with wild-type and ag cDNAs to identify inner-whorl-specific cDNAs. Those cDNAs that are specifically expressed in the wild-type but not in the ag mutant will be further analyzed, including the isolation and characterization of full-length cDNAs.

**PUBLICATIONS**


In Press


Our main interest continues to be regulation of Start and mitosis 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. Many key cell cycle events are regulated by protein kinase complexes formed between Cdc28 and a cyclin. There are now nine known cyclins with cell cycle roles and probably more to come. These fall into two broad groups: the G1 cyclins Cln1, Cln2, and Cln3 (which regulate Start) and the mitotic, B-type cyclins Clb1, Clb2, Clb3, and Clb4. Two other cyclins, Clb5 and Clb6, are very important for DNA replication, but they also have roles at Start and perhaps also in early mitosis.

The highlight of the past year was collaborative work with A. Amon and K. Nasmyth (IMP, Vienna) which showed that the G1 cyclins and the mitotic cyclins regulate each other. This interaction may largely explain the autoregulatory cell cycle oscillation.

**Autoregulation of Cell Cycle Oscillation**

A. Amon, K. Nasmyth (IMP, Vienna), M. Tyers, B. Futcher

In a normal cell cycle, the G1 cyclins shut off just as the mitotic cyclins are coming on. Perhaps this is because the mitotic cyclins repress the G1 cyclins. This turns out to be the case: a clb1 clb2 clb3 clb4 mutant cannot turn off the G1 cyclins. The mechanism of the repression of G1 cyclins by Clbs seems to be that the Clbs interact directly with Swi4, presumably inhibiting it in some way. Swi4 is a necessary transcription factor for the G1 cyclins, and thus, with Swi4 inhibited, the G1 cyclins turn off.

Another finding is that the CLBs have a positive feedback loop for their own transcription. CLB transcription seems to require cyclin–Cdc28 kinase activity. Initially, this is probably provided by the G1 cyclin complexes. Later, the Clbs themselves provide the kinase activity needed for their own expression.

These findings led to a model of how the cell cycle oscillation might work (Fig. 1). In early G1, cells are small, and CLN3 is on. In a size-dependent manner, CLN3 activates Swi4 and Swi6, and thereby up-regulates transcription of CLN1, CLN2, and other G1 cyclins. When the Cln–Cdc28 kinase is sufficiently active, Start occurs. Passage through Start (and Cln–Cdc28 kinase activity) allows expression of the CLBs. Perhaps the Cln kinases directly phosphorylate and activate a transcription factor for CLB expression. The Clbs, once on, repress transcription of CLN1 and CLN2 by inactivating Swi4. This destroys the CLN-Swi positive feedback loop and so leads to a loss of G1 cyclins (except for CLN3). Thus, the cells have moved from a G1 state (CLNs on, CLBs off) through an intermediate state (CLNs on, CLBs on) to a G2 state (CLN1 and CLN2 off, CLBs on). High Clb levels lead to mitosis. Completion of anaphase some-
how triggers proteolysis of the Clbs. As the level of 
Clb declines, the positive feedback loop for CLB 
transcription collapses, amplifying the effect of 
proteolysis on loss of the Clbs. Soon, all Clb protein 
has been lost because of proteolysis and weakening 
transcription, so Clb-Cdc28 kinase activity is lost, 
and the mitotic spindle depolymerizes. Cells go 
through cytokinesis and enter G1. Now that Clbs are 
gone, Swi4 protein is no longer inactivated. Swi4 can 
therefore be re-activated by the ever-present Cln3, 
leading to transcription of CLN1 and CLN2 and the 
next round of Start, and so on.

Mechanism of Cln3 Action
B. Cox, L. Littlepage (URP program)

Genetically, CLN3 is the most important G1 cyclin 
gene, and yet biochemically, the Cln3 protein is rarer 
than either Cln1 or Cln2, and the associated protein 
kinase is much weaker. The main role of CLN3 
seems to be to activate the Swi4 and Swi6 transcrip-
tion factors, which allow transcription of CLN1 and 
CLN2. Cln1 and Cln2 proteins, although more 
abundant than Cln3, are poor activators of Swi4 and 
Swi6.

To look for proteins associated with Cln1, Cln2, 
and Cln3, we attempted to use the two-hybrid screen 
developed by S. Fields and co-workers. However, all 
the Cln-Gal4 fusions tested were strong transcrip-
tional activators. Perhaps this reflects the normal role 
of Cln3: Perhaps Swi4 and Swi6 bind Cln3-Cdc28 
and bring the complex to the promoters of CLN1 and 
CLN2. Cln1 and Cln2 proteins, although more 
abundant than Cln3, are poor activators of Swi4 and 
Swi6.

Size, Growth, and Start
G. Tokiwa, M. Tyers, B. Schneider

The mRNAs and protein for the G1 cyclins CLN1, 
CLN2, and CLN3 are extremely unstable. This poses 
a problem for cells growing slowly under poor condi-
tions. If the G1 cyclins are being made slowly, but 
still degraded rapidly, how does enough cyclin ac-
cumulate to trigger Start? We are examining G1 
cyclin expression under different growth conditions 
to address this question.

One finding is that CLN1 and CLN2 expression is 
partly controlled by the Ras/cAMP pathway, which is 
known to respond to nutrient conditions. Better 
nutrients up-regulate the cAMP pathway, and this 
leads to down-regulation of CLN1, and to some ex-
tent CLN2. Evidence for this is that (1) addition of 
glucose to a culture growing on a poor carbon source 
represses CLN1, (2) CLN1 fails to respond to glucose 
in a strain lacking an intact cAMP signaling pathway, 
and (3) the effect of glucose can be mimicked by ad-
dition of cAMP to a cAMP-permeable strain.

We think that this is a balancing mechanism. 
When cells synthesize G1 cyclin rapidly, G1 cyclin 
gene expression can be low. When protein synthesis 
is slow, gene expression is de-repressed to com-
pensate. Consistent with this, cells lacking an intact 
cAMP signaling pathway have difficulty adapting to 
slow-growth conditions and must grow to extra-
ordinarily large sizes before going through Start, just 
as if they were having difficulty accumulating suffi-
cient G1 cyclin.

What Is Start, Anyway?
Q.-H. Yang

Originally, Start was defined as a unique "point" in 
the cell cycle at which cells become committed to 
division. Operationally, cells have passed through 
this "point" if they have budded and initiated DNA 
synthesis and become resistant to mating pheromone.

It seems that passage through Start depends on 
achieving a critical level of Cln–Cdc28 protein 
kinase activity. Presumably, this kinase phosphory-
lates one, or several, key substrates that commit the 
cell to division. Then it is this phosphorylation or 
phosphorylations that constitute Start: Start may be 
an event, or several events, rather than a point.

If there are several substrates, one important for 
budding, one for DNA synthesis, one for pheromone 
resistance, etc., then it should be possible to complete 
some of these events but not others; i.e., perhaps Start 
can be dissociated into its component events.

We have started to do this using a cln1 cln2 
GAL-CLN3 cdc34 strain. According to current
dogma, the CLNs are needed only for Start. A cdc34 mutant arrests as a budded cell that has not initiated DNA synthesis; i.e., it is past the "point" of Start (since it has budded), but its mutant defect prevents DNA replication. In our experiment, we grow the clnl cln2 GAL-CLN3 cdc34ts strain in galactose medium (to provide CLN3 from the GAL promoter) at 23°C (the permissive temperature for cdc34). The cells are shifted to 37°C (restrictive), and the cells arrest with buds (i.e., past Start, and so by definition "committed" to division). While at the arrest, the galactose medium is washed away and replaced by raffinose medium. CLN expression ceases, and Cln3 protein is lost by rapid proteolysis. The cells are now shifted back to permissive temperature. However, they do not progress in the cell cycle; they still do not replicate DNA. This indicates that the cells were not in fact irreversibly committed to DNA replication even though they had budded. Either there was a reversible commitment or budding and DNA replication are due to two separate events.

### Clb Proteolysis

W. Seufert

Mitotic cyclins are destroyed at the end of anaphase. This cyclin destruction is needed for the loss of Cdc28 kinase activity, which is needed for spindle depolymerization and exit from mitosis. As shown in Figure 1, loss of mitotic cyclin is a key re-setting step in the cell cycle, not only allowing mitotic exit, but also allowing re-expression of CLN G1 cyclins in the next G1 phase.

All known mitotic cyclins, including Clb1, 2, 3, and 4 in *S. cerevisiae*, have amino-terminal signals called "destruction boxes" which target the cyclin for ubiquitination and proteolysis. We have been investigating this proteolysis. Previously, it had been found by W. Seufert and S. Jentsch that the ubiquitin-conjugating enzyme Ubc9 was important for Clb destruction. Now, we have found that another ubiquitin-conjugating enzyme, Ubc1, is also involved. Strangely, the destruction of at least two different Clbs with rather similar destruction boxes is regulated independently. In our system, the destruction box alone does not seem to lead to cell-cycle-regulated proteolysis. Perhaps there are other signals for proteolysis somewhere else in the cyclins, and these are responsible for the timing of destruction.

### Mechanisms of Heat Shock Resistance

B. Elliott

Although many proteins are induced by heat shocks, it is not clear how these induced proteins are involved in heat shock resistance. In most cases, mutational inactivation of a heat-shock-induced gene does not cause cellular heat shock sensitivity. Heat shock sensitivity can be caused by mutations in the Ras/cAMP pathway, suggesting that it is a major regulator of resistance. To find out about mechanisms of heat shock resistance, we looked for mutants that lacked induced heat shock resistance. One mutant is of particular interest because it controls heat shock resistance independently of the Ras/cAMP pathway.

This mutant lacks the function of a gene called *TPS2*, which is thought to be involved in synthesis of trehalose, a storage carbohydrate. Our *tps2* null mutant, however, does make trehalose, and therefore lack of trehalose does not seem to be the cause of sensitivity. There must be alternative pathways for trehalose synthesis. *TPS2* is a very large gene, and it has two close homologs in yeast. Different alleles of these genes cause a bewildering array of defects in growth, glycolysis, carbohydrate and phosphate metabolism, and, as we now know, heat shock resistance. How these are all tied together is unclear.

### Yeast Telomerase

B. Steiner

Telomerase is an enzyme that adds repeats (in this case, G1_3T) to the ends of chromosomes to compensate for the loss of terminal sequences during DNA replication. It is possible that somatic mammalian cells lack this enzyme, and this may be one of the causes of cellular senescence.

Because the enzyme is rare and biochemical characterization has been difficult, we are taking a genetic approach to finding the genes involved. We have constructed a circular plasmid that can be linearized in vivo upon expression of the HO endonuclease. The linearized plasmid has very short telomeres at its ends and will only survive in the cells if these prototelomeres can be lengthened. We are using this approach to screen for mutants that cannot lengthen
the plasmid telomeres; these may be telomerase mutants.

PUBLICATIONS


In Press


TRANSCRIPTION AND CELL CYCLE REGULATION IN YEAST

K.T. Arndt A. Sutton C. Devlin T. Zhong
M. Luke F. Lin C.J. Di Como
F. Della Seta A. Doseff H. Chang (URP)

Our research focuses on the regulation of the G1 phase of the cell cycle. For most eukaryotic organisms, it is during G1 that decisions are made as to whether or not to initiate the cell cycle and divide. For these studies, we use the model organism Saccharomyces cerevisiae, which is commonly called budding yeast. Budding yeast cells that have executed the late G1 event(s) termed "Start" are committed to initiate DNA synthesis, form a bud, and divide. Start is the point where growth signals and mating factors control entry into the cell division cycle. The execution of Start seems to require some threshold level of G1 cyclin/CDC28 kinase activity, which is determined in large part by the rate and levels at which the G1 cyclin RNAs accumulate during late G1. However, very little is known about the mechanisms that determine if G1 cyclin RNAs accumulate and the rate at which G1 cyclin RNAs accumulate.

Many cell cycle events are controlled by the regulation of the phosphorylation of certain key cell cycle substrates. We have found that the SIT4 protein phosphatase, which removes phosphate groups from phosphoserine and phosphothreonine residues, is required during late G1 for the execution of Start, for bud formation, for the initiation of DNA synthesis, and for spindle pole body duplication. Our major goals are to determine the signals that control the ability of SIT4 to promote Start and the mechanisms by which SIT4 regulates Start and late G1.

SIT4 Is Required for G1 Cyclin Accumulation

K. Arndt, C.J. Di Como, H. Chang

SIT4 is required for the execution of Start because it is required for the expression of the CLN1 and CLN2 G1 cyclin genes. CLN1 and CLN2 proteins bind to CDC28, thereby activating the kinase activity of CDC28. When some threshold level of CLN/CDC28 kinase activity is reached, Start is executed and the cells are committed for DNA synthesis and the completion of the cell cycle. During early G1, the levels of CLN1 and CLN2 RNAs are very low. During late G1, the levels of CLN1 and CLN2 RNAs increase at a very rapid rate. The rate at which CLN1 and CLN2 RNAs accumulate is determined in part by the activity of the CLN3 gene. Hyperactive alleles of CLN3 cause a more rapid rate of increase of CLN1 and CLN2 RNA accumulation, whereas loss-of-function alleles of CLN3 cause a slower rate of increase.
Much evidence shows that CLN3 and SIT4 provide additive pathways for the activation of CLN1 and CLN2 expression. In fact, mutation of both SIT4 and CLN3 causes a near lethal effect that is due solely to a defect in CLN1 and CLN2 expression. Like a sit4 mutation, a mutation in any gene required in the SIT4 pathway for the activation of CLN1 and CLN2 expression should also be lethal in combination with a Δcln3 mutation. Therefore, to identify genes functioning in the SIT4 pathway for the activation of CLN1 and CLN2 expression, we used a colony sectoring assay to isolate mutants that could not grow in the absence CLN3. These mutants, termed ctr (for CLN three requiring), fell into 12 complementation groups that were divided into two classes: class A, where (like sit4 mutations) the synthetic lethality with Δcln3 is cured by expression of CLN2 from a SIT4-independent promoter, and class B, where the synthetic lethality with Δcln3 is only partially cured by heterologous expression of CLN2. We predict that CTR genes of class A function for CLN1 and CLN2 expression, whereas CTR genes of class B might function downstream from CLN1 and CLN2 (they require higher than normal levels of CLN2) or might function in a pathway parallel to that of CLN3, but that is not involved directly in CLN1 or CLN2 expression. Therefore, we have concentrated on the analysis of the class A group.

So far, we have analyzed ctr7 in the most depth. The CTR7 gene is the same as the BCK2 gene, previously identified in David Levin's laboratory by its ability, when overexpressed, to suppress partially the temperature-sensitive cell lysis defect (due to a defect in polarized cell growth) of pck1 mutants (PKC1 encodes a protein kinase C homolog) and mpk1 mutants (MPK1 encodes a MAP kinase homolog that functions downstream from PKC1). In summary, the following are our findings: Deletion of BCK2, like deletion of CLN3, causes no obvious growth defect but causes the cells to be more sensitive to α factor and to execute Start at a larger than normal cell volume. Overexpression of BCK2, like hyperactive alleles of CLN3, causes the cells to be more resistant to α factor and to execute Start at a smaller than normal cell volume. At the level of CLN1 and CLN2 expression, a Δbck2 strain or a Δcln3 strain still has cell-cycle-dependent expression of CLN1, CLN2, and SCB-driven lacZ gene (the rate of accumulation is slightly delayed). However, a Δbck2 Δcln3 SSD1-v strain (like a Δsit4 Δcln3 SSD1-v strain) has a severe growth defect and accumulates CLN1 and CLN2 RNAs during late G1 at extremely slow rates and low levels. Moreover, the strong growth defect of a Δbck2 Δcln3 SSD1-v strain is completely cured by low-level expression of CLN2 from a SIT4-independent promoter. In addition, expression of BCK2 from the pGAL promoter, in a CLN3 strain or a Δcln3 strain, induces CLN1, CLN2, and SCB: lacZ expression (to levels slightly higher than expression of the hyperactive CLN3-1 allele from the pGAL promoter). Therefore, BCK2 and CLN3 provide parallel activation pathways for CLN1, CLN2, and SCB-dependent expression during late G1. In the absence of either CLN3 or BCK2, the other gene provides a good pathway for the cell-cycle-dependent expression of CLN1 and CLN2 via SWI4-dependent SCB promoter elements. In the absence of both CLN3 and BCK2, expression of CLN1 and CLN2 is extremely low. We suggest that BCK2 functions in the SIT4 pathway for CLN1 and CLN2 expression by the following reasoning (see Fig. 1): BCK2 and CLN3 provide parallel pathways for the activation of G1 cyclin expression and a Δbck2 Δcln3 SSD1-v strain grows very slowly due to a defect in CLN1 and CLN2 expression. Likewise, SIT4 and CLN3 provide parallel pathways for the activation of G1 cyclin expression and a Δsit4 Δcln3 SSD1-v strain grows extremely slowly due to a defect in CLN1 and CLN2 expression. However, a Δbck2 Δsit4 SSD1-v strain grows at a rate identical to that of a Δsit4 SSD1-v strain, and both strains have the same slow rate of CLN1 and CLN2 accumulation during late G1. These findings, and others (e.g. unlike a Δcln3 Δswi6 SSD1-v strain which is viable, but like a Δsit4 Δswi6 SSD1-v strain which is inviable, a Δbck2 Δswi6 SSD1-v strain is inviable), suggest a model where BCK2 functions within the SIT4 pathway for G1 cyclin expression. Our future experiments will be directed toward determining the mechanism of BCK2 function and how BCK2 relates to SIT4 (the sequence of BCK2 provides no clues for its function).

Characterization of the In Vitro Phosphatase Activity of SIT4

M. Luke

In vitro, we have not been able to detect any SIT4 activity toward para-nitrophenol phosphate or a protein phosphorylated on tyrosine residues. However, using RCM-lysozyme phosphorylated by protein kinase A
(a gift from J. Kuret) as an artificial substrate, the SIT4 phosphatase activity is readily detectable in vitro. In this assay, the SIT4 phosphatase activity is optimal at pH 6 and does not require monovalent or divalent cations. The SIT4 phosphatase activity is similar to that of mammalian type-2A protein phosphatases in that it is inhibited by nanomolar concentrations of okadaic acid. Type-1 protein phosphatases require higher concentrations of okadaic acid for inhibition. This is consistent with the fact that SIT4 is more similar to the catalytic subunit of type-2A phosphatases than to that of type-1 phosphatases. This in vitro phosphatase assay has also been used to show that the activity of the SIT4-102 protein (a sit4-102 strain is temperature sensitive and arrests in late G1) is lower than that of wild-type SIT4 protein at 24°C and (unlike wild-type SIT4 which is more active at 37°C) is not detectable at 37°C. Therefore, sit4-102 strains arrest in late G1 because of the absence of SIT4 activity. In the future, we hope to use substrates for the in vitro assay that are true in vivo substrates of SIT4.

Identification of SIT4-interacting Proteins by the Two-hybrid Approach

F. Della Seta

To identify the cellular substrates and regulatory subunits of SIT4, we used the two-hybrid technique (developed by Stan Fields) to identify SIT4-interacting proteins. We have prepared fusions of SIT4 to lexA (a bacterial DNA-binding factor, with no eukaryotic activation domain) and the DNA-binding domain of GAL4 (residues 1-147). The lexA-SIT4 fusion was transformed into a strain that contains the HIS3 and lacZ (β-galactosidase) genes driven by promoters containing lexA DNA-binding sites as UAS sequences. Similarly, the GAL4(1-147)-SIT4 fusion was transformed into a strain that contains the HIS3 and lacZ (β-galactosidase) genes driven by a promoter containing GAL4 DNA-binding sites as UAS sequences. Neither the lexA-SIT4 protein nor the GAL4(1-147)-SIT4 protein activated transcription from the lexA or GAL4 DNA-binding site containing promoters, respectively. These strains were transformed with libraries containing yeast DNA sequences fused to the GAL4 transcriptional activation domain (which does not have DNA-binding function on its own). If the yeast sequences encode a protein that interacts with SIT4, then the GAL4 transcriptional activation domain should be tethered to the lexA-SIT4 or GAL4(1-147)-SIT4 fusions, resulting in the activation of transcription from lexA and GAL4 DNA-binding sites, respectively.

We have used the two-hybrid system to screen more than 10^7 transformants from yeast genomic and cDNA libraries. We have obtained 20 unique library plasmids (many isolated multiple times) that pass all the tests of specificity to SIT4. Our next step will be to determine which of the 20 library plasmids encode proteins that physically and/or functionally interact with SIT4. We hope that the analysis of the proteins interacting with the SIT4 will allow us to better define the in vivo SIT4 substrates and the functions of SIT4.

Function and Regulation of the SIT4 Phosphatase in the Yeast Cell Cycle

M. Luke, F. Della Seta

During most of G1, SIT4 exists mostly as monomeric uncomplexed SIT4. Very close to the time SIT4 is required for the execution of Start and for bud formation, SIT4 associates in separate complexes with two high-molecular-weight proteins, p155 and p190 (the complexes dissociate at about mid-mitosis). We propose that p155 and p190, like the high-molecular-weight targeting subunits of type-1 phosphatases, may target SIT4 to specific substrates during late G1.

In this model, we propose that growth signals cause the association of p155 and p190 with SIT4, thereby activating the SIT4-dependent processes of G1 cyclin expression and bud formation.

To date, our genetic approaches have not identified the genes encoding p155 and p190. To determine the function of p155 and p190, we need to obtain these genes. Therefore, we purified p155 and p190 to obtain partial peptide sequence and for antibody production. More than 100 µg each of p155 and p190 were purified by multiple large-scale immunoprecipi-
Identification of Genes Functioning in the SIT4 Pathway for Bud Initiation

A. Doseff

In certain strain backgrounds, deletion of SIT4 is not lethal but instead results in very slow growth with a greatly expanded G₁ phase. The ability to live in the absence of SIT4 is due to a single genetic locus, which we term SSD₁ (suppressor of sit4 deletion). SSD₁-v alleles allow viability in the absence of SIT4, whereas ssd₁-d alleles give death in the absence of SIT4. The SSD₁ locus is polymorphic in that some strain backgrounds have a SSD₁-v allele and other strain backgrounds have a ssd₁-d allele.

Deletion of SSD₁ in a wild-type SIT4 strain results in only subtle phenotypic alterations (especially in G₁ regulation). It seems that whatever SIT4 can do, SSD₁-v can do as well. For instance, SSD₁-v provides a pathway to CLN₁ and CLN₂ expression that is independent of SIT4. However, the SIT4 pathway is the major pathway to G₁ cyclin expression, whereas the SSD₁-v pathway is a minor pathway.

If CLN₂ is expressed from a SIT4-independent promoter, the cells can initiate and complete DNA synthesis in the absence of both SIT4 and SSD₁-v function. Therefore, SIT4 and SSD₁-v are required for CLN₁ and CLN₂ expression, which is required for Start and the initiation of DNA synthesis. However, independently of CLN₂ expression, SIT4 and SSD₁-v are also required for bud formation. Again, SIT4 provides the major pathway and SSD₁-v provides the minor pathway leading to bud formation.

Very little is known about the factors that regulate late bud emergence and polarized cell growth. That blocking protein synthesis in cells released from a Start arrest allows actin polarization to the site of future bud emergence but prevents bud emergence raises the possibility that some factor required for bud emergence does not pre-exist at Start but must be expressed during late G₁ at some time post-Start. Could SIT4/SSD₁ be required for the expression of this factor? Unfortunately, there are too many unknown processes involved in bud emergence to design experiments to address these questions directly. Therefore, we have initiated a screen that should identify genes that function in the SIT4 pathway for bud emergence.

We used a synthetic lethal screen to identify genes that function in the SIT4 budding pathway by isolating mutations that (like sit4) are lethal in the absence of SSD₁-v. From this screen, we isolated 75 mutants (termed LAS, for lethal in the absence of SSD₁) that fell into six complementation groups. That we isolated mutations in SIT4 four times confirms the design of the screen. LAS₁ (defined by the major complementation group) is a previously unidentified essential gene that encodes a predicted 64-kD protein with no strong similarity to known proteins. Overexpression of LAS₁ (from the pGAL promoter) causes a remarkable phenotype: Up to five extra small buds are formed in addition to the main bud (Fig. 2; the main bud of the cell is not visible in this slice). These projections are buds because actin cables extend into the projections and because SPA2 protein (which is localized to normal buds in wild-type cells) is localized to each small projection. These small buds remain small and do not get larger, suggesting that overexpression of LAS₁ promotes emergence of the bud, not continued growth of the bud. In addition, that these extra small buds occurred only on the mother cell suggests that the cells might have to execute Start to obtain the extra small buds. These findings (combined with the finding that HA epitope-tagged LAS₁ fractionates with nuclei) raise the possibility that LAS₁ is a positive regulator of bud emergence. Database searches showed that LAS₁ has weak similarity to the E2F transcription factor and that this similarity is better than that between E2F and SWI4 (which has been proposed to be related to E2F). However, the significance of the weak similarity of LAS₁ to E2F is not known.

Although mutations in LAS₁ interact genetically with mutations in SIT4, we do not yet know if LAS₁ and SIT4 function within the same pathway for bud formation.
FIGURE 2 Overexpression of LAS1 causes the formation of extra small buds. Thin-section electron microscopy (freeze substitution; performed by Bob Derby) of a pGAL:LAS1 strain grown on galactose medium; 50-60% of the cells had three to five extra small buds in addition to a main bud (which gives rise to the daughter cell). The main bud is not visible in this slice.

Isolation of Mutations That Cause Lethality in Combination with a sit4 Mutation

A. Sutton

One way to identify genes functioning downstream from SIT4 (within the SIT4 pathway) or in a pathway parallel to the SIT4 pathway is to identify mutations that cause inviability in the absence of SIT4 function. We obtained mutations in eight different complementation groups that cause lethality in the absence of SIT4 function. These genes are called PDL1 through PDL8, for phosphatase deficient lethality. So far, we have initiated the characterization of PDL1 and PDL3.

The PDL1 gene is the same as the previously identified CHS1 gene. CHS1 encodes one of three known chitin synthase enzymes in budding yeast. Chitin synthase is required for cell wall chitin synthesis at specific stages of the cell cycle. Unlike the pdll-1 mutation, which causes lethality in the absence of SIT4, deletion of CHS1 does not cause lethality in the absence of SIT4. This finding suggests that the pdll-1 allele is not simply a loss-of-function mutation. Although there are no readily observable phenotypic alterations associated with the pdll-1 mutation in a wild-type SIT4 mutant, the pattern of chitin deposition in the pdll-1 cell walls is aberrant: There is a very pronounced punctate staining throughout the mother cell and daughter bud. The pdll-1 mutation may alter the localization and/or enzymatic activity of CHS1. Experiments are currently under way to distinguish between these possibilities. In any case, that the pdll-1 mutation results in lethality in the absence of SIT4 suggests a role of SIT4 in cellular morphogenesis or cell wall biosynthesis.

The PDL3 gene encodes a previously unidentified essential protein with sequence similarity to serine/threonine protein kinases. We have made several mutations in PDL3 that separately alter residues which are highly conserved among protein kinases and are known to be required for catalytic activity. These mutations result in sharply reduced PDL3-dependent kinase activity in vitro. The pdl3 mutation causes an aberrant cell morphology (the cells are considerably longer than wild-type cells), implicating the PDL3 kinase in polarized cell growth. The pdl3 mutation also causes a delay in the G2 or M phase of the cell cycle. Northern analysis reveals that pdl3 mutant cells have a defect in their ability to accumulate mitotic cyclin RNAs during G2. Therefore, the PDL3 protein kinase may be involved in morphogenesis and may be required for activation of the CLB/CDC28 kinase. We have previously obtained evidence suggesting that SIT4 may have a direct role in budding. The fact that we have identified genes involved in budding and polarized cell growth in a synthetic lethal screen with a sit4 mutation provides further support for this hypothesis.

Overexpression of SIS2 Increases the Growth Rate of sit4 Mutants

C.J. Di Como

Yeast strains containing a mutation in the SIT4 gene have a very slow growth rate. SIS2 was isolated as a gene that, when present in high copy number, dramatically increased the growth rate of sit4 mutants. Moreover, loss-of-function mutations in SIS2 cause lethality when combined with a sit4 mutation. Additional genetic tests show that SIS2 functions in a pathway parallel to the SIT4 pathway (but not in the SSD1-v pathway).
The **SIS2** gene predicts a protein of 562 amino acids and a molecular mass of 62 kD. The **SIS2** protein contains an extremely acidic carboxy-terminal region where within a stretch of 59 amino acids, 51 residues are either glutamate or aspartate. Strains containing a deletion of **SIS2** are viable and have a normal growth rate, are not temperature sensitive for growth, accumulate normal levels of glycogen, have no obvious mating defects, and have a normal cell size.

Because **sit4** mutants have a defect in G1 cyclin RNA accumulation, we examined if overexpression of **SIS2** can stimulate the rate of **CLN1** and **CLN2** RNA accumulation during late G1 in a **sit4** mutant. We found that overexpression of **SIS2** greatly increased the rate of **CLN1** and **CLN2** RNA accumulation in **sit4** mutants. In addition, we looked at a **CLN1 cln2 cln3** (wild-type **SIT4**) strain, which accumulates **CLN1** RNA at slower rates due to the absence of **CLN2** and **CLN3**. We found that overexpression of **SIS2** greatly increased the rate of **CLN1** RNA accumulation for a **CLN1 cln2 cln3** strain. The ability of overexpressed **SIS2** to stimulate G1 cyclin expression suggests that overexpression of **SIS2** increases the growth rate of **sit4** mutants by stimulating the transcription of certain genes whose RNA levels are present at low levels due to the **sit4** mutation.

To determine the mechanism by which **SIS2** is able to stimulate transcription, we performed biochemical fractionation experiments. **SIS2** fractionates with nuclei. In addition, **SIS2** is released from the nuclear fraction upon treatments (DNase I or micrococcal nuclease) that digest DNA within chromatin but not by other treatments (RNase A). These results raise the possibility that **SIS2** might physically interact with chromatin. Indeed, strains with lower histone gene dosage of histone H2A and H2B are sensitive to higher levels of **SIS2** protein (due to even a single extra copy of the **SIS2** gene).

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The **SIS1** Protein: An Essential DnaJ Homolog Required for the Initiation of Translation and for Normal Regulation of the Heat Shock Response

T. Zhong

The budding yeast **SIS1** gene encodes an essential heat shock protein with similarity to the bacterial DnaJ heat shock protein. To understand the cellular functions of the **SIS1** gene, we isolated an extragenic Ts+ suppressor of the temperature-sensitive **sis1-85** mutant strain. This suppressor mutation occurred in the gene encoding a 60S ribosomal subunit protein. These results led to the finding that **SIS1** is required for the initiation of translation and associates with ribosomes. Recently, we found that **SIS1** may have an additional role: regulation of the heat shock response. We do not currently know if the heat shock regulation role of **SIS1** is related to the role of **SIS1** for the initiation of translation.

Heat shock normally results in the rapid transcriptional activation of heat shock genes and the preferential translation of heat shock messages. The transcription and translation of nonheat shock genes are repressed. We examined the levels of **SIS1** RNA in wild-type **SIS1** strains and in **sis1** mutant strains. Interestingly, at 24°C with no heat shock, the levels of **SIS1** RNA are fivefold higher in the **sis1** mutant strains than in a wild-type **SIS1** strain. Addition of wild-type **SIS1** to the **sis1** mutant strains represses the **sis1** RNA levels. Therefore, the cells can sense a defect in **SIS1** and induce the transcription of **SIS1**. Because the levels of **sis1** RNA are induced in **sis1** mutants, we examined the effect of **sis1** mutations on the RNA levels of other heat shock genes, including the **HSP70** (DnaK homolog) and **HSP90** gene families.

The **SSA4** gene is one of four highly related genes encoding a particular class of yeast HSP70 protein. In a wild-type strain, heat shock causes a 5-fold induction of **SSA4** RNA levels. The **SSA4** gene has no sustained heat shock response: By 90 minutes after the heat shock of a wild-type strain, **SSA4** RNA decreases to very low levels. In contrast, in an **sis1** mutant strain, heat shock causes an 18-fold induction in the level of **SSA4** RNA and after 90 minutes, the level of **SSA4** RNA is about 60% of the maximal heat shock level. Elevated expression of the **SSA4** gene has also been seen in **ssa1** **ssa2** mutant strains. The increase in the level of **SSA4** RNA under conditions of reduced **SIS1** function suggests a negative regulatory model of the control of the heat shock response. We also looked at the effect of **sis1** mutations on the expression of the two yeast **HSP90** genes (termed **HSC82** and **HSP82**). Importantly, the expression of the constitutively expressed **HSC82** gene and the expression of the heat-shock-inducible **HSP82** gene were not altered in **sis1** mutants. Therefore, **sis1** mutations selectively alter the heat shock response of only certain heat shock genes. Future studies will be
**Role of Type-2A Protein Phosphatase for Entry into Mitosis**

F. Lin

Budding yeast has two type-2A protein phosphatase genes, *PPH2α* and *PPH2β*. Disruption of either gene has no detectable effects, but deletion of both genes results in a slow growth phenotype. Only triple deletions of *PPH2α*, *PPH2β*, and a third type-2A-related gene, *PPH3*, are lethal. To investigate how type-2A protein phosphatases function for the cell cycle, we prepared a temperature-sensitive mutation in *PPH2β*, in a strain deleted for *PPH2α* and *PPH3*. For simplicity, we call this strain *pph2β-102*. When shifted to 36°C, the *pph2β-102* cells accumulate budded cells with a 2n DNA content and with a defect in mitotic spindles. These findings suggest that a major requirement for type-2A phosphatase is for advancement into mitosis.

A key factor required for progression from *G2* into mitosis is the B cyclin/CDC28 kinase. Because CLB2 is the major B cyclin in budding yeast, we examined the CLB2/CDC28 kinase activity (for histone H1) in wild-type and *pph2β-102* mutant cells. When released from a hydroxyurea arrest (which gives large budded cells with a 1n DNA content) and shifted to 36°C, wild-type cells are able to double the cell number and finish one cell cycle within 2 hours. In addition, the CLB2/CDC28 kinase activity increased dramatically as the cells entered and progressed through mitosis. In contrast, the *pph2β-102* cells completed DNA synthesis but failed to increase in number and failed to increase the CLB2/CDC28 kinase activity (in fact, the CLB2/CDC28 kinase activity decreased) even though the levels of CDC28 were normal and the levels of CLB2 protein were normal. One known pathway for the activation of B cyclin/CDC2 complexes (CDC2 is a homolog of CDC28) is via the dephosphorylation of a phosphotyrosine in the ATP-binding pocket of CDC2. However, changing the Tyr-19 residue of CDC2 (in the presumed ATP-binding pocket) to a phenylalanine residue did not cure the *G2* block and did not restore the kinase activity of the CLB2/CDC28 complexes isolated from a *pph2β-102* strain shifted to 36°C. Therefore, in budding yeast, the type-2A phosphatases are required, by a mechanism other than Tyr-19 dephosphorylation, to activate CLB2/CDC28 complexes.

**Alterations in GMP Synthase Can Increase *HIS4* Transcription in the Absence of the Normal Activators of Transcription**

C. Devlin

GCN4, BAS1, and BAS2 are transcription factors that bind to the *HIS4* promoter to activate *HIS4* transcription. In the absence of GCN4, BAS1, and BAS2, the yeast cells have a His- phenotype due to insufficient transcription of *HIS4* (which encodes an enzyme required for biosynthesis of histidine). We isolated *sit* mutations (suppressors of the initiation of transcription) that confer the ability of a *gcn4 bas1 bas2* strain to increase *HIS4* transcription and become His+. The *sit1* and *sit2* mutations occurred in the largest and second largest subunits of RNA polymerase II. Mutations in the *SIT7* gene, which encodes GMP synthase, were also isolated as His+ suppressors of a *gcn4 bas1 bas2* strain. Primer extension analysis showed that the *HIS4* RNA in the *sit7* strain initiated at four sites: One of the start sites was at the wild-type initiation site, and three of the start sites were upstream of the wild-type *HIS4* transcription initiation site. *HIS4* RNAs initiating at the site immediately upstream of the wild-type site were the most abundant. A *sit7-1* mutant strain has a slow-growth phenotype, is temperature sensitive for growth at 37°C, and is a partial inositol auxotroph. Addition of guanosine to the culture medium stimulated the growth rate of the *sit7-1* strain and reversed the His+ suppression effect. Therefore, limitation of guanine nucleotides can alter transcription of *HIS4* in the absence of the normal DNA-binding transcriptional activators. This effect is specific to guanine nucleotides because limitation for adenine nucleotides does not alter *HIS4* transcription. The mechanism by which limitation for guanine nucleotides alters transcription is under investigation.

**PUBLICATIONS**

SMALL NUCLEAR RNA GENES

The human small nuclear RNA (snRNA) genes form a unique family containing both genes transcribed by RNA polymerase II (e.g., the U1 and U2 genes) and genes transcribed by RNA polymerase III (e.g., the U6 gene). Yet all snRNA genes have very similar promoters that consist of an essential proximal sequence element (PSE) located around position -55 and an enhancer referred to as the distal sequence element (DSE) located around position -215. The RNA polymerase III snRNA promoters contain in addition a TATA box located around -28. Curiously, the TATA box is the determinant of RNA polymerase III specificity. When the U6 TATA box is mutated, the RNA polymerase III U6 promoter becomes transcribed mainly by RNA polymerase II, and when the U6 TATA box is inserted into the U2 promoter, the RNA polymerase II U2 promoter becomes transcribed by RNA polymerase III. Because the RNA polymerase II and III snRNA promoters are so similar, they offer a unique model system to identify the determinants of RNA polymerase specificity.

Tremendous progress has been achieved in identifying and cloning genes corresponding to transcription factors involved in RNA polymerase I, II, and III transcription. One of the key advances has been the realization that several of the factors that had been refractory to purification by standard chromatography are in fact large complexes that contain a common subunit, the TATA-box-binding protein (TBP). Thus, TFIIID, which recognizes the TATA box in promoters of mRNA-encoding genes and is also required for transcription from TATA-less mRNA promoters, is a large TBP-containing complex consisting of TBP and eight to ten associated polypeptides. SL1, a factor required for transcription of the rRNA genes by RNA polymerase I, is a TBP-containing complex consisting of TBP and three associated polypeptides. In addition, TFIIIB, a factor involved in RNA polymerase III transcription from TATA-less RNA polymerase III promoters such as the 5S and tRNA promoters, is also a TBP-containing complex.

As a step toward understanding the determinants of RNA polymerase specificity in snRNA gene transcription, we are identifying the factors involved in transcription of the U1, U2, and U6 snRNA genes. In particular, we have examined whether transcription of these genes requires TBP- or TBP-containing complexes.

Characterization of the TFIIIB Fraction

S.M. Lobo, M.L. Sullivan, N. Hernandez

Figure 1A shows the assembly of transcription factors on a typical RNA polymerase III promoter, the VAI promoter. Like the promoters of tRNA genes, the VAI promoter consists of an A box and a B box located downstream from the start site of transcription. These elements are recognized by the transcription factor IIIC, a large factor composed of at least five polypeptides. The binding of TFIIIC allows the recruitment of TFIIIB to the DNA, and this in turn allows the recruitment of RNA polymerase III. In yeast, it has been shown that after the recruitment of TFIIIB, TFIIIC can be stripped off the DNA by treatment with high concentrations of salt or heparin, leaving only TFIIIB on the DNA. TFIIIB on its own is then capable of directing several rounds of transcription by RNA polymerase III. Thus, at least in yeast, TFIIIC is an assembly factor whose main role is to recruit TFIIIB to the DNA, whereas TFIIIB recruits RNA polymerase III, presumably by direct protein-protein contacts.

Since TFIIIB is a key RNA polymerase III transcription factor in yeast, we wanted to characterize mammalian TFIIIB and evaluate its role in transcription of the VAI and U6 genes. Mammalian TFIIIB consisted of a crude phosphocellulose fraction (B fraction). We further fractionated TFIIIB by chromatography over a Mono-Q column and could show that
the activity separates into two components designated 0.38 m-TFIIIB and 0.48 m-TFIIIB, which are both required for transcription of the VAI gene. 0.38 m-TFIIIB is a TBP-containing complex, whereas 0.48 m-TFIIIB is uncharacterized. Thus, as shown in Figure 1B, we can now represent the TFIIIB fraction as consisting of two components, one of which (0.38 m-TFIIIB) is a TBP-containing complex.

An important question is whether the VAI and U6 promoters, which have completely different structures, recruit the same TFIIIB components. We have shown previously that transcription from the U6 promoter requires TBP bound to the U6 TATA box in addition to the TFIIIB fraction. As illustrated in Figure 1C, transcription reconstitution experiments indicate that U6 transcription requires only the 0.48 m-TFIIIB component in addition to TBP. The 0.38 m-TFIIIB TBP-containing complex is dispensable. This observation suggests that the role of the TBP-associated factors (TAFs) in the 0.38 m-TFIIIB complex is to bring TBP to the promoter through protein-protein interactions with TFIIIC and perhaps also through protein-DNA interactions. In the case of the U6 promoter, TBP can be recruited to the promoter by direct binding to the TATA box, and the 0.38 m-TFIIIB TAFs are therefore dispensable.

Characterization of the 0.38 m-TFIIIB Factor

R. Mital, N. Hernandez

To identify the exact components of the 0.38 m-TFIIIB TBP-containing complex, we are using a functional transcription assay coupled with immunoprecipitations. In the functional assay, we follow purification of the 0.38 m-TFIIIB complex by testing fractions for their ability to reconstitute VAI transcription when combined with a crude phosphocellulose fraction (C fraction) that provides RNA polymerase III and TFIIIC, as well as a crude phosphocellulose B fraction depleted of TBP-containing complexes by passage over an anti-TBP monoclonal antibody column. This depleted B fraction provides 0.48 m-TFIIIB but is largely devoid of 0.38 m-IIIB. To visualize polypeptides specifically associated with TBP in the 0.38 m-TFIIIB complex, we perform non-denaturing immunoprecipitations with different anti-TBP monoclonal antibodies from fractions at different stages of purification. After purification of the 0.38 m-TFIIIB activity over three columns, the non-denaturing immunoprecipitations consistently bring down TBP and a doublet of two polypeptides with apparent molecular masses of about 85 and 90 kD. This doublet is absent from denaturing immunoprecipitations, suggesting that it is not due to direct cross-reaction with the antibodies. This is confirmed by the observation that the doublet is observed in non-denaturing immunoprecipitations performed with different anti-TBP monoclonal antibodies from fractions at different stages of purification. After purification of the 0.38 m-TFIIIB activity over three columns, the non-denaturing immunoprecipitations consistently bring down TBP and a doublet of two polypeptides with apparent molecular masses of about 85 and 90 kD. This doublet is absent from denaturing immunoprecipitations, suggesting that it is not due to direct cross-reaction with the antibodies. This is confirmed by the observation that the doublet is observed in non-denaturing immunoprecipitations performed with different anti-TBP monoclonal antibodies that recognize different epitopes in TBP. The doublet is not seen in immunoprecipitations performed with an irrelevant antibody. We are therefore confident that this doublet is associated with TBP as part of the 0.38 m-TFIIIB activity. We are scaling up the purification to obtain sufficient amounts of material for protein sequencing.
The PSE-binding Factor Is a TBP-containing Complex Required for Transcription of snRNA Genes by RNA Polymerases II and III

C.L. Sadowski, R.W. Henry, S.M. Lobo, N. Hernandez

The U6 TATA box binds TBP and, in the context of the U6 promoter, is a dominant determinant for RNA polymerase III specificity. Since the RNA polymerase II snRNA promoters are recognized by RNA polymerase II by virtue of the absence of a TATA box, an interesting question is whether these promoters recruit TBP. To address this question, we depleted an extract of TBP by passage over an anti-TBP or an irrelevant monoclonal antibody column and asked whether U1 and U2 transcription was affected. We found that depletion of TBP specifically inhibited U1 and U2 transcription, suggesting that TBP is indeed required for RNA polymerase II transcription of these genes. Intriguingly, efficient transcription could be restored by addition of a fraction enriched in the snRNA-activating protein (SNAP), a PSE-binding activity (see below). Thus, either the SNAP fraction was contaminated with TBP or TBP is in fact part of the SNAP factor. We could show that the SNAP factor is indeed a TBP-containing complex by disrupting SNAP binding to a PSE-containing probe in a gel mobility shift assay with different anti-TBP monoclonal antibodies. Thus, the TBP-associated proteins in SNAP essentially reprogram the DNA-binding specificity of TBP and allow its recruitment to a new sequence, the PSE. As shown in Figure 2A, we imagine that the binding of SNAP to the U1 PSE allows the subsequent recruitment of at least some of the general factors involved in RNA polymerase II transcription of mRNA-encoding genes.

The RNA polymerase II and III snRNA promoters have exchangeable PSEs, suggesting that the same factor may be involved in transcription by the two RNA polymerases. We therefore tested in a reconstitution experiment whether U6 transcription required the SNAP fraction. We found that SNAP is indeed required for U6 transcription and cannot be replaced by TBP. This and other observations suggest that, as shown in Figure 2B, the U6 initiation complex contains at least two molecules of TBP—one bound to the TATA box and the other bound to the PSE as part of the SNAP complex. Consistent with this possibility, we can show that TBP and SNAP can co-occupy their respective binding sites on a probe corresponding to the U6 promoter. We are presently performing experiments to confirm the presence of two molecules of TBP in the U6 initiation complex.

FIGURE 2 SNAP is a TBP-containing complex present in the RNA polymerase II and III initiation complexes formed on snRNA promoters. (A) SNAP binds to the U1 PSE and presumably recruits some of the general RNA polymerase II transcription factors. (B) SNAP binds to the U6 PSE, and TBP binds to the U6 TATA box. As a result, the U6 initiation complex contains at least two molecules of TBP. We imagine that the protein surface resulting from interactions between TBP bound to the TATA box and SNAP mediates the specific recruitment of 0.48 m-TFIIB.

Purification of SNAP and Isolation of cDNA Clones Encoding Components of the SNAP Complex

R.W. Henry, C.L. Sadowski, N. Hernandez

We have used a gel mobility shift assay to purify the SNAP complex. The purification consists of an ammonium sulfate cut followed by four columns and a glycerol gradient. We have obtained protein sequences for all the major polypeptides that peak with DNA-binding activity in the final glycerol gradient and are in the process of generating polymerase chain reaction (PCR) probes and obtaining corresponding cDNAs.

HUMAN IMMUNODEFICIENCY VIRUS

Transcription from the human immunodeficiency virus type-1 (HIV-1) promoter is regulated in part by...
a viral trans-activator referred to as Tat. Tat acts through the TAR element, an RNA target encoded between positions +18 and +44 downstream from the start site of transcription. The mechanism of Tat action has been controversial. In transfection experiments, the HIV-1 long terminal repeat (LTR) directs the formation of a large number of short transcripts and a small number of full-length transcripts in the absence of Tat. In the presence of Tat, the number of short transcripts decreases, and the number of long transcripts increases. This and other observations prompted the hypothesis that Tat is an antiterminator that functions by promoting elongation of the short transcripts. However, in other systems, Tat seems to act by increasing the levels of initiation, as transcription close to the promoter is increased in the presence of Tat as measured in run-on experiments.

We have described the identification of a transcriptional element, the inducer of short transcripts (IST), which activates transcription from the HIV-1 and other promoters. However, the RNA molecules resulting from this activation of transcription are all short. This suggests that IST functions by directing the formation of transcription complexes that are unable to elongate efficiently and therefore give rise to prematurely terminated RNAs. As described below, we have located IST precisely and examined whether IST activity is required for Tat trans-activation.

Characterization of the Inducer of Short Transcripts
M. Sheldon, F.C. Pessler, M.L. Sullivan, N. Hernandez

IST is located between positions -5 and +82 relative to the HIV-1 start site of transcription. Analysis of a series of mutations encompassing the region from +1 to +82 defines IST as a bipartite element, located at positions -5 to +26 and +40 to +59. The most important region for activity is that proximal to the promoter, but the +40 to +59 region contributes to maximal activity. The RNA secondary structure is not important for IST function, and IST can be inverted without loss of activity, strongly suggesting that, unlike TAR which is an RNA element, IST is a DNA element.

Most of the IST does not overlap with the region encoding the TAR element. As a result, it is possible to debilitate IST with mutations that leave TAR intact. Such mutations reduce the formation of short transcripts to barely detectable levels in the absence of Tat, yet they do not inhibit Tat trans-activation. This suggests that (1) Tat can function by binding to TAR present on a long transcript and (2) Tat does not function by promoting elongation of short transcripts into long transcripts, since in an IST- mutant, there are few or no short transcripts to elongate. These observations strongly suggest that Tat does not act on an RNA polymerase paused around position +60 but rather on an RNA polymerase at the promoter. The effect of Tat on this polymerase could be either to increase the rate of initiation or to improve the elongation properties of the enzyme, or both. To confirm these conclusions, we are in the process of testing another mutation in IST which should completely abolish the formation of short transcripts. If this mutation does not inhibit Tat trans-activation, the antiterminator model can be definitively ruled out.

Identification of Trans-acting Factors Involved in IST Function
F.C. Pessler, N. Hernandez

We have identified a putative IST factor that in a mobility shift assay binds to probes containing an intact IST but not to probes containing a debilitated IST. This factor differs from previously identified factors that bind downstream from the HIV-1 start site, such as LBP-1 and TFII-I. So far, the factor has been purified over three successive chromatographic columns. We are pursuing the purification of the factor to be able to identify the polypeptides that compose it.

Identification of TBP Domains Required for Tat Trans-activation and IST Activity
P.S. Pendergrast, D.B. Morrison, M. Sheldon, N. Hernandez

The observation that HIV-1 constructs carrying severe mutations in the IST element can still be trans-activated by Tat strongly suggests that Tat acts on an initiation complex located at the promoter to
increase either the initiation rates or the elongation properties of the RNA polymerase. This raises the question of whether Tat is comparable to other well-studied trans-activators that bind to DNA targets in different promoters. To approach this question, we are examining which domains of the TATA-box-binding protein TBP are required for trans-activation by Tat. For this purpose, we have modified the HIV-1 TATA box to the sequence TGTAAA. This sequence is recognized by an altered specificity TBP (developed by K. Struhl and colleagues) that contains mutations in the DNA-binding domain. We have shown that changing the HIV-1 TATA box to TGTAAA inhibits basal transcription and Tat trans-activation and that both can be restored by cotransfection of a vector expressing the altered specificity TBP. We will now test a series of mutations in the altered specificity TBP.

**Direct Interactions between TBP and Tat**

D.B. Morrison, P.S. Pendergrast, N. Hernandez

Tat has been reported to interact directly with TBP. We are interested in determining whether mutated TBPs that cannot support Tat trans-activation have also lost the ability to interact directly with Tat. For this purpose, we are generating GST-Tat and GST-TBP affinity columns. Wild-type and mutant Tat and TBP proteins will then be tested for their ability to be retained on such columns.

**PUBLICATIONS**


**In Press**


**TELOMERASE AND TELOMERE LENGTH REGULATION**

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Chromosome stability is essential for living cells. Eukaryotes have linear chromosomes, and the telomeres that cap the ends protect chromosomes from degradation and recombination. In the 1930s, Muller and McClintock recognized that broken chromosomes that lack telomeres are unstable. Subsequently, experiments in protozoa, yeast, and mammalian cells have demonstrated the requirement for telomeres. Telomeres are highly conserved in eukaryotes. The DNA sequence contains simple tandem repeats of specific GT-rich motifs. The exact sequences are characteristic of a particular organism: for example, d(TTGGGG) in *Tetrahymena*, d(TTTTGGGG) in *Oxytricha*, and d(TTAGGG) in humans. The number of repeats on any given chromosome end may vary, giving telomeres a characteristic heterogeneous or "fuzzy" appearance on Southern blots.

*Tetrahymena* telomerase is a ribonucleoprotein (RNP) containing an essential endogenous RNA component. The enzyme synthesizes d(TTGGG) repeats de novo onto synthetic single-stranded d(TTGGG) primers. The nucleotides are added one base at a time. The 159-nucleotide RNA component contains within it the sequence CAACCCCAA.
which provides the template for d(TTGGGG) repeat synthesis. Since the initial identification of the Tetrahymena telomerase, telomerase activity has been found in three other ciliates, as well as Xenopus laevis, and immortalized human and mouse culture cells. The properties of telomerase in these species are very similar to those of the Tetrahymena enzyme. However, the mouse and Xenopus enzymes do not synthesize long products as do the other enzymes. The telomerase RNA component has only been cloned from one other organism: Euplotes telomerase RNA contains the sequence CAAAAACCCC-AAAACC complementary to the d(ITITGGGG) telomeric repeats in this ciliate.

Our model for the role of telomerase in telomere replication suggests that an equilibrium is established between telomere shortening and telomere lengthening during rounds of cell division. DNA replication leads to telomere shortening because DNA polymerase cannot replicate the molecular end of double-stranded DNA. Telomerase elongates the chromosome through de novo telomere sequence addition. DNA polymerase and primers then fill in the complementary C-rich strand.

Strikingly, length maintenance does not occur in primary human somatic cells. We found that as primary human fibroblasts are passaged in culture, telomere length decreases. Primary human cells have a limited life span in culture, and telomere shortening correlates with loss of replicative capacity. Cells from older donors have shorter telomeres and go through fewer divisions before senescence. Telomere shortening in tissues in vivo has been demonstrated for fibroblasts, leukocytes, and endothelial cells. Sperm cell telomeres do not shorten with age, suggesting that the germ line is protected from telomere loss. Although telomere loss is correlated with cell senescence, it is not clear whether shortening has any direct role in this process.

Telomere shortening is also found in human cancer tissues. Telomeres in Wilms' tumors and mammary and colon carcinomas were initially shown to be shorter than those in normal neighboring tissues. These data are consistent with the observation that telomeres shorten with each round of cell division. Tumor tissue has undergone many more rounds of division than has the normal neighboring cells. Clearly, telomere shortening cannot go on without major consequences on the cell. To determine what happens to cells when telomeres get very short, in collaboration with Drs. Harley and Bacchetti at McMaster University, we followed telomere length and telomerase activity throughout the process of cellular immortalization. Human embryonic kidney cells were transfected with SV40 T antigen and immortal variants were selected. Telomeres shortened progressively in the primary and extended life span clones. Telomerase was not detected in these cultures. At crisis, many of the cells died. However, in those clones that survived crisis, telomeres were stabilized at a very short length and telomerase activity was detected. These results suggest that primary human cells express little or no telomerase activity, but after immortalization, the cells reactivate telomerase and telomere length is maintained. If telomerase is required for the growth of immortalized cells, telomerase inhibitors may be excellent anticancer drugs. In the past year, our laboratory has focused on the biochemistry of Tetrahymena telomerase and regulation in mammalian cells and during development in Xenopus.

Primer Binding by Tetrahymena Telomerase RNP

L.A. Harrington, C.W. Greider [with undergraduate researcher C. Hull and rotation student J. Crittenden]

To study the kinetic parameters associated with telomerase, one must be able to isolate the different steps in the elongation cycle. All studies to date have used primer elongation as a measure of primer-binding efficiency. However, if the rate-limiting step is elongation, the intrinsic affinity of telomerase for different primers may not be measured by assaying elongation products. To dissociate primer binding from elongation and to test the role of the RNA component in binding, we identified and characterized a specific telomerase gel shift. When partially purified telomerase fractions were incubated with 32P-labeled d(TTGGGG)3, a labeled band appeared near the top of the gel (Fig. 1). Four lines of evidence indicated that telomerase was responsible for this shift: (1) The shift was competed by excess d(TTGGGG)3 but not by nontelomeric oligonucleotides; (2) the shift copurified with telomerase more than 1000-fold and cosedimented on a sucrose gradient; (3) the shift was destroyed by preincubation of telomerase with RNase or micrococcal nuclease; and (4) PCR analysis showed that telomerase RNA was present at a posi-
FIGURE 1 Identification of a d(TTGGGG)$_3$-specific mobility shift. Approximately 1 ng of the probe $^{32}$P-labeled d(TTGGGG)$_3$ was incubated with 10 μl of partially purified telomerase extract. For competition with unlabeled oligonucleotides, the indicated amount of competitor was added to the mobility shift mixture. (1) $^{32}$P-labeled 1 kbp marker; (2-6) competition with unlabeled d(TTGGGG)$_3$ from 2500 ng (lane 2) to no competitor (lane 6). (7-11) Competition with oligo 3; (12-16) competition with pBR. A large arrow indicates the position of the d(TTGGGG)$_3$-specific complex. The sequences of oligonucleotides used in this experiment are as follows: pBR, 5' -AGCCACTATCGACTACGCGATCAT-3', oligo 3, 5' -GCACTAGATTTTGGGG.

UV Cross-linking of Telomerase Components

L.A. Harrington

We used UV cross-linking to identify potential components of the telomerase enzyme. After testing several different nucleotide analogs for cross-linking, we found that the aryl azide, N$_3$RdUTP, gives high cross-linking efficiency with telomerase. Telomerase was incubated under reaction conditions with $^{32}$P-labeled dGTP and N$_3$RdUTP. The analog was incorporated into reaction product in place of dTTP. After cross-linking for 5–10 minutes, proteins were resolved on an SDS gel. Several radioactively labeled bands were apparent; however, only the band at approximately 100 kD was specific to UV treatment. RNase treatment either before or after telomerase elongation with N$_3$RdUTP abolished the labeling at 100 kD. We used our most highly purified telomerase fractions and found that the 100-kD labeled product peaked with activity in the final sucrose gradient (Fig. 2). These data suggest that a polypeptide of approximately 100 kD is a component of telomerase.

Reconstitution of Active Telomerase from Proteins and RNA

C. Autexier

In the past year, we have developed a functional reconstitution assay for telomerase. We have used this assay to study mutations in the telomerase RNA and to identify a previously unknown specialization in the RNA template region. Using a method of RNP "unfolding" with EDTA, we defined a specific complex on an acrylamide/agarose gel that was competed with unlabeled telomerase RNA but not with other unlabeled RNAs. After defining salt and temperature optima for this exchange reaction, we then used these conditions in functional assays. Removal of endogenous RNA with micrococcal nuclease (MNase) completely abolished telomerase activity. Incubation of the enzyme with EDTA and a T7 transcript of the telomerase RNA, followed by a brief heating step and re-addition of Mg$^{2+}$, resulted in restoration of telomerase activity. To examine the properties of the reconstituted telomerase, we tested primer specificity after reconstitution. Only telomeric sequence oligonucleotides were elongated, the addition products were similar to wild-type enzyme, and activity was RNase-sensitive. Primers containing permuted versions of the telomeric sequence d(TTGGGG)$_3$ were elongated with the addition of the correct next nucleotide in a manner identical to that of the wild-type enzyme (Fig. 3). For example, d(TTGGGG) had TTG added first, whereas d(GGGGTT) had GGG...
added first. To define the sequence added, ddTTP was used to chain-terminate the elongation products. These experiments demonstrated that the reconstituted telomerase has similar specificity to that of the wild-type enzyme. Since the synthetic T7 transcript used does not contain any modified bases, any modification that may be present in the wild-type RNA must not be essential for activity.

We used the reconstituted enzyme to study the role of nucleotides in the template region of the RNA. Our initial experiments suggest that all nine nucleotides in the template region 5′-CAACCCCAA-3′ do not provide templating information as previous models had predicted. Five different mutations were made in the "template region" by site-directed mutagenesis: 5′-UAACCCCAA-3′ (43U), 5′-UAACCCUA-3′ (4349U), 5′-CGGCCCAA-3′ (4445G), and 5′-CAACCCCGG-3′ (5051G). To define the sequence added onto primer oligonucleotides, mutant telomerase enzymes were subjected to a variety of tests. First, since each mutation introduces a new nucleotide into the template region, elongation was carried out in the presence or absence of this nucleotide. For example, the mutant 5′-UAACCCCAA-3′ (43U) did not generate long products when dATP was omitted from the reaction. Second, the nucleotide specified by the template mutation was added as a radioactive precursor. For example, 32P-labeled dATP was incorporated by the mutant 5′-UAACCCCAA-3′ but not the wild-type telomerase. Third, dideoxynucleotides were added to

**FIGURE 2** Copurification of the 100-kD cross-linked protein with telomerase elongation activity. *(Left)* Telomerase was purified using five chromatographic steps, loaded onto a sucrose gradient. Fractions were assayed for elongation and cross-linking in the presence of N3RdUTP. *(Lanes 1–11)* Telomerase activity in the presence of N3RdUTP for the fraction loaded onto the gradient (L), and gradient fractions 1–10 (fraction numbers are indicated at the top of the figure, and lane numbers at the bottom). The peak in telomerase activity occurred in fractions 4 and 5. *(Lanes 12–22)* The same samples as those in lanes 1–11, after cross-linking and SDS-PAGE analysis. *(Right)* Arrow indicates the position of the 100-kD cross-linked protein.
FIGURE 3 Reconstitution of primer specificity and cleavage. Telomerase assays were performed with telomerase pretreated as indicated. (Lane 1) Native telomerase; (2) native telomerase preincubated under similar buffer conditions as reconstitution reactions; (3) telomerase treated with nuclease. The primer d(GGGT)₃ was used in lanes 1–3. (Lanes 4–21) Permutations of Tetrahymena telomeric oligonucleotides were used to assay the reconstituted telomerase. The sequence of each oligo is shown in the figure. Elongation reactions were carried out in the presence of [α-³²P]dGTP and dTTP, [α-³²P]dGTP and ddTTP, or [α-³²P]dGTP, dTTP and ddCTP as described in the figure. Lanes 19–21 are a darker exposure of the reactions in lanes 4–6 to show that long products were synthesized. Numbers indicate the number of nucleotides added onto the input primers. (P) Size of the 18-mer input primer.

chain-terminate the elongation products at specific sites so that the sequence of the added products could be deduced. For example, addition of ddCTP to elongation reactions with the mutant 5'-CGGCCCCAA-3' (4445G) and the primer d(GGTTG)₃ resulted in the addition of three nucleotides dG, dG, and ddC. Wild-type enzyme was not effected by the addition of ddCTP. A summary of the results obtained with each type of mutant is shown in Figure 4A.

The mutant RNA we assayed in these reconstitution experiments fell into two classes: The two mutations in the 3'-most three residues of the template region, CAACCUAA-3' (49U) and CAACCCCCGG-3' (5051G), did not change the sequence added, whereas the other mutations all resulted in incorporation of new nucleotides. On the basis of these results, we suggest a model in which the 3' three residues are involved only in primer alignment, whereas the 5' six residues provide the template information for the enzyme (Fig. 4B). We are currently assaying further template mutations to test this model of telomerase action.

FIGURE 4 Template mutations in the telomerase RNA. (A) Telomerase RNA template mutants. Incorporation of specific nucleotides and deoxyribonucleotides specifies the sequence added onto the telomeric primer. (B) Model for template specificity. In wild-type telomerase only the 5'-most six residues provide template information.

Regulation of Telomerase during Mouse Embryonic Stem Cell Differentiation

M. Scharring

Our previous work with both human and mouse cells suggested that telomerase is present in stem cells but
inactivated in many somatic cell lines. To examine telomerase expression during differentiation, we studied mouse embryonic stem (ES) cell lines. ES cells that were grown in the presence of LIF and remained undifferentiated had telomerase activity. After the removal of LIF and the addition of retinoic acid and cAMP analogs, the cells differentiated into a neuronal cell type, and no telomerase activity was detected in the differentiated cultures. These studies indicate that telomerase is regulated during development and differentiation and that ES cells offer a useful system to study the regulatory pathways.

Identification of Telomerase in *Xenopus* Oocytes

L.L. Mantell

Since telomerase is involved in the synthesis of telomere repeats, telomerase activity is expected to be present primarily in the nucleus. We manually dissected nuclei, or germinal vesicles (GV), from stage VI *Xenopus* oocytes and prepared nuclear lysate. A specific telomere primer elongation activity was found predominantly in the nuclear fraction. The activity was specific for telomeric primers and was abolished by RNase pretreatment of the extracts. The elongation products synthesized were very short as seen with the mouse telomerase, although permuted sequence primers confirmed the specificity of the added sequence. To further test if the sequence TTAGGG was added onto the input telomeric primers, dideoxynucleotides were substituted for deoxynucleotides to terminate the elongation reaction at specific positions. These results confirmed that *Xenopus* oocyte nuclear extracts contain telomerase activity that synthesizes TTAGGG repeats de novo.

We assayed telomerase activity at each stage of oogenesis. S-100 extracts were prepared from approximately 200 oocytes from stage I to stage VI and purified over a DEAE-agarose column. Most of the *Xenopus* telomerase activity was recovered in a 0.2 M NaCl wash from a DEAE-agarose column. Extracts representing all stages of oocyte maturation had telomerase activity. To determine whether telomerase was active during *Xenopus* development, we obtained fertilized oocytes and allowed development to occur. S-100 extracts were prepared from embryos at different developmental stages and assayed for telomerase; activity was detected in matured eggs and at all stages of embryogenesis from blastula to neurula.

To examine whether telomerase activity is regulated during the cell cycle, S- and M-phase-specific extracts were prepared from *Xenopus* eggs. For the S-
phase extract, cycloheximide was added to the buffer to prevent any new synthesis of cyclin that may induce MPF activity. Telomerase activity was detected in both S- and M-phase extracts that were purified either by DEAE-agarose chromatography or by 15% (NH4)2SO4 precipitation or after simple dilution without further purification (Fig. 5, top). As a control for the specificity of the S- and M-phase extracts, MPF activity was tested in these extracts. A synthetic polypeptide substrate, specific for p34cdc2 protein kinase, was used to assay MPF activity (courtesy of Dr. Marshak, CSHL) (Fig. 5, bottom). As expected, the M-phase extracts had significant MPF activity, whereas the S-phase extracts had very little.

Telomerase activity was detected in total testes extracts. *Xenopus* testes are composed of primary and secondary spermatocytes, spermatids, mature sperm, and supporting somatic cells. To determine if telomerase activity is present in sperm cells, mature sperm were separated from the testes tissue, the mature sperm were washed and pelleted five times, and activity was detected in extracts prepared from approximately 10^6 mature sperm cells. Telomerase activity was present also in the extract prepared from testes after the removal of most mature sperm. The telomerase activity detected in this extract may be from somatic cells or from immature spermatocytes, spermatids, and the remaining mature sperm.

Telomerase regulation in humans has suggested that this enzyme may have a role in the development and life span of mammalian tissues. The ability of immortal cell types to divide indefinitely seems to require the presence of telomerase. It is not yet clear whether this same regulation occurs in *Xenopus*. Although telomerase regulation may differ in some respects between *Xenopus* and mammalian cells, by understanding the differences and similarities, we will gain insights into the essential components of both systems.

**PUBLICATIONS**


STRUCTURE AND COMPUTATION

This section includes two major groups that heavily use computers and networks to solve important biological problems related to cell processes such as signal transduction, growth control, and development. The structural and sequencing studies described below are designed to benefit from and to complement the genetic and biochemical experiments performed at the laboratory. Gene sequence analysis and atomic models of kinases, phosphatases, DNA-binding enzymes, and other proteins will help us comprehend the roles of genes and proteins in cell division and in cell transformation and will ultimately lead to new and better treatments of human afflictions such as cancer, diabetes, AIDS, and Alzheimers' disease.

The members of the W.M. Keck Structural Biology Laboratory elucidate the three-dimensional atomic structures of proteins and protein-DNA complexes. Dr. Xiaodong Cheng has solved the crystal structure of HhaI DNA methyltransferase in a binary complex with S-adenosyl-L-methionine cofactor and in a ternary complex with both bound DNA and cofactor. Astonishingly, in the latter structure, a 13-mer oligonucleotide has a cytosine base flipped out of canonical base-pairing so that its C5 is positioned near the conserved active site cysteine sulfhydryl and the bound cofactor. Dr. Jeff Kuret has been instrumental in the structure determinations of casein kinase 1 with Dr. Cheng and of cAMP-dependent protein kinase with Dr. Jim Pflugrath. The collaborative efforts Dr. John Anderson and Dr. Cheng have yielded the structure of a PvuII endonuclease/DNA complex. Dr. David Barford, a CSHL Junior Fellow within the Keck Laboratory, determined the first known structure of a protein tyrosine phosphatase. All the crystallography efforts used data collection software developed by Dr. Pflugrath.

Members of the Computational Biology group also develop software and conduct basic research to help molecular biologists, geneticists, and other scientists to determine, map, and analyze the sequences and functions of genes and proteins. Dr. Tom Marr leads a group that has created the Genome Topographer software, which is an integrated and comprehensive tool to visualize and analyze genetic objects and databases. Dr. Dick McCombie and colleagues develop sequencing technology as they sequence the genome of Schizosaccharomyces pombe.

MACROMOLECULAR CRYSTALLOGRAPHY

J.W. Pflugrath  J. Horton  Y. Liu
J. Keller  E. Yoo

Structural Studies of the Neurotrophic Factor S100β

Y. Liu, J.W. Pflugrath [in collaboration with D. Marshak, Cold Spring Harbor Laboratory]

S100β is a member of a family of dimeric acidic Ca**+-binding proteins involved in cell differentiation, cell cycle progression, and calcium signal transduction. S100β is synthesized and released from astrocytes in the brain, and its disulfide-linked dimeric form has neurotrophic activity on cerebral cortical neurons in vitro. S100β is homologous to proteins with EF-hand calcium-binding motifs, such as calmodulin and intestinal calcium-binding protein.
We are attempting to determine the crystal structures of S100β at pH 7 and pH 8 from existing crystals of S100β that diffract beyond 1.5 Å resolution. Our attempts to solve the structure by molecular replacement with intestinal calcium-binding protein as a model have failed. We have also failed to produce any heavy atom derivatives by either soaking or cocrystallization. Since these two conventional methods have failed, we have expressed S100β in the Escherichia coli strain DL41(DE3), a methionine auxotroph. When these bacteria are grown in the presence of selenomethionine (Se Met), the recombinant S100β expressed contains five selenium atoms that scatter X-rays anomalously. Thus, a multiwavelength anomalous dispersion experiment can be performed at a synchrotron to yield phases and an electron density map. Up to now, the purified Se Met-S100β protein has not yielded crystals.

Yeast cAMP-dependent Protein Kinase

J.W. Pflugrath, J. Kuret

Protein kinases, which catalyze the transfer of a phosphate from ATP to serine, threonine, and tyrosine residues of protein substrates, are key regulatory enzymes that participate in many biochemical pathways. As catalysts, they can amplify intra- or extracellular signals. By virtue of their broad yet limited substrate specificity, they are able to regulate more than one substrate protein at a time. We wish to understand how protein kinases achieve the specificity necessary to regulate cell processes, a fundamental problem in biology, and how kinases are regulated at the structural level. The more than 200 known protein kinases share a common catalytic core based on sequence comparison and recent structure determinations. Despite the similarities in the catalytic subunits, protein kinases exhibit several different modes of regulation.

We have crystallized and solved the structure of the catalytic subunit of the Saccharomyces cerevisiae cAMP-dependent protein kinase. The crystals belong to space group P6_5 22 with cell dimensions a = b = 61 Å, c = 320 Å. The structure has two domains with a wide cleft between them where ATP binds. A shallow groove on the large domain was shown to be the protein substrate-binding site (Knighton et al., Science 253: 407 [1991]).

Casein Kinase II

J. Horton, J.W. Pflugrath [in collaboration with D. Marshak, Cold Spring Harbor Laboratory]

Casein kinase II (CKII) is a highly conserved, ubiquitous serine/threonine heterotetrameric protein kinase composed of two catalytic (α and/or α') and two regulatory (β) subunits. This kinase is found predominantly in cell nuclei and primarily, in mammals, in the brain. It is believed that CKII is involved in the phosphorylating cascade originated by M-phase-specific kinase (Cdc2 kinase) and participates in mitogenic signaling, particularly in neural development.

From similar conditions, we have grown three different crystal forms of the regulatory subunit (CKIIβ) of this kinase. SDS-PAGE analysis suggests that one is of the full-length protein (residues 1-215), one of a truncated form (residues 1-189), and another that probably contains a mixture of both full-length and truncated proteins. Truncated CKIIβ is found in most preparations and copurifies with the full-length protein; it is not obvious if the truncation is the result of premature termination or proteolysis. The position of the CKIIβ truncation was determined by carboxy-terminal sequencing and mass spectrometry in collaboration with Applied Biosystems.

Presently, we are attempting to characterize these small fragile crystals. All crystal forms appear to be temperature-sensitive. We are searching for conditions to stabilize them and/or to obtain new, better crystals.

Cell Cycle Protein Regulators

J.R. Horton, J.W. Pflugrath

We wish to understand the underlying molecular mechanisms of regulation and activity of proteins that control the proliferation and differentiation of eukaryotic cells. Toward this end, we hope to determine the three-dimensional structure of components of the cell cycle regulation system that contain human CDK4 and cyclin D1. Importantly, aberrations of the protein subunits of this system have been directly linked to human cancers. In normal cells, cyclin D1 and CDK4 form quaternary com-
plexes with PCNA (proliferating cell nuclear antigen), a known subunit of DNA polymerase δ, and another protein, p16. (Recently, p16 has been named INK4, for inhibitor of CDK4, since it binds specifically to CDK4 and inhibits in vitro its ability to phosphorylate Rb.) However, in cells transformed by the expression of viral oncoproteins, CDK4 is no longer involved in the quaternary complex but in either a binary complex with p16 and/or a ternary complex with p16 and cyclin D1. Thus, p16/INK4 may be an important negative regulator of cell proliferation.

We have been working toward purifying and crystallizing the protein subunits of the CDK4–cyclin D1–PCNA–INK4 complex. Unfortunately, although the gene for p16 has been put into high-expression systems in *E. coli* and the yeast, *Pichia pastoris*, there is little or no evidence of a large amount of the protein after induction. Additionally, the protein is only partially soluble in *E. coli*; thus, only 0.25 mg of the protein can be recovered per liter of bacterial culture. We have set up crystallization trials with what little protein we have to no avail. We are investigating ways of increasing the amounts of p16 that can be obtained in these systems.

In autumn 1993, during sequence analysis, we noticed that p16 is almost entirely made up of four ankyrin-like repeats. Ankyrins are a family of proteins that are thought to coordinate interactions between various integral membrane proteins and cytoskeletal elements; some ankyrins have greater than 20 repeats in a distinct domain. Several other cell cycle proteins also have ankyrin-like repeats in them, and we think that such repeats must have important functions in the regulation of the cell cycle.

To learn something about the three-dimensional structure of p16, if we find that we cannot obtain large enough amounts of p16 for crystallization, we may design a protein with a number of ankyrin-like repeats and crystallize it.

Although a large amount of cyclin D1 is expressed in *E. coli* (40–50 mg per liter of culture), most, if not all, of this protein is found in inclusion bodies. After refolding procedures, nearly all of the protein remains insoluble or forms soluble aggregates. We have been successful in properly refolding very small amounts of this insoluble cyclin D1 and have partially purified it; however, we think that this is not the avenue for acquiring the large milligram quantities of protein needed for crystallization studies and subsequent structure determination. We are investigating obtaining soluble, recombinant cyclin D1 from other expression systems, either in a *P. pastoris* system or in a baculoviral system. The *E. coli* strain harboring the cdk4 gene expresses this protein nearly as robustly as cyclin D1. However, this protein is also found entirely in inclusion bodies. In the coming year, we will test the same avenues as those of cyclin D1, so that we will obtain large soluble homogeneous quantities of CDK4 for crystallization. Ultimately, we wish to cocrystallize cyclin D1 and CDK4 as well as their complexes with associated proteins found in normal and transformed cells.

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**Structural Analysis of a Bifunctional Enzyme: 6-Phosphofructo-2-Kinase/Fructose-2,6-Bisphosphatase**

J.W. Pflugrath, E. Yoo, J. Keller [in collaboration with S. Pilkis and Y. Lee, SUNY, Stony Brook]

Protein phosphorylation is the major mechanism by which intracellular events are controlled by extracellular stimuli. We are studying protein kinases (see above), but how phosphorylation modulates the activity of protein substrates is just beginning to emerge. The activity of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (6PF2K/F26BP) is controlled by phosphorylation at Ser-32 by the cAMP-dependent protein kinases. This single bifunctional liver enzyme catalyzes both the synthesis and degradation of fructose-2,6-bisphosphate (Fru-2,6-P2). The 6PF2K activity is inhibited by phosphorylation, whereas the bisphosphatase activity is increased. Fru-2,6-P2 is a regulatory metabolite whose steady-state level determines glycolytic/gluconeogenic flux in the hepatocyte and glycolytic flux in extrahepatic tissue: When the concentration of Fru-2,6-P2 is low, net glucose synthesis occurs in tissues capable of gluconeogenesis. The homodimeric enzyme is composed of 54.5-kD monomers of 470 amino acids forming two discrete domains: an amino-terminal kinase domain (residues 1–249) and a carboxy-terminal bisphosphatase domain (residues 250–470). Sequence and modeling analysis shows that the amino-terminal kinase domain is similar to that of the glycolytic enzyme 6-phosphofructo-1-kinase and that the carboxy-terminal bisphosphatase domain is similar to that of yeast phosphoglycerate mutase. Work by Pilkis and colleagues has begun to elucidate the chemical basis for catalysis and substrate/product...
binding of the bisphosphatase half of the enzyme. A three-dimensional model of this enzyme would be an invaluable asset in interpreting the kinetic, mutational, and phosphorylation results. A model would also aid the design of inhibitors and/or activators that could lead to the control of Fru-2,6-P2 levels which in turn help regulate the glycolytic gluconeogenic pathway.

Both rat and human liver 6PF2K/F26BPs have been cloned and expressed in E. coli by the Piliks laboratory. The rat liver bifunctional enzyme has been expressed as the holoenzyme of 470 residues and also as its distinct and active domains: the kinase and the bisphosphatase. We have crystallized the bisphosphatase domain in four different crystal forms. Two forms grow in the presence of inorganic phosphate and the other two forms grow in the absence of phosphate. The crystals diffract to 1.8 Å resolution, although they are very sensitive to temperature changes and radiation damage. Native data sets for two crystal forms to 2.8 Å resolution have been collected and heavy atom screening is under way. To understand the enzyme mechanism in atomic detail, we will soak in (or crystallize the protein in the presence of) the substrate Fru-2,6-P2, which can act as an inhibitor at high concentrations, and the product fructose-6-phosphate. Numerous site-directed mutants are available for crystallization as well. Attempts are also under way to crystallize the entire protein and the kinase domain separately.

**Area Detector Software**

J.W. Pflugrath [in collaboration E.M. Westbrook, Argonne National Laboratory]

At its simplest, X-ray diffraction data collection consists of producing a set of reflections from a crystal. One mounts the crystal on the goniometer where it will be bathed by the source. The source photons (or neutrons!) are diffracted by the crystal rotated on the goniometer. These diffracted photons are detected in a detector that will be scanned or read periodically to create images. The pixels in the images will be assigned to individual Bragg reflections and integrated to yield an estimated intensity and standard deviation. Thousands of diffracted intensities from numerous crystals must be measured in order to determine a crystal structure of a macromolecule. To address this problem, we have developed the device-independent package MADNES to collect and process diffraction images from many different types of area detectors. MADNES is used widely in the crystallographic community. In 1993, more than 60 publications cited the use of the MADNES package.

MADNES is the application software used with the 1024 by 1024 pixel CCD area detector developed at Argonne National Laboratory and installed at beamline X8C of the National Synchrotron Light Source located at Brookhaven National Laboratory (BNL) here on Long Island. In the past year, we have maintained and extended the software at the X8C beamline. For example, the device handlers for the CCD detector hardware have been re-implemented in a client-server mode; i.e., the detector hardware need not share the same computer platform to which the other system hardware such as shutter, goniometer motors, and ion chamber counters are attached. This is a forward-looking development for when larger CCD detectors with more advanced electronics come online. MADNES was also implemented on the High Flux Beam Reactor neutron diffraction beamline at BNL and on three different imaging plate diffractometers.

**PUBLICATIONS**


In Press

During the year of 1993, two talented young scientists arrived at this X-ray laboratory: Ruiming Xu with a solid background in physics came from the Institute for Theoretical Physics at Stony Brook, and Margaret O’Gara with a background in chemistry came from the University of Galway in Ireland. Ruiming began working on yeast casein kinase-1 in an effort to determine its three-dimensional structure. Margaret started to work on Hhal methyltransferase to illustrate further the mechanisms of DNA recognition and methylation on a detailed steric chemical level. Diane Alonso, an URP from Scripps College at California, also applied her energy on the methyltransferase using the techniques of molecular genetics. The latest structure solved in this laboratory is PvuII endonuclease. Our progress on three different enzymes are described below.

Structure of Yeast Casein Kinase-1

R. Xu, T. Malone, X. Cheng [in collaboration with J. Kuret, Cold Spring Harbor Laboratory; R.M. Sweet, Brookhaven National Laboratory]

We have determined the crystal structure of the catalytic domain of casein kinase-1 (CK1) from *Schizosaccharomyces pombe* in complex with MgATP at 2.0 Å resolution. The current R-factor is 20.7%. The truncation consists of the amino-terminal 298 residues and is fully active in vitro. CK1 is a family of protein kinases common to all eukaryotic cells and phosphorylates many proteins in vitro, including SV40 T antigen, p53, and glycogen synthase. CK1 employs a catalytic domain that differs enzymologically from most other protein kinases. CK1 isoforms selectively recognize the sequence SpXXS, where X is any amino acid, and the underlined serine residue is the phosphate acceptor. The presence of acidic residues amino-terminal to a phosphorylatable hydroxyamino acid is the minimal requirement for recognition of synthetic peptides by CK1. Replacement of the acidic amino acid with phosphoserine at a position three residues amino-terminal to the phosphorylatable serine results in a synthetic substrate with superior kinetics. Thus, the substrate selectivity of CK1 appears to be directed toward phosphate groups rather than unmodified amino acids.

The structure contains nine α helices and seven β strands, designated A to I for helices and 1 to 7 for strands from the amino to the carboxyl terminus of the amino acid sequence (Fig. 1). The loops are designated by their flanking secondary structures. The molecule folds into two parts, and we named them the beta domain (amino acids 1–86) and the alpha domain (amino acids 91–298), connected by a single loop L-5B (amino acids 86–91). There is a deep cleft between the two domains, which binds MgATP and peptide substrate. The overall architecture of CK1 is quite similar to that of the cAMP-dependent protein kinase and the cyclin-dependent kinase-2.

The beta domain contains one third of the protein from the amino terminus. It has five twisted antiparallel strands (β1–β5) and one helix (αA). A characteristic glycine-rich sequence motif among most protein kinases, G19-T-G21-S-F-G24-V, forms a part of the β1-turn-β2 structure over the MgATP-binding site. G19 is the last amino acid of strand β1, and V25 is the first amino acid of strand β2. The adenine base and ribose ring of ATP are inserted into a hydrophobic pocket, whereas the triphosphate group is covered by glycine-rich loop L-12. An Mg²⁺ ion bridges the α and γ phosphates.

The alpha domain contains five long helices (C,E,F,H,I), three short helices (B,D,G), two short strands (6,7), and one long loop (L-7D). Four of five long helices (αC, αE, αH, and αI) form an antiparallel bundle with the fifth long helix (αF) almost perpendicular to the bundle. These five helices form a structural framework for this domain. The remainder of the domain contains three short helices (αB, αD, and αG) and two short strands (β6 and β7) and loops including the 10-residue loop L-C6, the 30-residue long loop L-7D, and the 12-residue loop L-EF. These short secondary structures and the loops containing conserved residues are clustered around the surface.
of the cleft and may be crucial for the function of the enzyme.

**Structure of PvuII Endonuclease**


We have determined the structure of the PvuII endonuclease (R·PvuII) complexed with cognate DNA at 2.6 Å resolution. The final crystallographic R-factor is 18.9%. R·PvuII, which forms part of the restriction-modification system of Proteus vulgaris, cleaves duplex DNA with the sequence 5'-CAGC- TG-3' between the central guanine and cytosine residues in both strands to generate blunt-ended DNA products. R·PvuII is the smallest restriction endonuclease found so far, containing 157 amino acids. Two views of the structure are shown in Figure 2.

The cleft between the U-shaped dimer of R·PvuII provides the binding site for DNA. The DNA in the structure is a 12-base duplex containing the recognition sequence (CAGCTG) with single-base (T) 5' overhangs. Contacts between the protein and backbone phosphates span 12 base pairs of the duplex. The bound duplex has the characteristic shape of B-form DNA (Fig. 2, top). The minor groove of the DNA faces the bottom of the cleft and the major groove faces the open end (Fig. 2, bottom).

The monomer structure contains five α helices and eight β strands, designated A to E for helices and 1 to 8 for strands from the amino to the carboxyl terminus of the amino acid sequence (Fig. 2, bottom). The molecule of the monomer is folded into three parts, and we named them the protein-interface region, the catalytic region, and the DNA-recognition region.

The first 46 amino-terminal residues form the dimer interface through two helices connected by a loop (αA, loop L-AB, and αB). The 21-residue helix αA is bent due to a proline. The two αA helices of the dimer cross over each other, and as a result, the amino terminus of one monomer is close to the car-
FIGURE 2 Structure of PvuII endonuclease. Two subunits are shown with a ball-and-stick model of the bound cognate DNA segment. (Top) Front view of the protein-DNA complex. A hydrogen bond between the side chains of H85, which both lie in the major groove of the DNA, closes off the DNA-binding cleft. (Bottom) Side view of the protein-DNA complex from an angle as indicated in the top drawing.
The recognition region comprises two substructures from different locations in the primary sequence, each containing one strand and one helix. Strand β4 and helix αC run antiparallel with helix αD and β7. The resulting two antiparallel β strands make specific interactions with DNA bases in the major groove. A similar mode of base-specific recognition was seen in the structure of Met repressor, in which two antiparallel β strands from different monomers contact bases in the major groove. The side chains of amino acids in the loop L-AB, which spans the minor groove, make water-mediated contacts to the central G-C base pair and backbone phosphates.

**Structure of Hhal Methyltransferase**

X. Cheng, K. McClory, D. Alonso, M. O’Gara
[in collaboration with R.J. Roberts, New England Biolabs, Inc.]

We have solved the structure for a chemically trapped covalent reaction intermediate between the Hhal methyltransferase (M·Hhal), S-adenosyl-L-homocysteine (AdoHcy), and a duplex 13-mer DNA oligonucleotide containing methylated 5-fluorocytosine at its target. M·Hhal forms part of a type II restriction-modification system from *Haemophilus haemolyticus*. The enzyme recognizes the specific tetranucleotide sequence, 5'-GCGRG-3' and methylates the C5 position of the first cytosine residue.

We have crystallized a complex formed upon incubation of M·Hhal, S-adenosyl-L-methionine (AdoMet), and a 13-mer oligonucleotide containing the recognition sequence GFGC in which the target cytosine was substituted by 5-fluorocytosine (F) (Fig. 3a). The substrate forms a 12-base-pair duplex with single-base 5' overhangs. Reaction with this substrate results in the formation of an intermediate, which contains a covalent linkage between the sulfur atom of Cys-81 of the enzyme and the C6 atom of the target cytosine ring. In this intermediate, the methyl group has transferred to the C5 position of cytosine, but elimination is inhibited because the fluorine on C5 cannot be released as F+. During the course of this reaction, AdoMet is converted to AdoHcy, which is still present in the complex. The structure has been solved at 2.8 Å resolution by molecular replacement. The final crystallographic R-factor is 17.4%.

In contrast to the endonuclease that acts on the phosphate backbone, the methylase requires access to a base in order to carry out its chemistry. How this is accomplished is revealed remarkably by the structure. M·Hhal binds DNA as a monomer, and the cleft between the two domains provides the binding site for DNA as predicted previously (see last year’s Annual Report) (Fig. 3b). Overall, the structure of the protein component is very similar to that in the ab-
In light of the dramatic structural change that has occurred in forming the intermediate in DNA methylation, the mechanistic details of the reaction have suddenly become much more complicated. The structure has raised more questions than it has answered. For instance, it is not certain that the contacts between DNA and protein that are seen in this structure are the ones responsible for sequence-specific recognition during the initial binding event. It seems likely that this initial recognition is tied to the mechanism for flipping out the target cytosine and that both take place as a concerted event. There is also the problem of returning the methylated cytosine residue into the helix. Clearly, studies either with mutant proteins or with cytosine analogs that fail at some point in the reaction pathway will be useful in dissecting the mechanism further. Currently, such studies are under way.

Replacement of the catalytic Cys-81 of M·Hhal methyltransferase with serine abolished catalysis (see last year’s Annual Report by S. Mi and R.J. Roberts in the Nucleic Acid Chemistry section). This mutant behaves as the sequence-specific DNA-binding protein but loses the activity of methylation. The mutant protein can be trapped as an intermediate with DNA and cofactor due to the lack of subsequent methylation. Crystals of this mutant protein complexed with DNA have been obtained.

As mentioned above, two major changes take place upon DNA binding: the complete disruption of the target base pair and a conformational change of the active-site loop. Gln-237 from the small domain occupies the position left by the target cytosine after it has flipped out of the helix. The polar groups along the Watson-Crick pairing edge of the orphan guanine are all involved in intermolecular hydrogen bonds with the backbone and side chain atoms of Gln-237 whose alignment is stabilized through hydrogen bond formation with Ser-87 from the large domain (Fig. 3e). The three glycines flanking Gln-237 appear to be crucial in positioning the side chain of Gln-237 for its deep penetration into the helix to ensure the stacking and hydrogen bonding. Interestingly, Gln-237 and Ser-87 are not conserved amino acids among the cytosine-C5-methyltransferases, establishing that hydrophobic interactions also contribute to these protein-DNA interactions. For this reason, we examined the role of Gln-237 in DNA binding and substrate specificity by site-directed mutagenesis. (For detailed analysis and results, see report by S. Mi and R.J. Roberts.)

The bound 13-mer DNA fragment has close to the characteristic conformation of B-form DNA, with standard Watson-Crick hydrogen bonds between the base pairs, except for a completely disrupted G-C base pair that contains the target cytosine. Remarkably, the target cytosine (both the base and sugar rings) has flipped completely out of the DNA helix (Fig. 3d). The exclusion of the cytosine from the helix is accompanied by substantial distortions of the phosphodiester backbone on the same strand. This can be seen by comparing the oligonucleotide structure with that of an unbound DNA decanucleotide containing the M·Hhal recognition sequence GCGC (Fig. 3d). Both phosphates flanking the target cytosine are shifted significantly away from their corresponding positions in the unbound oligonucleotide, whereas further distortions extend along the phosphodiester backbone in the 5' direction. These changes increase the interstrand phosphorus-phosphorus distances and allow the target cytosine to flip through the minor groove and out of the helix. The DNA is contacted from both the major and the minor grooves, but almost all base-specific interactions between the enzyme and the recognition bases occur in the major groove, through two glycine-rich loops from the small domain.

The binding of M·Hhal to its DNA substrate causes extensive conformational changes. The three essential components of the methylation reaction, the target cytosine, the catalytic nucleophile, and the methyl donor, are brought into close proximity with one another, allowing the methylation reaction to proceed. Around the active site, the protein forms extensive contacts with both the DNA and the cofactor mainly through well-conserved amino acids. The complex offers a particularly clear example of the induced-fit mechanism of enzyme-substrate interactions. The entire story is of great interest in understanding protein-DNA interaction and the catalysis of methylation.

The entire story is of great interest in understanding protein-DNA interaction and the catalysis of methylation.
Gln-237 was replaced by all other amino acids except Ile and Tyr. The result of substitution can be divided into three classes. The first class retains full DNA-binding and methylation activities, including Glu, Lys, Arg, and Leu. The second class greatly reduced both activities, including Met, Asn, Ser, His, Thr, Ala, and Pro. Substituting with Met retained slightly higher residual activities than others in this class. The third class completely abolished the binding and methylation activity, including Gly, Cys, Val, Asp, Tyr, and Phe. The three substitutions with charged amino acids in the first class retained the potential to form hydrogen bonds with the orphan guanine and/or Ser-87, consistent with the fact that Gln-237 functions by hydrogen bonding. Substituting with Asp, however, abolished both activities completely, suggesting that the size and shape of the side chain of Gln-237 may be critical for proper hydrophobic interactions with the neighboring DNA bases. This may also explain why substitution with Leu retained full activities and Met retained partial activities, whereas aromatic rings eliminated activities.

In summary, the binding of the enzyme to substrate DNA results in large conformational changes of both the DNA and the protein. The first distortion appears to be the flipping of the target cytosine, accompanied by a glutamine (Gln-237) occupying its space and providing hydrogen bonds to its partner guanine. Subsequent closing of the active-site loop on the target brings an additional amino acid (Ser-87) into the DNA helix to stabilize the conformation of Gln-237. The two amino acids restore the stack in the DNA.

FIGURE 3 (See facing page for parts d and e and legend.)
FIGURE 3 Structure of Hhal methyltransferase. (a) Target cytosine base with a fluorine atom substituted for a hydrogen atom at its C5-position. (b) Stereoview looking down the axis of DNA helix in which the DNA can be seen to lie between the large domain (on the upper right) and the small domain (on the lower left). (c) Stereoview showing the superimposition of the protein Cα chains from ternary complex of DNA-M·Hhal·AdoHcy and the binary structure of M·Hhal·AdoMet. The Cα chains of M·Hhal are shown as a light line (binary complex) or a heavy line (ternary complex). The 20-residue active-site loop in the large domain undergoes a large conformational change upon DNA binding. The extreme movement is about 25 Å toward the cleft. The small domain is also shifted toward the cleft with a movement of about 3 Å. (d) Stereoview showing the DNA conformation (as heavy line) from the minor groove. The structure of normal B-DNA is shown as a light line. Excluding the flipped-out target cytosine and the unpaired 5' bases, the average helical twist is 31.6° and the mean rise per base is 3.6 Å, implying 10.9 bp per turn. (e) Gln-237 makes three hydrogen bonds (two from side chain atom O1 and one from main chain amide) with N1, N2, and O6 atoms of the orphan guanosine, which originally paired with the target cytosine. A hydrogen bond links Gln-237 to Ser-87.
PUBLICATIONS

In Press

PROTEIN CHEMISTRY
R. Kobayashi  J. Murtha
B. Schwender

Our research goal has focused on developing methods for microcharacterization of biologically important proteins. In the beginning of this year, James Murtha joined the laboratory as a part-time assistant and Brian Schwender joined in July. James helped to synthesize amino acid derivatives for the modified Edman degradation and to explore a new staining dye for protein after gel electrophoresis. Brian has been constructing a protein database for the yeast Saccharomyces cerevisiae as a part of the Quest Protein Database Center. We have been identifying protein spots on two-dimensional gels by analyzing their internal amino acid sequences (see the Quest Protein Database Center section).

PROTEIN SEQUENCING
The collaboration with other scientists at Cold Spring Harbor Laboratory has been extended lab-wide since the end of 1992 and has been a major activity in our laboratory. This year’s collaborators were Drs. Arndt, Bar-Sagi, Beach, Garrels, Greider, Hernandez, Krainer, Kuret, Moran, Patterson, Spector, Stenlund, Stillman, Tanaka, Tonks, and Wigler. Most of the collaborations were for protein sequence analysis so that each researcher could clone their genes for further study and determine the phosphorylation sites of the proteins. The technique for internal amino acid sequence analysis of protein was improved this spring. The change was to remove the SDS after polyacrylamide gel electrophoresis prior to enzymatic digestion of the protein instead of using SDS in the digestion and separating peptide fragments by high-performance liquid chromatography (HPLC) using an anion-exchange column. This change improved the recovery of peptides when 1-2 μg of protein is studied.

This year, we successfully obtained amino acid sequence information for more than 60 proteins those of which were DNA replication factors, transcription factors, including p300, RNA splicing factors, and cell cycle control proteins including p21.

Cell cycle control protein p21 was found in normal human cells but not in many transformed cells. This protein was isolated and purified from 400 tissue culture dishes by Dr. Yue Xiong in the Beach laboratory at Cold Spring Harbor. About 2 μg of p21 was finally obtained after polyacrylamide gel electrophoresis. The protein band was excised and subjected to in-gel digestion with Achromobacter protease I (lysylendopeptidase) in the presence of 0.1% SDS. The peptide fragments after the digestion were separated by HPLC. The HPLC profile is shown in Figure 1. Each peptide fragment was sequenced by automated sequencers. Based on the peptide sequence, oligonucleotide primers were designed and synthesized, and the gene encoding p21 was then cloned in the Beach laboratory. Excitingly, Dr. Beach and his colleague found that p21 is a universal inhibitor of cyclin kinases and that overexpression of p21 inhibits the proliferation of mammalian cells.
METHODOLOGICAL STUDY OF PROTEIN SEQUENCE ANALYSIS

We have been trying to develop a highly sensitive method for protein sequencing by combining modified Edman degradation and chemiluminescence detection. However, this research has not progressed much this year, although we synthesized and purified many of the standard amino acid derivatives for a new protein-sequencing method. We expect to spend more time in the future in developing a new method in 1994 in order to sequence peptides at the sub-picomole level.

PUBLICATIONS


Casein kinase-1 (CK1) is a ubiquitous eukaryotic protein kinase that phosphorylates acidic substrate recognition sequences efficiently. Once considered a single entity, it is now known to consist of subspecies that together comprise a distinct branch of the eukaryotic protein kinase family. Family members consist of a highly conserved, ~290-residue amino-terminal catalytic domain, joined to a carboxy-terminal region that is not conserved among family members and that varies in size from 40 to 180 amino acids. Although the full role of the carboxy-terminal domain is not understood fully, part of its function is to target the enzyme to specific regions of the cell.

Last year, we reported the isolation of four new CK1 genes from fission yeast, their expression in bacteria, and their enzymological properties in vitro. We also reported the rational design and crystallization of a carboxy-terminal deletion mutant of Ckil, a cytoplasmic isoform of CK1. The truncation, termed CkilΔ298, consists of the 298-residue catalytic core of Ckil and is fully active in vitro. Three different crystal lattices were obtained: one form of CK1 alone and two different forms of the CK1/MgATP binary complex. This year, the first of these structures, a binary complex diffracting to 2.0 Å resolution, was solved.

Casein Kinase-1 Tertiary Structure
R.-M. Xu, G. Carmel, P. Kearney [in collaboration with X. Cheng, Cold Spring Harbor Laboratory, and R.M. Sweet, Brookhaven National Laboratory]

The methods used to determine the crystal structure of CK1 are discussed in the Macromolecular Crystallography section of this volume. The overall structure of CK1 is described there as well. Here, we focus on the functional aspects of structure.

On the basis of kinetic measurements, CK1 selectively binds synthetic peptide substrates containing a phosphoamino acid located three residues amino-terminal to the phosphate acceptor site (position P-3). This requirement differs dramatically from that of cAPK, which selectively binds a positively charged residue in positions P-2 and P-3. Not surprisingly, the key residues involved in substrate binding in cAPK, Glu-230, Glu-127, and Glu-170, are not conserved in CK1, being replaced by Tyr-210, Ser-91, and Asp-135, respectively. Despite these differences, the peptide-substrate-binding cleft found on the face of the α lobe of cAPK is found in a similar location on CK1. As described above, all known forms of CK1 are positively charged at neutral pH. Calculation of the local electrostatic potential on the surface of CK1 reveals that the positive charge is enriched in the substrate-binding cleft, in a position suitable to make contact with the peptide substrate amino-terminal to the phosphorylatable hydroxyamino acid.

Interestingly, we have identified a sulfate anion in the positively charged peptide-substrate-binding region, where it may mimic the noncovalent binding of a phosphate ion or phosphohydroxyamino acid residue as it does in the crystal structures of other phosphoproteins (Fig. 1). Clearly, this sulfate does not mediate crystal contacts. In CK1, sulfate binds the main-chain nitrogen of Gly-220, the ε-amino nitrogen of Lys-229, and is approximately 3.4 Å away from the guanidino moiety of Arg-183. Each of these residues is conserved throughout the CK1 family of enzymes. Gly-220 and a lysine or arginine at position 229 are found in subdomains IX and X of all known CK1 isoforms, whereas Arg-183 is conserved in all isoforms except mammalian CK1β. Although Gly-220 flanks a kinase insert sequence unique to CK1, Arg-183 occupies the same position as does Glu-203 in cAPK. In cAPK, this position is an important determinant of substrate selectivity that makes contact with P-3 and P-6 of PKI. In addition to suggesting the structural basis of substrate selectivity, the S1 site may mediate the inhibition of CK1 by phosphatidylinositol-4,5-bisphosphate.

Our model of CK1 also suggests functions for structural motifs conserved throughout the CK1 family of kinases. First, the motif Asp-Leu-Leu-Gly-Pro-Ser-Leu-Glu-Asp-94 found in subdomain V is conserved in all CK1 isoforms because it makes direct
FIGURE 1 Structural basis of CK1 substrate selectivity. An α-carbon trace of CK1 is shown. The side chains Arg-183 and Lys-229 come together with the main-chain nitrogen of Gly-220 to create a high-affinity site for sulfate and presumably for phosphate as well. This site suggests a mechanism whereby CK1 binds synthetic peptide substrates containing phosphoamino acids in the P-3 position with an affinity greater than that of identical peptides containing simple acidic residues in this position.

Contact with the nucleotide substrate while spanning a single-residue deletion relative to other protein kinases. In addition to supplying a hydrophobic-binding pocket, the backbone amide and carbonyl groups of Asp-86 and Leu-88 hydrogen bond with N1 and N6 of the base. Moreover, this sequence bridges the two lobes and spans the presumptive hinge region. Although the CK1-bound conformation of ATP is almost identical to that found in cAPK, the side chains and water molecules that make contact with it differ greatly between the two enzymes.

One Mg\(^{++}\) was identified on the basis of its bonding distances and coordination geometry. The ion makes direct contact with nucleotide, bridging its α and γ phosphates, and binding the side chain of Asn-136. This position corresponds to the low-affinity M1 site of cAPK. Although cAPK contains a second Mg\(^{++}\) bridging the β and γ phosphates (the M2 site), no density for this site was observed in CK1. It is possible that the M2 site exists in CK1 but is obscured by dynamic motion. This appears unlikely, because in the model of the protein kinase ERK2, the γ-phosphate of ATP is completely disordered and yet the M2 site is well-resolved. In cAPK, occupation of the low-affinity M1 site is accompanied by a reduction in phosphotransferase velocity. Our results
suggest that the primary role of Mg\textsuperscript{++} is to provide an
electron sink for the γ-phosphorus and that it can supply this function by directly binding the γ-
phosphate in either the M2 or M1 conformation. As
demonstrated through kinetic measurements, the
binding of a second Mg\textsuperscript{++} increases the affinity of
ATP for kinase by decreasing its dissociation rate.
Because the dissociation rate of the ADP product is
also reduced, and because this is the rate-limiting
step, the overall rate of phosphoryltransfer is dimin-
ished. Thus, the occupation of both M2 and M1 by
Mg\textsuperscript{++} appears to be inhibitory. Our results suggest
that it is the binding of the first Mg\textsuperscript{++} rather than the
occupation of a specific site that activates the protein
kinase and that the binding of additional ions to either
site may lead to inhibition through decreased product
dissociation. Again, our results suggest that the loca-
tion of the highest-affinity Mg\textsuperscript{++}-binding site will
vary among protein kinases.

The most intriguing conserved feature of CK1 is
the triplet Ser-Ile-Asn-188 of helix αD where it re-
places the triplet Ala-Pro-Glu found in cAPK, Cdk2,
and most other protein kinases. In both cAPK and
Cdk2, the glutamic acid residue of the triplet forms a
salt bridge with Arg-280 in cAPK and Arg-274 in
Cdk2 that are located in loops homologous to L-HI.
Surprisingly, we find that the Ser-Ile/Val-Asn motif
adopts the same secondary structure and is in the
same location as the Ala-Pro-Glu-208 sequence of
cAPK. However, major differences exist among the
kinases in the structure of L-HI. As a result, sub-
domain VIII no longer makes direct contact with sub-
domain XI via a salt bridge as it is thought to do in all
other protein kinases. Instead, Ser-186 and Asn-188
hydrogen bond to Arg-198 and Asp-199 of helix αE,
respectively, whereas the side chain of Ile-187 is
buried. In addition, the helices flanking L-HI are sta-
bilized by salt-bridging between Glu-202 of αE and
Arg-261 of αH. Therefore, the Ser-Ile/Val-Asn motif
appears to be conserved in the CK1 family because of
the unique fold of L-HI, rather than adopting an un-
usual conformation by itself.

If this is the case, what is the function of loop L-
HI? We suspect that the conformation of L-HI is
unique because it interacts directly with the long loop
L-9D, in which are scattered several conserved
residues. Several features of L-9D suggest that it has
a regulatory role. First, as described above, Lys-159
and Lys-176 form part of a potential phosphoryla-
tion site. Although both of these lysine residues interact
with Glu-194 in our dephospho structure of CK1,
they may bind a phosphate moiety in phospho-CK1
homologous to Thr-197 of cAPK. Second, L-9D
resembles the regulatory T-loop of Cdk2. However,
the T-loop in Cdk2 is rotated approximately 180°
with respect to L-9D in CK1, so that upon super-
imposition, the two loops lie opposite each other. Be-
cause our model is of fully active CK1, whereas
Cdk2 is inactive, the two conformations may cor-
respond to the active versus inactive states of these
enzymes. Indeed, it has been suggested that upon
Cdk2 activation, the small helix amino-terminal to
the T-loop (αL12) unwinds, removing the T-loop
from the active site, whence it may adopt a con-
formation similar to that of L-9D in CK1. Similarly,
an in vivo regulatory mechanism may exist for CK1
that stabilizes L-9D in a conformation similar to that
observed for the T-loop in inactive Cdk2. Interest-
ingly, all CK1 isoforms retain a threonine residue (Thr-
166; Fig. 2) at the apex of the hairpin (L-9Da) that is
homologous to a regulatory phosphorylation site in
Cdk2 (Thr-160).

Although CK1 and Cdk2 share similarities in
structure, they may be regulated in different ways.
Our model points to a second potential mechanism
for CK1 regulation. Included in L-9D is the sequence
His-Ile-Pro-Tyr-Arg-Glu-Lys-175, a motif found in
the head regions of kinesins, the motor proteins of
microtubules. The head region contains microtubule-
binding sequences, of which His-169-Lys-175 may
be a component. Isoforms of CK1 copurify with
cytoskeletal proteins and may associate with them in
part through the His-169-Lys-175 sequence. If this is
the case, then the role of L-9D may be to regulate the
reversible association of CK1 with the cytoskeletal
fraction.

In summary, CK1 is a conserved member of the
eukaryotic protein kinase family despite its unusual
primary structure. Its most divergent motifs appear to
shape its unique substrate specificity and participate
in its regulation. The genetically tractable CK1
homologs of fission yeast will prove useful in testing
these hypotheses and correlating structure with in
vivo function.

New CK1 Crystal Forms

G. Carmel, R.M. Xu

During the 1980s, a family of selective, low-
molecular-weight casein kinase inhibitors were de-
Potential mechanism of CK1 regulation. CK1 contains a loop (L-9D) that resembles the T-loop of Cdk2. The loop begins with a 10-residue hairpin and terminates with 15 residues in extended conformation. In the structure of inactive Cdk2, the homologous loop (T-loop) rises from the α lobe to make contact with the glycine-rich flap. Thus, the T-loop is positioned to control Cdk2 activity by reversibly blocking its active site. In CK1, L-9D is in an open conformation, leaving the active site unblocked. Shown here is the T-loop of Cdk2 superimposed on the backbone trace of CK1. The view is rotated 120° relative to Fig. 1.

veloped. The first of these was N-(2-aminoethyl)-5-choroisoquinoline-8-sulfonamide, also known as CKI7. It is one of a large family of naphthalene and isoquinoline sulfonamide derivatives that differ in potency and specificity. Selectivity for individual protein kinases has been achieved by varying the chemical substituents at two locations on the isoquinoline ring. For example, CKI7, which has a chlorine at ring position 5 and ethylamine sulfonamide at ring position 8, inhibits CK1 with a $K_i$ of 9.5 μM but is weakly inhibitory for all other kinases tested. As with other isoquinoline sulfonamides, CKI7 is a competitive antagonist of the nucleotide substrate, but it has weak activity against other ATP-using enzymes such as ATPases and adenylate cyclase. As a result, individual isoquinoline sulfonamides have emerged as important pharmacological tools for dissecting signal transduction pathways.

To determine the structural basis of isoquinoline sulfonamide specificity, we crystallized CK1 in complex with CKI7. The crystals grow as hexagonal bipyramids up to 0.8 mm in length (Fig. 3) and diffract X-rays to at least 3.5 Å resolution. This is our most recent crystal, and as of this writing, its space
FIGURE 3 Crystals of CK1 in complex with an isoquinoline sulfonamide inhibitor. The maximum dimension is 0.6 mm.

The "Open" Conformation of the cAMP-dependent Protein Kinase

J. Kuret, G. Carmel

Last year, we described the preliminary structure of the unliganded form of yeast cAMP-dependent protein kinase, performed in collaboration with J. Pflugrath (CSHL). This summer, its refinement was completed, bringing the crystallographic portion of the project to a conclusion after 4 years of effort. The final model, which includes data to 2.8 Å resolution, has an R-factor of 20.9% and deviates from ideal bond lengths and interbond angles by 0.018 Å and 2.8°, respectively.

No electron density is apparent for the two amino-terminal residues or for 18 residues located carboxy-terminal to the catalytic core. The stereocchemical quality of the model is good: 73.1% of 264 nonproline, nonglycine residues fall within the most favorable region of the Ramachandran plot, with an additional 25.0% falling in additionally allowed regions. The model is in excellent agreement with the structure of unliganded mammalian cAPK, published earlier in the year.

Comparison of this structure with its fully liganded mammalian homolog reveals a highly conserved protein fold composed of two globular lobes. Within each lobe, root mean square deviations in Cα positions average ~0.8 Å. In addition, a phosphothreonine residue is found in the carboxy-terminal domain of each enzyme. Further comparison reveals that ligand binding is accompanied by two major

FIGURE 4 Differences in backbone φ and ψ angles between unliganded TPK1 and fully liganded mammalian cAPK.
conformational changes. The first consists of a 15.8° rigid-body rotation of one lobe relative to the other along an axis parallel to a large α-helix located in the carboxy-terminal lobe (Helix E) and results in closure of the active site cleft. The rotation is mediated in part by the single strand that connects the two lobes and can be described by rotations in backbone dihedral angles $\phi$ and $\psi$. The largest changes in this region are a 65° increase in the $\phi$ angle of Gly-126 accompanied by a 70° decrease in the $\psi$ angle of Gly-125 (Fig. 4). Because Gly-125 is highly conserved throughout the protein kinase family, we suspect that the hinge motion observed in cAPK will emerge as a common feature of these enzymes. The second conformational change affects only the glycine-rich nucleotide-binding loop, which moves $\sim$3 Å to further close the active site and trap the nucleotide substrate.

**PUBLICATIONS**


In Press


**COMPUTATIONAL BIOLOGY**

T. Marr  
L. Catapano  
W. Chang  
S. Cozza  
D. Cuddihy  
E. Cuddihy  
E.C. Reed  
J. Salt  
W. Li  
M. Zhang  

Our group does basic research and computer software development in a new emerging area often referred to as "genomics" or "genome informatics." Through the efforts of the international Human Genome program, highly detailed molecular maps of human and model organisms are rapidly becoming available to all researchers in the broad areas of molecular cell biology. Data are often released on a daily basis from the large (by typical molecular biology standards) genome centers around the world, but primarily in the United States, Great Britain, and France. It is our thesis that gene finding and functional analysis could be greatly accelerated if these data were provided to the numerically dominant (and intellectually important) small, hypothesis-driven research laboratories in a simple, consistent, integrated, and timely manner.

Currently, one must use several different sources of data to devise informative experiments, such as the published scientific literature and scientific meetings, and a myriad of often difficult-to-use computer programs to accomplish even relatively simple investigations. The consequence of the current situation is that most biologists are not able to analyze fully the significance of their data and therefore are potentially missing crucial new findings. This situation is exacerbated by an ever-growing number of journals, scientific meetings, and specialized databases using ad hoc nomenclature and data representational and computer access methods. A major goal of our software development is to be able to move from genetic map, to physical map, to sequence and function using what appears to the user as one computer program. This type of "virtual program" ultimately is intended to be the primary source that an investigator can use to plan the next experiment, rather than being dependent on the relatively inefficient primary scientific literature and scientific meetings. The field is naturally evolving in this direction and "publication" will take on new meaning in the near future as fast, inexpensive computers and high-speed, wide-area networks become widely used.

We have a major computer software project in our group that has been under development for several years. This software package, called Genome
Topographer (GT) represents the results of many years of studying and participating in the experimental side of genome mapping and genomic analysis. We are in particular focusing on methods that accelerate finding genes involved in complex, multigene diseases, such as in what is expected for bipolar affective disorder (see H. Feilotter in the Neuroscience Section), and on methods for gene function prediction (using DNA and protein sequence analysis). In this way, our system will provide continuity to the process of gene mapping through directional cloning and functional analysis, which is currently lacking.

Since genetic, molecular, and biochemical information is essential in most cases to study gene function and dysfunction, Genome Topographer provides uniform representation (and database storage) of such data for human and major model organisms (including mouse, fruit fly, nematode worm, and the fission and budding yeasts). The types of data that we collect (and store) from multiple, independent databases from international computer networks and from the scientific literature include (1) cytogenetic maps, (2) genetic linkage maps, (3) physical maps (clone contigs, restriction maps, PFG maps, genomic sequences), and (4) loci, including genetic markers (mutations, polymorphism, genotype, disease state), DNA sequence (cDNA, fragments, translated protein sequence, alternate splicing, features unique to DNA sequences), protein sequence (features unique to protein sequences, motifs), locus effects (cell cycle, tissue, development stage expression patterns), locus interactions (nucleic acid-nucleic acid, nucleic acid-protein, protein-protein).

The premise of Genome Topographer is that a comprehensive, integrated presentation of the data be essential for their understanding. Therefore, a need arises for tools that will automate certain aspects of this process. Two maps that share certain elements, for example, can generally be compared by eye (and then annotated). However, this can become tedious if the amount of data grows exponentially. Simple tools that will compare two maps and highlight inconsistencies in the ordering of, or distance between, common elements can be of great help in many circumstances. Furthermore, "no single experimental procedure can be used to map a genetic object as complex as a mammalian chromosome. The most successful projects employ a variety of mapping techniques, some clone-based and others involving direct analysis of genomic DNA" (M.V. Olson and P. Green, unpubl.). Our own work of mapping the fission yeast *Schizosaccharomyces pombe* genome (to 13-kb resolution; Mizukami et al. 1993) was a combination of bottom-up and top-down methods: hybridization data that can be viewed either as STS/SCL (single-copy landmarks) content mapping or more broadly as "multi-origin chromosome walking" (Olson and Green 1993), integrated with genetic and restriction maps of the genome. It is this diversity of data that poses a challenge: The tools must "understand" the parameters of the underlying data types and origins (i.e., which laboratory), otherwise, they will not function optimally.

We have targeted specific areas of explosive growth where tools can be built that are likely to be of great help in the task of data integration. These include the Saccharomyces cerevisiae 3-kb resolution EcoRI/HindIII restriction map (Riles et al., Genetics 134: 81 [1993] and in prep.), the complete sequences of yeast chromosomes (Oliver et al., Nature 57: 38 [1992] and in prep.), Caenorhabditis elegans genomic sequencing (Waterston et al., Science 252: 1651 [1992]), and expressed sequence tag sequencing (Adams et al., Nature Genet. 1: 114 [1992]). In addition, such a tool that compares two maps locally can be adapted into a search engine that localizes one region inside a much larger one if the common elements have high enough specificity. For example, when a restriction-digested clone or sequenced locus is "drag-and-dropped" onto a restriction map or a genomic sequence, its precise location can be determined and displayed on the computer screen in "near real time" (i.e., without appreciable delay).

The construction of novel, highly effective computer tools for scientific discovery requires a solid quantitative and theoretical understanding of the problems at hand. In our case, we are studying DNA at multiple levels of resolution and protein sequences, including motifs. To gain insight into biological mechanisms that might be responsible for generating patterns we see in the data, we have initiated theoretical studies in genome dynamics (e.g., gene splicing recognition sites, inheritance patterns of segments of DNA that are identical by descent, and mechanisms that generate long-range statistical patterns in DNA sequences). We have a small group of theorists working on these problems, two of whom are physicists (one classical theorist, M. Zhang, and one complex systems theorist, W. Li) and one theoretical computer scientist (W. Chang), who specializes in the theory and practice of complex combinatorial calculations.
Below, we report on some of our new work in this area.

**Length Distribution of DNA Segments That Are Identical by Descent**

W. Li, M. Zhang, T. Marr

It is well known that a child inherits half of the genome from the father and half of the genome from the mother and that two siblings on average have half of their haploid genome identical by descent. These estimates, however, are very rough and are not adequate for many situations. The lengths of fragments inherited and their statistical distribution within and between individuals in an extended family unit are fundamental quantities in genetics that must be understood. Understanding these fundamental quantities has direct implications in many experimental strategies for mapping complex genetic traits, for biochemical difference cloning methods, and for cytogenetic difference analysis. For example, several new experimental methods under development are able to take DNA from two (or more) individuals in a family and pool them in a hybridization reaction, allowing those pieces that are largely identical-by-descent (IBD) to cross-hybridize. Such a reaction could be highly effective when comparing normal and, for example, transformed cells in the same person, by removing the IBD segments by stringent hybridization and selectively amplifying the differences hypothesized to be primarily associated with the transformed cell condition (see M. Wigler in the Molecular Genetics of Eukaryotic Cells Section). In any case, it is of interest to know the probability of two individuals sharing 100% IBD, 90% IBD, and so on, because of the discrete (intact and/or recombined segments from distinct chromosomes) nature of genetic inheritance in sexually reproducing organisms. The set of probabilities for each extent of IBD gives us a statistical distribution. We have carried out extensive numerical simulations and mathematical analyses on this topic.

In the simple situation, assuming there is only one crossover event per chromosome per meiosis, the IBD segments are always composed of contiguous stretches of DNA originating from particular individuals and matings. We have obtained the size distribution of such fragments for an arbitrary number of generations. Mathematical analysis shows that this distribution contains two discrete components (nothing IBD or all IBD) and one continuous component (0% < IBD segment < 100%). The distribution density after \( N \) rounds of meiosis follows:

\[
p(x)_N = \begin{cases} 
1 - \frac{(N+2)}{(N+1)} & \text{if } x = 0% \\
(2 - x)^{(N-2)} \frac{(N)(N+3-x)(N+1)}{(N+2)} & \text{if } 0% < x < 100% \\
\frac{1}{4^N} & \text{if } x = 100%
\end{cases}
\]

For example, when \( N = 1 \), this equation says that the probability of inheriting all of one particular haploid chromosome is 1/4, that for inheriting zero of that particular haploid chromosome is 1/4, and that for inheriting \( x \) percent is proportional to a constant value, 1/2. We have more general forms of this basic equation and circumstance available that address issues related to what we refer to as horizontal and vertical IBD, which refer to siblings of arbitrary genetic relation and of descendants of arbitrary genetic relation. We also have an expression relating to the question of the probability of inheriting a particular segment, for example, a segment containing a disease gene. We are continuing our work in this area by examination of the situation of multiple crossovers, the nature of the genetic disease (e.g., autosomal dominant, single gene vs. recessive expression, or quantitative genetic traits).

**Detecting Local Similarity between Protein Sequences**

W. Chang, T. Marr

The fundamental problem of protein sequence comparison is judging whether two or more sequences share structural and/or functional features, based on similarities observed in their amino acid sequences. Current understanding of the underlying processes of structure and function is not sufficient for a completely rigorous solution to this problem. Nevertheless, two developments in particular have combined to produce methods that are reasonably rigorous and successful. The first of these attacks the central problem of assigning a score to aligning a single pair of residues, according to chemical properties or statistical analysis of allowed mutations in known homologous sequences. The second is the rigorous treat-
ment of optimal alignment of regions by dynamic programming.

The widely used PAM matrices (Dayhoff et al., *Atlas of Protein Sequence and Structure*, vol. 5 [suppl. 3] 345–352 [1979]) were calculated on the basis of 1600 accepted point mutations in 71 groups of closely related proteins. This model of amino acid substitution assumes that the nonlethal mutations follow the same rate and distribution as in the original data, extrapolated to evolutionarily distant sequences and beyond. Generally, 50% identity over a putative domain (not the entire sequences) is considered extremely significant; in the PAM scale, this corresponds to about 90 PAMs. Occasionally, a particular 33% identity (120 PAMs) alignment is claimed to be significant. Recent advances (Altschul, *J. Mol. Biol.* 219: 555 [1991]) have led to the important understanding that PAM-X scores measure the likelihood that the aligned portion of two sequences are X PAMs apart evolutionarily. Thus, one ought to use the PAM 120 matrix to find 33% similar homologs.

Given a scoring matrix, one can find the highest scoring local alignment between two sequences (Smith and Waterman, *J. Mol. Biol.* 147: 195 [1981]). However, this alignment can have an arbitrarily low (but positive) mean score per residue. If

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**FIGURE 1** Overview of high-scoring local alignments. Query sequence is drawn along the top edge. Top window shows identities (=) and conservative substitutions (+). Middle window displays scores and score-per-residue. All windows scroll.
an alignment is significant, one would expect its mean score per residue to be fairly high (indeed characteristic of X PAMs). If the observed mean is much lower than expected, then the two sequences must be much more distant or unrelated, i.e., not what we are looking for. What we want is a method that finds all sufficiently long local alignments with a given mean score per residue. We have devised such an algorithm (Chang and Marr 1994) and are currently implementing and testing it in conjunction with other tools we have built.

A Suite of Tools for the Analysis and Visualization of Protein Sequences

W. Chang, D. Cuddihy, T. Marr

A similarity search of a given protein sequence in a large database can produce a massive amount of data that must be scrutinized for useful information. Therefore, the importance of a concise and effective visual display of the data cannot be overstated. We
are developing an interactive display, with scrolling windows and multiple views of the data, that uses character-cell graphics. It works effectively through both high-speed Ethernet and dial-up phone connections. The user-interface is kept simple, even though fairly powerful facilities are available for selectively hiding portions of the data (such as low-scoring alignments). Redundant data (such as identical alignments against several database entries) are combined but not discarded. Such filtering can pare down a BLAST or FASTA report by a factor of ten, without losing any information. Access to databases and powerful search facilities are available on-line as well. For example, several alignments can be merged to produce a PROSITE-type pattern, which is then compared against the entire database in just seconds. Version 1.0 (see Figs. 1 and 2) will be ready for release the first week of March, 1994.

We also implemented a search engine based on dynamic programming algorithms for finding high-scoring local alignments (Smith and Waterman, J. Mol. Biol. 147: 195 [1981]; Gotoh, J. Mol. Biol. 162: 705 [1982]; Waterman and Eggert, J. Mol. Biol. 197: 723; Huang and Miller, Adv. Appl. Math. 12: 337 [1991]). Our highly efficient implementation of Smith-Waterman-Gotoh (called GSW) is the fastest available for a uniprocessor computer, taking just 10 minutes on a DEC Alpha to compare a 400-residue sequence against the SwissProt database. (The fastest Email-server, blitz@EMBL-Heidelberg.de by S.S. Sturrock and J.F. Collins of the University of Edinburgh [1993], runs on a 4000-processor MasPar and is 25 times faster.) Dynamic programming has advantages over the highly tuned heuristic method FASTA (Pearson, Genomics 11: 635 [1991]) in that the latter has difficulty characterizing its findings and nonfindings. GSW is about one-third the speed of FASTA (most sensitive setting, -o and ktup=1). The drawback of using BLAST is that it only finds gapless alignments. A search engine based on a new method (Chang and Marr 1994) of finding alignments with appropriately high score per residue is under development.

PUBLICATIONS


In Press


GENOME SEQUENCE ANALYSIS

W.R. McCombie  N. Kaplan  D. Lombardi  A. Melnikov

The comparison of the structures of different eukaryotic genomes will be important in our understanding of the structure and function of the human genome. In addition, the complete genome sequence of an organism will have a dramatic impact on the understanding of that organism and on the way it is used as a model system. Yet, while the rate at which DNA sequence data can be obtained and to a lesser extent analyzed has changed rapidly in recent years, even the smallest eukaryotic genome presents a major challenge for DNA sequencing. One of the major goals of our laboratory is to sequence and analyze the
Sequencing Technology Development

D. Lombardi, N. Kaplan, W.R. McCombie

We are developing sequencing technology in three primary areas: (1) development of computer software tools and databases to facilitate the management of large-scale sequencing projects, (2) implementation of hexamer strings on automated sequencers, and (3) optimization of double-stranded template-based sequencing strategies and technologies. Once these new sequencing technologies are optimized, we will use the database tools we have developed to compare them with more traditional strategies.

An important capability in large-scale sequencing projects is the ability to rapidly and accurately process and store the large amounts of data generated by automated sequencers. This requires the ability to handle data generated from multiple types of instruments in different formats and to reformat the data into still other formats for assembly and analysis. Although theoretically simple, the demands of this process when the thousands of samples processed are multiplied by several different formats can cause a severe bottleneck in the sequencing process. We have begun addressing this problem by developing and implementing a series of software tools. This work has been done in collaboration with Tom Marr (CSHL).

We have chosen to use several commercially or academically available software packages and to develop the necessary software to integrate them and ultimately place them all under the control of a common graphics user interface. This will allow these packages to be used by individuals who do not have extensive computer experience. Software developed this year allows data to be transferred to a central Sun computer that functions as a file server from either a Macintosh used for data acquisition by the ABI sequencer or the IBM compatible computer that the Pharmacia sequencer employs. This is carried out by a simple "drag and drop" function with the mouse. Once on the Sun, a program has been written that reformats the data to the format used by the ted and Staden Xbap graphical assembly packages used on the Sun and simultaneously transfers a copy of the data to both a Sun (Xbap) and pc (Macintosh or IBM native format) backup. A second program then autoloads the data one directory at a time (currently up to 36 sequence files) into the ted editor (developed by LaDeana Hillier at the C. elegans genome center at Washington University). Once the ted editor is used to remove poor-quality data at the 3' end of the sequence and vector at the 5' end, the edited files are stored on the Sun. After a group of sequences is processed to this stage, programs written at CSHL are used for the next steps, which are again carried out in a batch mode on all the sequence files in a directory simultaneously. Copies of the edited data are made in Staden format, Intelligenetics format (IG), and Fasta (Blast) format. These files are automatically moved to separate directories for each format. This puts copies of the data in a separate directory for Staden assembly, IG assembly and homology searching of GenBank, and the protein databases (Fasta format). Furthermore, the data are automatically placed in subdirectories based on what project it is a part of, and a record of failed sequences is written out to a separate directory location. Each project is typically a cosmid being sequenced. The next phase of the software development will be the implementation of user interface screens that will be displayed on the Macintosh and allow the above-described functions to be carried out using the Macintosh graphics interface. This will eliminate the need for directly logging into the Sun and executing Unix commands to carry out the data handling. The third phase will be the development of software to transfer automatically various statistical data derived from the sequencing process into a Sybase database. This will enable us to evaluate quantitatively the impact of technology advances made in the laboratory.

An important technology that we are testing in the laboratory is the implementation on automated sequencers of sequencing using strings of contiguous hexamers rather than a single 18 mer as a primer. This has the potential of making low-redundancy primer walking possible on a large scale with minimal reagent and management costs associated with de novo primer synthesis. This is because all 4096 possible hexamers can be synthesized in advance and used when needed. This is made possible by the presence in the reactions of saturating amounts of Escherichia coli single-stranded DNA-binding protein (SSB) (Kieleczawa et al., Science 258: 1787 [1992]).
The reactions require an initial extension step at 0°C to stabilize the hexamers, followed by a termination step in which fluorescent analogs of deoxynucleotides are incorporated at 37°C.

Last year, we were able to get some priming of the reactions using ABI sequenase terminator chemistry and hexamer primers, but there was a relatively high error rate on M13 templates. This year, we have made further progress in adapting this chemistry to the ABI373. We found that a tenfold increase in hexamer concentration relative to what we used previously and what had been used for 35S sequencing by Kieleczawa et al. (Science 258: 1787 [1992]) appears to result in better, more reproducible signals. We have tried four different hexamer strings on M13. All of them worked and the best results to date are read lengths of about 400 bases with slightly over 1% errors in the range from 20 to 400 bases from the priming. Our future efforts will be to determine and ultimately improve the reproducibility of these reactions using a wider range of templates. In addition, we will be testing the reactions on double-stranded templates and testing ways to remove the SSB from the reactions other than the organic extractions currently employed. While we are working to improve the utility of hexamer primed reactions, we have begun sequencing the *S. pombe* genome using a nucleation and walk strategy. This will allow flexible incorporation of primer-walking-based sequencing (such as the use of hexamer strings) into our overall sequencing strategy. We will do this when hexamer-string-based reactions have been further tested on control templates. In the meantime, we have begun testing the effect of different variables of template isolation and sequencing reactions on our *S. pombe* templates but have no conclusive results yet.

**S. pombe Genome Sequencing**

N. Kaplan, A. Melnikov, D. Lombardi, W.R. McCombie

Previous work in David Beach’s and Tom Marr’s laboratories has provided a high-resolution physical map of the *S. pombe* genome and a set of mapped, overlapping cosmid clones that represent virtually the entire genome of the organism with only a few small gaps (Mizukami et al., Cell 73: 121 [1993]). These clones make the *S. pombe* genome ready for sequencing, and we have begun that project.

Cosmid 359 is the most telomeric cosmid mapped to chromosome II of *S. pombe*. We subcloned cosmid 359 by randomly fragmenting the DNA with nebulization, which is a new technique that appears to give more discrete fragmentation than sonication. Fragments in the 1.5–3.0-kb range were subcloned into pGEM vectors using blunt-end end-cutting enzymes to prepare the vector. We began sequencing these subclone libraries and found a large percentage of the subclones had damage to the vector, which included the sequencing primer-binding sites, that prevented them from being successfully sequenced. We developed a polymerase chain reaction (PCR) assay to screen for functional clones rapidly and continued sequencing cosmid 359 using the screened clones. Simultaneously, efforts continued to make libraries with a higher percentage of sequenceable clones so that screening would be unnecessary. The results of the sequencing of cosmid 359 are not yet complete, but several interesting genes have already been found based on putative homology with previously known genes.

As of February 1994, cosmid 359 is present in a single contig of slightly over 38,000 base pairs. There is a vector insert junction present at the centromeric end of the insert, but a small amount of sequence, including the vector-insert junction, is apparently missing at the telomeric end of the insert. This sequence was probably transferred out of the assembly with the vector sequences, and we are in the process of searching those data for the junction in order to recover it. There are about 750 fragments of sequence data in the cosmid 359 contig. Most of these are from the shotgun phase of the project. However, a number of gaps in the cosmid were filled by using custom primers and either sequenase dye-deoxyterminators or Taq dye-deoxyterminator chemistry. We are currently in what is commonly called the finishing phase of a shotgun-sequencing project with cosmid 359. In this stage, the disagreements between the multiple sequence fragments present at a given point in the consensus sequence are resolved. It is also when regions of insufficient redundancy are resequenced or sequenced on the complementary strand. When completed, we will have double-stranded coverage on all of the cosmid, meaning that both strands are sequenced at least once at every base. In addition, during the finishing phase, compressions and other problem areas are resolved. This involves picking custom
primers to resequence areas and sequencing areas with sequenase rather than Taq (or vice versa) to eliminate problems caused by the sequence at a particular site that interferes with sequencing by one of the enzymes. When this phase is completed, the final sequence will be analyzed for gene content in collaboration with Tom Marr's and David Beach's laboratories. This will include using software to predict the presence of coding regions and splice sites. Although the predictions of splice junctions and open reading frames require accurate sequences with the ambiguities resolved, the analysis of the sequence based on homology with previously known genes does not. We have begun this aspect of the analysis and have identified potential homologs of eight genes in cosmid 359. These are shown in Table 1 along with their positions in the contig.

In other large-scale sequencing projects, including both genomic sequencing and cDNA tagging, between 30% and 50% of the genes have detectable homology with genes already identified in the various protein and DNA databases. Assuming that a similar percentage of S. pombe genes could be identified in this manner, the eight detected putative homologs would imply 16–24 genes present in this 38-kb region of the S. pombe genome. This is in good agreement with the estimate of one gene per 2 kb in S. pombe. Once the sequence of cosmid 359 is finished, it will be analyzed not only for potential genes, but also for compositional patterns and other features that can be determined and compared to the results from Saccharomyces cerevisiae.

As the shotgun phase of cosmid 359 was being completed, the next two cosmids toward the centromere (cosmids 1198 and 1683) were begun. Due to problems with the construction of subclone libraries for cosmid 359, a new method of library construction was tested. One of us (W.R.M.) traveled to Baylor College of Medicine to learn new techniques in the laboratory of Dr. Richard Gibbs, who has been working extensively on solving this subcloning problem. The latest generation of these techniques are described by Andersson et al. (Analytical Biochem., in press). In addition to learning techniques at Baylor, we were able to show them the nebulization technique and combine it with their subcloning procedures. The modified procedure as we carried it out at Baylor began with the fragmentation of the cosmid with nebulization and the repair of the fragments to make them blunt-ended. Adapters with a long single-strand overhang were then ligated to the insert fragments and 2–5-kb fragments were fractionated and purified from an agarose gel. The vector was prepared quite differently from that with cosmid 359 or other standard strategies. The vector DNA was cut with two enzymes at positions in the universal cloning site region to minimize the amount of intact vector in the preparation. The actual vector used for the subcloning was then prepared by PCR amplification of this doubly digested vector. A small (5 pg) amount of cut vector was amplified. The primers consisted of sequences complementary to the forward and reverse sequencing primers so that the amplification began from those two points. In addition, each primer had a 5' extension that produces the 3-base repeating sequence found in the insert adapter overhang region. However, the vector primers included uracil in place of thymidine in the repeat portion of the primer. Amplification was carried out in the presence of thymidine and no uracil in the dNTP mix so that only the ends of one strand of the amplification product contained uracil. In previous work done at Baylor with M13 amplification, it was necessary to purify the proper vector fragment from an agarose gel. We found that the pBluescript that we amplified was much more homogeneous and so we eliminated the gel purification step. An aliquot of the amplified vector was then treated with uracil-d-glycosylase, which cleaved at the three uracils present on one strand at each end of the vector, producing overhangs complementary to the insert adapter overhang. The vector and insert were then annealed in the absence of ligase for 1 hour and used to transform E. coli

### TABLE 1 Potential Homologs of Genes in Cosmid 359

<table>
<thead>
<tr>
<th>Potential homolog</th>
<th>Positive segments (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine racemase</td>
<td>3–4</td>
</tr>
<tr>
<td>Hypothetical protein permease</td>
<td>5–6</td>
</tr>
<tr>
<td>Agglutinin attachment subunit</td>
<td>13</td>
</tr>
<tr>
<td>Multidrug resistance gene</td>
<td>19–21</td>
</tr>
<tr>
<td>Inorganic phosphate transporter</td>
<td>29–30</td>
</tr>
<tr>
<td>AMP deaminase</td>
<td>31–32</td>
</tr>
<tr>
<td>Aminotriazole resistance</td>
<td>33–34</td>
</tr>
<tr>
<td>β-glucosidase</td>
<td>37–38</td>
</tr>
</tbody>
</table>

The 38-kb contig from cosmid 359 was divided into nonoverlapping 1000-bp segments. Each of the segments was used to search a nonredundant protein database at the National Center for Biotechnology Information using Blast. Potential homologies are noted above, along with the segments in which they were found. Segment distances are from the most telomeric end of cosmid 359; hence, the potential homolog of alanine racemase is 3–4 kb from the telomeric end of cosmid 359.
strain XL1-Blue. The long overhang allows the problems associated with ligation of blunt-end fragments of material purified from agarose gels to be eliminated. Cosmid 1198 and 1683 subclone libraries had very few blue colonies when transformed, and test sequencing indicates that probably less than 5% of the clones have damaged ends and will not sequence. This compares with 60–70% of the cosmid 359 clones (prior to screening) that were made using vector prepared by *SmaI* or *EcoRV* cleavage. We are continuing the shotgun phase of both of these cosmids without screening. In addition to drastically improving the efficiency of sequencing, this technique also has increased the amount of useful data obtained from each clone. In cosmid 359, insertion in the vector *SmaI* site led to 60 bases of vector being sequenced with the forward primer and 90–100 bases of vector being sequenced with the reverse primer. With cosmids 1198 and 1683, the vector is only amplified from the sequencing primer-binding sites; hence, only the adapter (less than 20 bases) is present between the primer-binding site and the insert. This results in an extra 40 and 80 bases of useful sequence, respectively, when forward and reverse primers are used. This improvement alone will result in an extra 30,000 to 48,000 bases of raw sequence being determined at no extra cost in the course of sequencing a typical cosmid. Cosmids 1198 and 1683 are well into the shotgun phase. In keeping with our overall strategy to reduce gradually the amount of random sequencing and increase the directed sequencing, the shotgun phase of cosmids 1198 and 1683 will be concluded when about 500–550 sequence fragments are obtained from each. Cosmid DNA from the next two cosmids has been isolated and is being tested. It will soon be used to make the next set of subclone libraries according to the procedure of Andersson et al. (1994).
The Neuroscience Section is focused on molecular-genetic mechanisms of neuronal events that underlie developmental and behavioral plasticity. Insight into these processes promises effective therapeutic approaches to cognitive dysfunction, such as that associated with normal aging or with Alzheimer’s disease. This year marked the first full year of effort by members of this new section. Already significant progress has been made, driven in part by a growing synergy of ideas among the neuroscientists: (1) The Enikolopov, Marshak, and Nawa groups have discovered different aspects of gene expression or regulation of protein phosphorylation that appear to mediate the effects of neurotrophic factors on the growth and differentiation of neurons and neural circuits. (2) The Silva, Tully, and Zhong groups are pursuing various aspects of the regulation of gene expression, signal transduction and synaptic transmission that are involved with animal learning and memory formation. (3) As part of the inter-institutional Dana Consortium, the Feilotter, Marr, and Witkowski groups are pursuing the identification of genes involved with bipolar affective disorder (manic depression) in humans.

To balance the departure of one neuroscientist, Dr. R. Davis, two new colleagues recently have accepted positions. Drs. R. Malinow and H. Cline will join the Laboratory early in 1994 to contribute their expertise in vertebrate hippocampal synaptic plasticity and vertebrate developmental plasticity, respectively, to the fledgling Neuroscience effort.

NEURONAL GROWTH AND DIFFERENTIATION

D.R. Marshak
N. Chester
J. Diaz-Nido
L. Peña

P. Recksiek
A. Rossomando
M. Vandenberg

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 subsequently to 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, as well as learning which growth factor molecules control these switches within cells. Specifically, we are interested in the action of a growth-stimulating protein, S100β, that is produced by astrocytes in the brain. 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.

Mechanism of Action of S100β

L. Peña, J. Diaz-Nido, D.R. Marshak

Our work on the action of S100β in Alzheimer’s disease and development has centered on studying protein kinases. One avenue of investigation has been to examine the direct influence of S100β on protein
kinase C. We found that S100β inhibited the phosphorylation of GAP43 by recombinant protein kinase C (Sheu et al. 1994). These studies suggest that protein kinase C might be a direct target of S100β action in neurons. Another effort grew out of the observations by members of this program that S100β can have mitogenic actions as well as differentiative actions. Our studies have focused on the interactions between protein kinases involved in mitogenic pathways and those involved in differentiative pathways. These protein kinases include casein kinase II, the cyclin-dependent kinases, MAP kinases, MAP kinase kinases, and glycogen synthase kinase 3.

Last year, we found that casein kinase II (CKII) phosphorylated a cyclin-dependent kinase, p34cdc2, in human cells on Ser-39. Since CKII is elevated in normal differentiated tissue and decreased in degenerating tissue, we sought to study the effects of this phosphorylation event. On the basis of mutation data, it appears that phosphorylation of Ser-39 in p34cdc2 prevents cyclin binding, disabling the activation of the enzyme. In Alzheimer's disease, decreases in CKII may lead to inappropriate activation of p34cdc2 or other cyclin-dependent kinases. In the developing rat brain, J. Diaz-Nido found that CKII catalytic subunits are differentially expressed, with the α' subunit appearing much later in development and correlated with synaptogenesis in the cortex. These observations suggest that CKII may have an interesting developmental role in neuronal function. Decreases in CKII in degenerative disease might lead to neuronal dysfunction.

Regulation of cdc2 and MAP Kinases in PC12 Cells
A. Rossomando, D.R. Marshak

Another area of signal transduction in developing neurons is that which responds to growth factors known as neurotrophins, such as nerve growth factors (NGFs). Using rat pheochromocytoma (PC12) cells, we examined the regulation of MAP kinase and p34cdc2 kinase in response to NGFs. The p34cdc2 is down-regulated as the cell differentiates. The MAP kinase is rapidly activated and then deactivated but not suppressed to zero levels during differentiation. Epidermal growth factor, which promotes proliferation of these cells, has somewhat different effects on these protein kinases. These experiments suggested that regulation occurs on some level between these kinase pathways.

To further analyze this phenomenon in human cells, we studied cycling HeLa cells. We found that the cell cycle controlled the pathway that culminates in the activation of MAP kinase (A.J. Rossomando et al. 1994). This enzyme is stimulated by MAP kinase kinase (also known as MEK). We found that both MAP kinase and MEK were not active in mitosis in human cells. Furthermore, MEK is phos-phorylated in a cell-cycle-dependent manner by p34cdc2, which is most active during mitosis. The phosphorylation was localized to threonine residues near the carboxy-terminal end of MEK, and the phosphorylation of those residues resulted in the complete inactivation of MEK. These results indicate that cell division regulates the MAP kinase pathway at the level of MEK. This is significant to neuronal survival, since the action of neurotrophins appears to act through MAP kinases. Abnormal activation of p34cdc2 might lead to inactivation of the pathways that support neuronal survival and subsequent cell death.

Regulation of S100β by β-amyloid
L. Peña, P. Recksiek, D.R. Marshak

Further work has been done on the basis of our observation that S100β gene expression can be stimulated by Aβ peptides derived from amyloid precursor protein (Peña et al. 1993). In Alzheimer's disease and Down's syndrome, severely afflicted brain regions exhibit up to 20-fold higher protein levels of S100β, and astrocytes surrounding neuritic plaques exhibit higher protein levels of S100β. A major constituent of the plaques is β-amyloid protein, which has been reported to have both neurotrophic and neurotoxic effects in vitro. We examined the responses of central nervous system glia to Aβ peptide. Primary astrocyte cultures, obtained from neonatal rats and rat C6 glioma cells, were synchronized by serum deprivation and treated with AβA(1-40), a synthetic fragment of β-amyloid. A weak mitogenic activity was observed, as measured by [3H]thymidine incorporation. Northern blot analysis revealed increases in S100β mRNA within 24 hours, in a dose-dependent manner. Nuclear run-off transcription assays showed
that βA(1-40) specifically induced new synthesis of S100β mRNA in cells maintained in serum but caused a general elevation of several mRNA species in cells maintained under serum-free conditions. At the protein level, corresponding increases in S100β protein synthesis were observed in response to the βA(1-40) peptide, as measured by immunoprecipitation of 35S-labeled cellular proteins. The data indicate that S100β expression can be influenced directly by β-amyloid.

**Phosphorylation of Amyloid Precursor Proteins**

D.R. Marshak [in collaboration with P. Greengard, Rockefeller University]

Control of the processing of the amyloid precursor protein (APP) is central to the generation of the βA4 peptide that is found in plaques. We studied cell division cycle control of APP processing in HeLa and PC12 cells (Suzuki et al. 1994). During mitosis, the forms of APP produced are qualitatively different from that during G1 phase. In particular, the immature form of APP is altered, the level of secreted, amino-terminal domain is decreased, and the level of the truncated, intracellular carboxy-terminal fragment is increased. These events are correlated with phosphorylation of the cytoplasmic domain of APP. We demonstrated that Thr-668 of APP is phosphorylated in vivo in a cell-cycle-dependent manner and that in vitro, the same site is phosphorylated by p34cdc2 kinase. These data are consistent with our working hypothesis that an abnormal mitogenic signal, resulting in the elevation of p34cdc2 kinase, can contribute to the neuronal degeneration seen in Alzheimer's disease.

**Distribution of S100β in Human Brain Diseases**

D.R. Marshak, M. Vandenberg [in collaboration with W.S.T. Griffin, University of Arkansas]

The proximity of S100β-containing astrocytes to neuritic, amyloid plaques in the brains of Alzheimer’s disease patients suggests a functional role for S100β in the pathology of the disease. Gliosis and elevated S100β accompany several neurodegenerative diseases, and the elucidation of the role of this growth factor in degeneration is important to our understanding of the etiology of the diseases. In addition, basic research in glial-neuronal interactions will help us learn the fundamental processes that control brain development and adult function. In collaboration with W.S.T. Griffin, radioimmunoassays for S100β have been done on a variety of brain regions from autopsy tissue of Alzheimer’s disease patients compared to age-matched controls. Temporal lobe regions that are clearly affected by amyloid deposition and degeneration have elevated S100β in reactive glia. In a separate study, samples from patients suffering from AIDS dementia were observed to have increased S100β, but not to as high a level as in Alzheimer’s disease.

**PUBLICATIONS**


In Press


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, the 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.

A major and important story contributed by our laboratory in 1993 was the characterization of the protein kinase A gene of Drosophila and an elaboration of its role in learning and memory. With molecular and behavioral techniques, we demonstrated that this gene is preferentially expressed in neural centers called mushroom bodies and that mutations in the gene cause dysfunction in olfactory learning. Progress on other projects in the laboratory is described below.

**DROSOPHILA PROTEIN KINASE A**

The fly stock, MB581, was isolated in an enhancer detector screen to search for mushroom body genes and was shown to contain a transposable element in the unique gene for the catalytic subunit (dc0) of protein kinase A (PKA). In situ hybridization to tissue sections and immunohistochemical analysis demonstrated that dc0 is preferentially expressed in the mushroom bodies. Thus, the preferential expression of PKA in the mushroom bodies, along with the rutabaga-encoded adenyl cyclase (AC) and the dunce-encoded cAMP phosphodiesterase (PDE), lends strong support for a role of the cAMP cascade in these neurons to serve learning/memory processes.

Molecular characterization revealed that the transposon in MB581 was inserted in exon 1, 71 bp downstream from the transcriptional start site of dc0, thus disrupting the gene. This disruption reduces the amount of dc0 RNA to barely detectable levels as assayed by in situ hybridization with homozygous mutant embryos. The insertion produces recessive lethality and a 35–40% reduction in PKA activity in heterozygous adults due to the reduction in the level of the DC0 protein. A similar decrease in activity and a lethal phenotype are exhibited by a different hypomorphic allele of this gene (B10) isolated in a screen by EMS mutagenesis (D.K., unpublished). The reduction in PKA activity in B10 is likely the effect of a missense mutation on the structure of the protein and/or activity of DC0. Surprisingly, heteroallelic MB581/B10 animals can be obtained that appear normal externally and normal with respect to brain structure. However, they exhibit only 20–25% of the PKA activity of control strains.

The effect of reduced PKA activity on behavior was investigated by determination of the learning/memory phenotype of MB581 and B10 heterozygotes and the MB581/B10 heteroallelic animals using an olfactory classical conditioning paradigm. Initial learning of MB581 is slightly reduced compared with the control strains and B10 heterozygotes. Interestingly, memory assayed 30 minutes or later is normal. Thus, the 35–40% reduction in PKA activity may have a mild effect on learning but is not limiting for memory. The MB581/B10 heteroallelic animals exhibit a 50% reduction in both initial learning and memory. This behavioral effect is not due to the inability of the mutants to perceive the stimuli demonstrated by control experiments in which both heterozygotes and the heteroallelic animals are indistinguishable from the control strains. These results demonstrate unambiguously that PKA plays a cardinal role in learning and memory processes mediated by the mushroom bodies.

**CELL ADHESION MOLECULES AND LEARNING**

Among the enhancer detector lines, one line named MB2225 has its P-element insertion site cytogeneti-
cally at 4AB. This is a locus housing the gene for a *Drosophila* cell adhesion molecule, fasciclin II (fasII), which was originally identified as a molecule involved in the early development of the nervous system. When tested for their ability to associate an odor with electric shock, flies from this line showed defective association and memory at all time points tested up to 3 hours after training.

To clarify the relationship between fasII and MB2225, the genomic fragment next to the P-element insertion site in MB2225 was obtained by plasmid rescue. Using this fragment for Southern hybridization and DNA sequence analysis, the insertion site in MB2225 was mapped to the first exon of the fasII gene at a position between the transcription start site and the open reading frame. Therefore, MB2225 was expected to be a fasII mutant. To confirm this, the protein expression was analyzed by immunohistochemistry using antibodies raised against the transmembrane form of the FasII protein. In head sections of wild-type adult flies, the protein is expressed mainly in the mushroom bodies and the ellipsoid body of the central complex. This expression is missing in MB2225. Because of the hypothetical role for mushroom bodies in learning, the FasII expression in these structures is consistent with the possibility that FasII functions in adult behavior in addition to its developmental functions.

**Lightminded, A PUTATIVE NEW LEARNING AND MEMORY MUTANT**

Enhancer detector line 2063 shows preferential expression of β-galactosidase in the mushroom bodies. Flies homozygous for the P-element insert are deficient in learning and memory in a classical negatively reinforced learning paradigm. Initial learning and retention over the 180 minutes tested are lower than that of wild type. Control experiments confirmed that mutant flies perceive the stimuli used for conditioning (olfactory cues and electrical shock) in a manner indistinguishable from that of wild type, suggesting a defect specific for the learning/memory process. Another P-element line with an insert only 200 bp from 2063 has also been shown to be deficient in learning/early memory, and this may be allelic to the first.

We have isolated genomic DNA flanking the P-element insertion site and used it to screen a head-specific cDNA library. Two classes of transcripts have been identified. Several head-specific RNAs are detected by the cDNA probes, some of which are absent or strongly reduced in the mutant. Future work will include characterizing these sequences and their spatial expression pattern.

**MAMMALIAN HOMOLOGS OF Dunce**

The study of *Drosophila* has yielded much of our current understanding of the cellular mechanisms underlying behavior. A number of genes that appear to be critical for the processes of learning and memory formation have been identified in this species, including the *dunce* gene which encodes a cAMP-specific phosphodiesterase. We are now in a position to ask whether these genes are structurally and functionally conserved in other species. We have thus begun to examine the mammalian homologs of the *Drosophila dunce* gene. Four distinct *dunce* homologs have so far been reported in mammals; we used polymerase chain reaction (PCR) to partially clone three of the mouse *dunce* homologs from rat clones obtained from M. Wigler’s laboratory (CSHL). Antibodies have been generated to each of the mouse *dunce* gene products, and we are now in the process of determining specifically where in the brain the Dunce proteins occur.

The most advanced information is for mouse *dunce* 2. This gene is exquisitely expressed in olfactory receptor neurons as detected by RNA in situ hybridization. Similarly, immunohistochemical results with isoform-specific antibodies have demonstrated that the protein is expressed in the olfactory receptor neurons but is compartmentalized in an interesting way. The protein is found in the soma and axon bundles that project to the olfactory bulb, but not in the cilia, where the initial signal transduction events have been proposed to occur. This suggests that the protein is involved in the propagation of the signal downstream from the initial signal transduction events. Thus, it may be involved in axon potential propagation by modulating ion channels, thus participating in olfactory acuity, sensitization, or adaptation processes.

**PUBLICATIONS**


Qiu, Y. and R.L. Davis. 1993. Genetic dissection of the
Although significant progress continues all along a wide front of molecular neurobiological analyses of learning and memory in fruit flies, our most important result is the discovery of a "molecular switch" for long-term memory (LTM). The switch turns out to be a transcription factor homologous to the vertebrate CREB family. As in vertebrates, dCREB2 is activated via phosphorylation by cAMP-dependent protein kinase. Thus, we expect that one or more CREB family members also will be involved with LTM in vertebrates. In a more general sense, our results represent a clear example of the power that gene discovery in Drosophila brings to the study of complex, "emergent" organismal functions like learning and memory.

Long-term Memory in Fruit Flies

T. Tully, M. Del Vecchio, S. Boynton

After one "standard" training cycle of Pavlovian conditioning (CS+ for 60 sec, rest for 45 sec, CS- for 60 sec, rest for 45 sec), memory retention of conditioned odor responses normally decays to about 18% of initial values within 24 hours and to 5% of initial values within 4 days. Recently, with the automation of our training apparatus, we have demonstrated much longer-lasting memory after extended training (Fig. 1). Ten training cycles with a 15-minute rest interval between each ("spaced" training) produced memory that decayed only to 35% of initial values within 7 days. Interestingly, ten cycles of training without a rest interval between each cycle ("massed" training) produced a moderate 24-hour memory, which nevertheless decayed to zero within 4 days (as was the case for one cycle of training; data not shown). Thus, spaced training was required to produce long-lasting memory—a property of memory formation ubiquitously observed in vertebrates.

A second property of LTM in vertebrates is that it is stable to disruption. To this end, we have exposed third instar larvae to extended training procedures and have demonstrated that conditioned avoidance is retained through metamorphosis (Tully et al. 1994). This retention of conditioned avoidance was observed only for the group of larvae for which odor and electric shock were temporally paired, indicating that memory through metamorphosis derived from associative learning. Moreover, memory through metamorphosis was observed after eight, but not three, training cycles, a property of long-lasting memory also observed in adults (data not shown). Interestingly, adults were tested 8 days after larval training and yielded a memory score similar to that seen for 7-day retention in adults trained as adults. Taken together, these data indicate that a stable LTM survives substantial neuronal reorganization during metamorphosis. The morphological questions raised by this observation will be fun to address in future experiments.

A third property of memory formation in vertebrates is that the induction of LTM can be blocked by protein synthesis inhibitors. Thus, we fed flies the protein synthesis inhibitor cycloheximide (CXM; 35
FIGURE 1 Memory retention after ten cycles of massed (circles) or spaced (squares) training in wild-type Can-S (closed) or mutant radish (open) flies. Some groups of Can-S and radish flies also were fed the protein synthesis inhibitor cycloheximide (triangles). N = 6 PIs per group.

mm in glucose for 17 hr), trained them with either massed or spaced procedures, put them back on CXM-laced glucose for 24 hours, and then measured memory retention. We found that 24-hour memory after spaced training in CXM-fed wild-type flies was only 60% that of glucose-fed controls, a level of memory retention similar to that of glucose-fed flies 24 hours after massed training (see Fig. 1). In contrast, CXM had no effect on conditioned avoidance immediately after one cycle of training (PIs = 81 ± 1 and 85 ± 2 for glucose- or CXM-fed flies, respectively; N = 6 PIs each), demonstrating that the drug did not produce any nonspecific performance deficits under these feeding conditions. More interestingly, CXM also had no effect on 24-hour memory after massed training (data not shown), suggesting that the massed training procedure induced only a "CXM-insensitive" memory, whereas spaced training induced both CXM-insensitive and CXM-sensitive components of 24-hour retention.

The fact that CXM did not affect 24-hour memory after massed training was reminiscent of past experiments, in which we showed that an earlier anesthesia-resistant phase of memory (ARM) was not disrupted in CXM-fed flies. This observation suggested that 24-hour memory after massed training—or after spaced training in CXM-fed flies—might be ARM (and nothing else). Recently, Dr. E. Folkers in W.G. Quinn's laboratory at the Massachusetts Institute of Technology demonstrated that ARM is disrupted in mutant radish flies (Folkers et al., Proc. Natl. Acad. Sci. 90: 8123 [1993]). We therefore reasoned that if 24-hour memory after massed training was ARM and radish disrupted ARM, then 24-hour memory after massed training in radish flies would be zero. In fact, we found such to be the case (Fig. 1). We then went on to show that the 24-hour retention deficit after massed training and the ARM deficit both "co-map" to the radish cytological region (data not shown). Such a result indicates formally that the radish mutation is responsible for both phenotypic defects.

Surprisingly, spaced training of radish mutants yielded completely normal memory retention 2–7 days afterward and 1-day retention 56% of normal. Moreover, the 1–7-day decay rate for radish mutants was zero (Fig. 1). The presence of such a memory curve after spaced training of radish flies suggested that LTM might be normal in these mutants even though ARM was disrupted. We predicted that 24-hour memory after spaced training in radish mutants would be blocked completely by CXM. In fact, we found such to be the case (Fig. 1). Taken together, these results indicate the following:

1. ARM and LTM are genetically distinct, functionally independent components of memory.
2. Spaced training of wild-type flies induces ARM and LTM, which then act in an additive fashion from 1 to 7 days after training to produce the observed memory retention curve.
3. Massed training in wild-type flies induces only ARM.
4. The radish mutation blocks ARM, leaving LTM intact.
5. CXM blocks LTM, leaving ARM intact in wild-type flies and yielding no 24-hour memory whatsoever in radish mutants.

A "Dominant-negative" Form of CREB Transcription Factor Specifically Blocks LTM in Adult Transgenic Flies

J. Yin, M. Del Vecchio, T. Tully [in collaboration with J. Wallace and W. Quinn, Massachusetts Institute of Technology; B. Wilder and N. Perrimon, Harvard Medical School]

Early biochemical and molecular work in other laboratories has established that the learning/memory
genes *dunce* and *rutabaga* encode a cAMP phosphodiesterase and a Ca\(^{++}\)/CaM-dependent adenylyl cyclase, respectively, thereby implicating the cAMP signal transduction pathway in olfactory learning in *Drosophila*. Subsequent work with transgenic flies expressing a "dominant-negative" inhibitor of cAMP-dependent protein kinase (PKA) or with mutants in *DCO*, the gene encoding the catalytic subunit of PKA, also has shown that disruptions of PKA produce learning/memory defects. In the *Aplysia* model system, blocking the PKA-dependent phosphorylation of a potassium channel leads to learning defects. In analogous fashion, fruit fly *Shaker* mutants have defective potassium channels and abnormal learning and memory. Given such clear evidence for the involvement of the cAMP signal transduction pathway with learning and memory in fruit flies and in *Aplysia* and given the general observation from vertebrate work that protein synthesis is required for LTM formation, Dr. J. Yin cloned two *Drosophila* homologs of the vertebrate transcription factor, cAMP response element-binding protein (CREB). He discovered that one of these genes, *dCREB-2*, produces several different RNA isoforms, one of which (CREB-A) acts as an activator of a CRE-activated β-galactosidase reporter gene and the other one of which (CREB-B) acts as a "dominant-negative" blocker of the activator form in cell culture (data not shown). In a manner analogous to that of the mammalian CREM gene, the activator splice form differs from the blocking form by the coordinate inclusion of three exons (data not shown). Dr. Yin then generated a transgenic strain carrying an hsp70-driven CREB-B construct on a wild-type background.

We have outcrossed these CREB-B transgenic flies to "equilibrate" their genetic background with that of wild-type Canton-S flies and then have assayed 24-hour memory retention after spaced or massed training with or without a 30-minute heat shock (37°C) 3 hours before training. Figure 2 shows that 24-hour memory after spaced or massed training was normal when hsp-CREB-B was not induced. After heat-shock induction of hsp-CREB-B, 24-hour memory after massed training was normal in the CREB-B transgenic flies, but 24-hour memory after spaced training was disrupted to a level similar to that of 24-hour memory after massed training. (This "profile" of results for 24-hour memory after massed or spaced training has been replicated with a second, independently derived hsp-CREB-B transgenic strain. We also have shown that olfactory acuity, shock reactivity, and immediate learning are normal in hsp-CREB2-B transgenic flies in the absence of heat shock or 3 or 24 hours after heat shock; data not shown.) That learning and memory after massed training were normal and that memory after spaced training was reduced to a level similar to that of memory after massed training in the CREB-B transgenic flies is an outcome identical to that of CXM-fed wild-type flies, suggesting therefore that heat-shock-induced expression of the CREB dominant-negative isoform blocked the formation of LTM.

We have corroborated this conclusion in two ways: First, we have shown that 7-day memory after spaced training in induced hsp-CREB-B transgenic flies is zero, as expected if LTM is not present (cf. Fig. 1). Second, in a manner analogous to that for *radish* mutants fed CXM, we have shown that 24-hour memory after spaced training is zero (PI = -4 ± 3, N = 6) in *radish* induced hsp-CREB-B "double mutants." Third, the performance deficit in hsp-dCREB2-B transgenic flies was not due to non-specific effects of heat shock or of the hsp-driven transformation vector (which includes a "miniwhite" gene), since 24-hour memory after spaced training was normal in induced *ala1* transgenic flies carrying a dominant-negative inhibitor of Ca\(^{++}\)/CaM-kinase in a similar hsp-driven vector. (Immediate learning and 3-hour retention after one cycle of training also were normal in the *ala1* transgenic flies, suggesting that
Ca\textsuperscript{++}/CaM-kinase is not involved with olfactory learning in flies; data not shown.)

Such phenotypic analyses of memory formation in \textit{radish}, transgenic \textit{hsp-CREB-B} flies, and transgenic \textit{ala1} flies and past work on \textit{amnesiac}, \textit{dunce}, and \textit{rutabaga} (Tully and Gold 1993), \textit{latheo} (Boynton and Tully 1992), and \textit{linotte} (Dura et al. 1993) have yielded a working model of memory formation after Pavlovian conditioning (Fig. 3). Experience-dependent information is acquired via associative learning (LRN) and then is processed through as many as three memory phases—short-term memory (STM), middle-term memory (MTM), and anesthesia-resistant memory (ARM)—on its way to becoming fully consolidated as long-term memory (LTM). We have shown herein that (1) \textit{radish} blocks the formation (or expression) of ARM, leaving LTM intact, and (2) CXM or the CREB-B blocker disrupts the formation (or expression) of LTM, leaving ARM intact. We have argued previously that \textit{amnesiac} disrupted MTM (Tully et al., Cold Spring Harbor Symp. Quant. Biol. 55: 203 [1990]), and we have shown that such a disruption affects both ARM and LTM (data not shown). Thus, MTM most likely is involved with the induction of both ARM and LTM. Comparisons of the overall memory retention curves of \textit{dunce} and \textit{rutabaga} with those of \textit{amnesiac}, \textit{latheo}, or \textit{linotte} suggest that these mutations first disrupt STM. Finally, since initial learning levels are low, but the memory decay rates of \textit{latheo} and \textit{linotte} mutants appear to be normal, these mutations most likely disrupt the initial acquisition of information.

This experimental and conceptual approach toward a genetic dissection of learning and memory formation is unique. We have developed all the behavioral assays and continue to test and to modify our working model. Our discovery that LTM in \textit{radish} mutants forms normally in the absence of ARM represents a fundamental contribution to the psychology of memory. Behavior-pharmacological analyses of memory formation in vertebrates led to the belief that ARM represented the earliest appearance of LTM. We have shown instead that ARM and LTM are functionally independent and genetically distinct. Our discovery that a dominant-negative form of CREB blocks LTM is the first demonstration in any intact animal that transcription factor regulation of gene expression is involved in memory formation. Germane to our model of memory formation is the fact that such regulation of gene expression itself must be induced by earlier learning/memory processes. We believe that further understanding of the molecular biology of extant and yet to be discovered "upstream" genes promises to shed light on some anatomical or biochemical aspects of these early inductive events leading to the formation of LTM. In addition, we now will be able to bring the full power of \textit{Drosophila} molecular genetics to bear on identifying "downstream" CREB-regulated genes specifically involved with the formation of LTM.

\textbf{Molecular Genetics of \textit{latheo}, \textit{linotte}, \textit{nalyot}, \textit{golovan}, and \textit{dnOS}}


\textbf{latheo AND linotte}

We have cloned and sequenced cDNAs corresponding to the putative messages for \textit{latheo} and \textit{linotte}. In both cases, deduced amino acid sequence analyses have revealed no convincing homologies with other known proteins. To establish beyond doubt that we have identified the correct messages, we have generated germ-line transformants carrying \textit{hsp70} promoter-driven \textit{lat\textsuperscript{+}} or \textit{lio\textsuperscript{+}} transgenes. Currently, we are breeding strains that carry these transgenes on \textit{lat} or \textit{lio} mutant backgrounds to determine whether the transgenes rescue the mutant learning deficits. With such knowledge in hand, we will continue in earnest the biological detective work necessary to understand how the Lat and Lio proteins function.

Phenogenetic analyses of \textit{latheo} mutants have provided some clues to the nature of this gene. We

FIGURE 3 Working model of information flow from initial Pavlovian olfactory learning (LRN) through short-term memory (STM), middle-term memory (MTM), and anesthesia-resistant memory (ARM) or long-term memory (LTM). Putative blocks in the pathway by mutations in different learning/memory genes also are shown. In particular, we have shown that the protein synthesis inhibitor cycloheximide (CXM) or a heat-shock-induced CREB blocker (CREB-B) disrupts LTM, leaving ARM intact, whereas the radish mutation disrupts ARM, leaving LTM intact.
have shown that excision of the lat^{P1} P element results about 10% of the time in homozygous-lethal mutations. Using the lethal excision alleles, we then were able to identify one lethal complementation group to which all of the lethal lat excisions mapped and to which three independently isolated EMS-induced lethal mutations mapped. Finally, we showed by assaying memory retention in adult-viable lat^{P1}/lethal genotypes that the behavioral deficits associated with the original lat^{P1} P-insert mutation also co-mapped to the same complementation group (Boynton and Tully, Genetics 131: 655 [1992]).

We also determined that homozygous-lethal latheo genotypes died during the pupal stage of development, thereby prompting us to look at larval anatomy just before this lethal period. We discovered that late third instar mutant larvae had no imaginal discs and underdeveloped central nervous systems (CNSs). Imaginal discs and CNSs from second instar mutants were normal, however. Apparently, the third instar defect was caused by a failure of cell proliferation, as determined from bromodeoxyuridine fluorescent labeling studies. Interestingly, we did see some disorganized imaginal disc structures and abnormal cell proliferation patterns in mutants homozygous for one (apparently hypomorphic) lethal excision allele. Immunocytochemical analysis using anti-ELAV antibodies (which label all terminally differentiated neurons in the Drosophila CNS and PNS) revealed an overabundance of ELAV+ cells in these mutant CNSs. Taken together, these developmental studies suggest that (1) latheo may be involved with the differentiation of "adult-specific" cells during late third instar and (2) a failure of cell division in late third instar mutants may represent the null (neural) phenotype of latheo.

These developmental results also prompted us to take a closer look at neuroanatomical structures in the adult brains of viable latheo mutants (cf. Boynton and Tully, Genetics 131: 655 [1992]). Under the supervision of Dr. M. Heisenberg at his laboratory in Würzburg, we quantified the neuropilar volumes of the mushroom body, the central complex, and the medulla from frontal sections of adult heads using a "planimetric" method with serial 7 μm head sections from adult-viable latheo genotypes (which show the behavioral deficits). We detected a 20% (significant) reduction in the mean volume of the mushroom body but normal central complex and medulla neuropil in learning-defective lat^{P1}/lat^{P1} mutants, compared to learning-normal lat^{P1}/+ flies. These mean relative volumes were 80% reduced in mushroom body, 20% reduced in central complex, and normal in medulla of sluggish, behavior-defective lat^{P1}/Df(1)vg56 mutants (cf. Boynton and Tully, Genetics 131: 655 [1992]).

These anatomical data run parallel to our behavioral data (cf. Boynton and Tully, Genetics 131: 655 [1992]). In general, the more severely mutant genotype produced more widespread anatomical defects. Consideration of the specific structures, however, is even more informative. Mushroom body is an anatomical structure involved with olfactory learning in bees and in fruit flies. Learning-defective lat^{P1}/lat^{P1} mutants only show anatomical defects in the mushroom body. Thus, the correspondence of behavioral defects and anatomical defects in latheo variants is consistent with the known functions of the affected brain structures and with known anatomical defects in other learning- or behavior-defective mutants.

Analysis of the deduced amino acid sequence of the putative linotte message using BLASTA has revealed a weak homology (25% identical, 40% conserved) over a 130-amino-acid domain in the carboxy-terminal half the rutabaga adenyl cyclase. Interestingly, this region of the rutabaga cyclase presumably resides on the cytoplasmic side of the cell membrane and shares no sequence homology with vertebrate adenyl cyclases (the common functional domains of cyclase are in the amino-terminal half of the rutabaga cyclase). On the basis of this sequence homology, we have bred a rut^{1}; lio double mutant. Such double mutants of two inessential genes are themselves viable and yield a Pavlovian learning score of 16 ± 4 (N = 4), suggesting that the rutabaga and linotte genes lie in different "learning" pathways. This result has prompted the breeding of a series of double-mutant combinations with linotte and other extant learning/memory mutants to generate additional data in support of the "two pathway" notion.

nalyot AND golovan

We most likely have identified two additional learning/memory genes, which we have named nalyot and golovan from our P-element mutagenesis initiated at Brandeis in 1987. The original P-insert mutants for each gene have been outcrossed to a wild-type genetic background for more than ten generations in a manner that allows recombination around the sight of the P insert. Such outcrossed mutants, homozygous for either the nalyot or golovan P inserts, still yield significantly lower 3-hour retention scores, indicating
beyond reasonable doubt that the P inserts are responsible for the defective behaviors. Mutant nalyot and golovan flies also show normal olfactory acuity and shock reactivity; thus, the performance deficits in the Pavlovian experiments most likely result from defects in associative learning/memory. Finally, we have mapped each P insert to cytologically distinct locations using the P-element sequence as a probe, and we have begun molecular cloning of nalyot (lack of funds prevents us from pursuing golovan).

With these new discoveries, we now have identified four new autosomal genes (latheo, linotte, nalyot, and golovan) from a total of 2200 P-element transposant lines, yielding an empirical estimate for a mutation frequency of 1/550, compared to a mutation frequency of 1/500 from the EMS mutageneses of the Benzer and Quinn groups (W.G. Quinn, pers. comm.). This observation shows clearly that our behavioral screen for new genes involved with associative learning and memory is efficient.

dNOS

We have cloned the Drosophila homolog of the vertebrate nitric oxide synthase (NOS), which also is the first demonstration of a NOS in any invertebrate. Conservation of the protein sequence and of the splicing pattern suggests that we can expect broad similarities in NO biochemical pathways between vertebrates and invertebrates. Knowing the homologies in properties of learning and memory between vertebrates and Drosophila, we intend to apply the genetic tools available in fruit flies to an investigation of the role of dNOS in learning and memory.

The dNOS message contains one long open reading frame (ORF) of 4350 bp encoding a protein of 1350 amino acids. This conceptual Drosophila protein shows a significant degree of amino acid sequence homology with the vertebrate NOS proteins: It is 38% identical to rat brain NOS, 36% identical to bovine endothelial NOS, and 28% identical to mouse macrophage NOS. The Drosophila sequence contains all of the known functional domains of vertebrate NOS: heme-, calmodulin-, FMN-, FAD-, NADPH-binding. In the NADPH-binding domain, all amino acids that have been shown to be contact points with the dinucleotide in crystals of ferrodoxin NADP+ reductase are perfectly conserved in the putative Drosophila protein. Moreover, the region between the heme- and calmodulin-binding sites is 70% identical between rat and Drosophila proteins and is likely involved with binding of arginine and possibly cofactor tetrahydrobioppterine.

Computer searches of protein sequence databases with the deduced amino acid sequence of dNOS revealed significant homologies only with other NOS proteins and CPR. There were limited sequence homologies with other nucleotide-binding proteins, but such were restricted to those domains containing a particular binding site. Comparisons of dNOS with individual vertebrate NOS isoforms have revealed that (1) the Drosophila protein does not contain a myristylation site close to the amino terminus, which is present in the endothelial NOS, (2) the fly protein does not seem to have a deletion in the middle part of a protein as is the case for macrophage NOS, (3) DNOS contains a PKA consensus site that is absent from the macrophage enzyme but present in brain and endothelial forms, and (4) dNOS is expressed predominantly in the head, which is analogous to the vertebrate brain NOS.

There are at least two splice forms of brain NOS transcripts in mice. One has a 315-bp fragment spliced out that results in a protein 105 amino acids shorter than the other. The deletion occurs in a region that is highly conserved among different NOS genes in vertebrates and likely contains the arginine-binding site. It is not known at present whether the shorter protein has NOS activity or what function it could play. This region is very well conserved in dNOS: It is 70% identical to the rat brain isoform, 67% to the macrophage isoform, and 65% to the endothelial isoform. We have used RT-PCR technology and appropriate primers against Drosophila head mRNA to test if a similar shorter isoform is present in fruit flies. We have obtained two DNA fragments: one of the size of the unspliced form and another of expected size similar to the shorter spliced form. Sequence data revealed that the shorter fragment originates from Drosophila mRNA and would be produced if the splicing pattern in fruit flies was identical to that in mice, i.e., it has a 315-bp in-frame deletion.

Behavioral Properties of Learning and Memory in Normal and Mutant Flies

N. Arora, S. Knabe, Z. Asztalos, C. Jones, T. Tully

In the past, we have described initial results from a new procedure (and apparatus) that we developed to
assay habituation of the jump reflex to olfactory cues (cf. Boynton and Tully, Genetics 131: 655 [1992]). This year, we have succeeded in developing the complementary behavioral assay: sensitization of the jump reflex. In the habituation experiments, flies were dishabituated by strong mechanical vibration in a vortexer. In our new sensitization experiments, we have used this same strong stimulus on naive flies to induce a sensitized response to odors. Initially, this has allowed us to compare dishabituation and sensitization induced by the same stimulus. Preliminary results suggest that vortexing naive flies produces sensitization, whereas vortexing habituated flies produces dishabituation rather than (or in addition to) sensitization. With assays for both forms of nonassociative learning (habituation and sensitization) in hand—both using the odor-induced jump reflex—quantitative comparisons among normal and mutant flies now are feasible. Moreover, we may be able to use electric shock as a sensitizing stimulus, thereby allowing us to compare nonassociative forms of learning with the same stimuli used in our Pavlovian (associative) learning experiments. These three basic learning tasks and their associated controls represent an extremely powerful repertoire of integrated behavioral assays with which to accomplish a genetic dissection of behavioral properties of learning and memory.

To this end, we continue to study the genetic dissection of associative and nonassociative learning by latheo mutations. Combined with our previous work showing that habituation and the US pre-exposure effect were normal in latheo mutants, our discovery of defective mushroom body brain structures in latheo mutants suggests that conditioned odor avoidance (associative) and habituation to olfactory cues (nonassociative) involve different anatomical regions of the fly brain. We currently are evaluating this possible example of anatomical dissection.

We also continue to develop separate assays for conditioned excitation and conditioned inhibition of odor avoidance responses. At the moment, we have two candidate learning/memory mutants showing defective conditioned inhibition. More thorough analyses of these and other mutants promise to yield biochemical and/or anatomical distinctions among these fundamental forms of associative learning.

**PUBLICATIONS**


In Press


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**REGULATION OF NEUROTRANSMITTER EXPRESSION AND ITS IMPLICATIONS IN NEURAL PLASTICITY**

H. Nawa K. Mizuno R. Lundsten M. Waga

Neurons communicate with each other using a large variety of neurotransmitters and neuropeptides. Despite this enormous diversity, each functional neural pathway can be recognized by the particular set of transmitters, neuropeptides, and receptors employed. Clearly, this "chemical coding" of pathways is critical for determining neural function and behavior. The long-term objective of our laboratory is to define the molecular and cellular mechanisms that regulate the synthesis and release of neurotransmitters and neuropeptides in the nervous system. In particular, we are interested in intercellular protein factors such as cytokines and growth factors, which can regulate the synthesis and release. The alteration
in neurotransmitter/peptide expression and release presumably changes the mode of neurotransmission at each neuron and consequently modulates neural functions such as cognition and memory.

We have identified multiple neuronal differentiation factors in heart-cell conditioned medium (CM), which control neurotransmitter/peptide expression in peripheral neurons. Our previous observations suggest the possibility that many distinct, diffusible factors can influence the development of neurotransmitter/peptide phenotypes in the peripheral nervous system. Recently, we found that the heart cell CM also has effects on neurotransmitter/peptide levels in cultured neurons prepared from the central nervous system (CNS). Subsequent biochemical analyses suggest that the responsible component resembles brain-derived neurotrophic factor (BDNF), which belongs to the nerve growth factor family, named neurotrophin. BDNF increases and maintains the expression of putative inhibitory neuropeptides, neuropeptide Y, and somatostatin in cultured cerebral cortical neurons. Our finding demonstrates that, as in the peripheral nervous system, neurotransmitter phenotypes in the CNS are plastic and can be regulated by various diffusible protein factors.

Our current efforts have been focused on the four projects: (1) regulation of BDNF protein production in nerve and muscle, (2) characterization of neurotransmitter/peptide differentiation activity of neurotrophins in vivo, (3) physiological consequences of regulated neurotransmitter production, and (4) identification of novel neurotransmitter differentiation factors. These studies will elucidate the mechanism of how such a complex but highly ordered expression of neurotransmitters/peptides is controlled during development and how it contributes to neural plasticity such as learning and memory processes.

Effects of BDNF on Neuropeptide Expression in Adult Rat Brain

H. Nawa, M. Waga

Hippocampal neuropeptides are known to be altered after brain seizure induction. BDNF mRNA is increased following seizure but with an earlier time course than that of the neuropeptides. Because the increase in BDNF mRNA precedes the neuropeptide changes after seizure, it is possible that BDNF mediates the peptide alterations. To test this hypothesis directly, BDNF was continuously infused into the hippocampus of adult rats from an osmotic pump. To test the regional specificity of any observed effects of BDNF administration on neuropeptide ex-
pression, infusions into the neocortex and striatum were also studied. To control for the specificity of BDNF, NGF and vehicle were also infused into different animals. Peptide and mRNA alterations were assessed by Northern analysis, immunohistochemistry, and radioimmunoassay. BDNF elevated peptide and mRNA levels for neuropeptide Y and cholecystokinin in hippocampus and neocortex, and somatostatin in cortex. BDNF increased mRNA only for neuropeptide Y and cholecystokinin in striatum. In contrast, BDNF decreased dynorphin peptide and mRNA in hippocampus. NGF’s effects were limited to mRNA increases, without corresponding peptide changes, for neuropeptide Y in hippocampus and striatum, substance P in neocortex, and cholecystokinin in striatum. The distinct and more limited effects of NGF infusion demonstrate that BDNF’s effects are not nonspecific results of protein infusion into the brain. These findings indicate that BDNF may have a regionally specific role in modulating neuropeptide expression in the adult brain as well as in various pathophysiological states.

Influence of Neurotrophins on Development of Striatal GABAergic Neurons

K. Mizuno, H. Nawa

Striatal neurons are derived from a developmental lineage different from that of neocortical neurons. In our previous study, all neurotrophins failed to alter GABA content in cultured neocortical neurons. However, we have found that neurotrophins can influence GABAergic properties of the striatal neurons both in vivo and in vitro. Among neurotrophins, BDNF and neurotrophin-5 (NT-5) specifically elevated cellular GABA content in striatal culture without altering neuronal survival. An increase in GABA content in the striatum was also observed following BDNF injections into the cerebroventricle of neonatal rats. The increase of GABA levels in culture resulted from a ninefold increase in holo-enzyme activity of the GABA synthetic enzyme glutamic acid decarboxylase (GAD) and a threefold increase in GABA uptake activity. In BDNF-treated striatal cultures, the newly differentiated neurons extended elaborate neurites and exhibited strong GAD immunoreactivity. These alterations were reflected by the up-regulation of mRNA encoding GAD_{67} and the neuronal GABA transporter GAT-1. BDNF treatment also promoted other phenotypic differentiations of striatal neurons: BDNF increased the frequency of parvalbumin-immunoreactive neurons and calbindin-immunoreactive neurons and neuropeptide content of neuropeptide Y and somatostatin. These observations suggest that BDNF may contribute to phenotypic differentiation of GABAergic neurons in the developing striatum.

Production and Secretion of BDNF Protein

M. Waga, R. Lundsten, H. Nawa

Despite numerous previous studies on BDNF mRNA, regulation of the production and secretion of BDNF protein has been poorly understood because of a lack of its quantitative measurement. Recently, we attempted to establish an ultrasensitive enzyme-linked immunosorbent assay (ELISA) for BDNF protein. The ELISA system enables us to measure trace amounts of BDNF protein in tissues, cells, blood, and cerebrospinal fluid. Using the ELISA system, we address the question of how BDNF protein production and secretion are regulated.

We purified anti-BDNF antibodies with a BDNF-linked affinity column; half were biotinylated with NHS-Biotin. Nonlabeled anti-BDNF antibody was coated on a 96-well microtiter plate for ELISA, and the biotinylated antibody was used for the detection of BDNF in combination with avidin-labeled peroxidase. Determination of optimal concentrations of the antibodies decreased the sensitivity of ELISA less than 3 pg/sample. This ELISA system exhibits no apparent immunocross-reactivity to other neurotrophins (i.e., NGF, neurotrophin-3, and -5) nor other cytokines (i.e., basic fibroblast growth factor, leukemia inhibitory factor, tumor growth factor, interleukin 1β, stem cell factor, CNTF, insulin, and activin). BDNF content was measured in various brain regions of adult rats. The highest concentration was found in the hippocampus. The striatum and septal area contained lower levels of BDNF protein. This observation at the protein level is quite consistent with the reported regional distribution of BDNF mRNA, suggesting the accuracy of the measurement. The BDNF level (20 ng/g tissue) is ten times higher than the NGF levels (2 ng/g tissue) in the hippocampus (Korsching et al., EMBO J. 4: 1389 [1985]), although Maisonpierre et
al. (Neuron 5: 501 [1990]) reported almost equal mRNA levels for NGF and BDNF in the hippocampus. Controversial observations suggest that production of each neurotrophin might also be regulated at posttranslational levels.

**PUBLICATIONS**


In Press


**THE BIOLOGY OF LEARNING**

A. Silva R. Burchuladze K. Peter Giese
B. Frenguelli Z. Wang
C.-M. Chen

The study of learning and memory is at a crossroads: Much information is available on potentially relevant behavioral, anatomical, physiological, and molecular phenomena; still, the functional connections among these findings remain illusive. Thus, we have developed a new strategy to integrate information pertinent to learning and memory. The essence of this approach is the combined biochemical, physiological, anatomical, and behavioral study of genetically altered mice. The goal is to determine how mechanisms at all of these levels of analysis interact to process and retrieve information.

In the past, much attention has focused on long-term potentiation (LTP), a long-lasting increase in synaptic strength, as a mechanism of memory. At the center of this hypothesis is the idea that memories are stored as patterns of strengthened synapses in the brain. However, little is known about the mechanisms that determine what and how quickly we learn. Perhaps other synaptic mechanisms, such as short-lived changes in synaptic strength, are involved in the filtering and processing of information to be stored. Indeed, in the last year, we found evidence consistent with this idea.

Our electrophysiological studies identified a novel synaptic mechanism involving the α-calcium-calmodulin kinase II (αCaMKII): Depending on the frequency and intensity of activation, this kinase can both enhance and dampen the potentiation of neurotransmitter release. Mice heterozygous for a targeted mutation of the αCaMKII gene lack this fine-tuning mechanism. Strikingly, behavioral studies of these mice showed that they can learn but that they do it slower than normal controls. In contrast, our previous studies of mice with impaired LTP showed that they were simply unable to learn.
αCAMKII: A BIDIRECTIONAL MODULATOR OF PRESYNAPTIC PLASTICITY

Synaptic weight changes are at the core of information processing and storage in neural networks. However, little is known about the biochemical machinery that mediates these changes. Previous studies showed that the α isoform of αCaMKII is required for LTP of synapses in the CA1 region of the hippocampus. We have found in the last year that this kinase also has a crucial role in hippocampal short-term plasticity. Field and whole-cell recordings in the CA1 region of mutants and controls indicate that the αCaMKII has the surprising capacity to either potentiate or depress synaptic function depending on the pattern of neuronal activation.

In the studies described below, we used mice both homozygous and heterozygous for a targeted disruption of the αCaMKII gene (henceforth referred to simply as homozygotes and heterozygotes). Biochemical analysis of the homozygotes showed that they completely lacked the αCaMKII protein and that forebrain homogenates from these mutants had half of the total Ca++-calmodulin-induced kinase function measured in vitro. Western analysis showed that in the heterozygotes, the amount of αCaMKII protein isolated from forebrain synaptosomes is roughly half of that in control littermates (Fig. 1). Similar results were obtained with hippocampal protein isolated from heterozygotes and controls. Consistent with the loss of half of the αCaMKII in heterozygous mice, in vitro kinase assays with hippocampal homogenates revealed a proportional decrease (mean ± s.d. of 71% + 7.8%; n = 9) in the amount of kinase activity. These protein studies were done with Alan Smith, a visiting scientist in the laboratory.

To determine how the decrease in αCaMKII affected synaptic function, Paul Chapman, another visiting scientist in our laboratory, started by studying paired-pulse facilitation (PPF) in the CA1 region of hippocampal slices maintained in vitro. PPF is a presynaptic facilitation revealed by the second of a pair of pulses. Figure 2A shows that heterozygous fields, as well as homozygotes, have smaller PPF than controls at all interpulse intervals tested. Changes in synaptic inhibition could explain the blunting of PPF in the mutants, a possibility that we excluded by finding consistently depressed PPF in recordings of mutant slices done in the presence of γ-amino-butyric-acid (GABA) channel inhibitors of the A and B types.

Whole-cell recordings of CA1 cells, carried out by Bruno Frenguelli, from heterozygotes and controls confirmed these results. Thus, changes in membrane potential in the mutants could not account for their depressed PPF, since these recordings were done under voltage clamp. Furthermore, our whole-cell recordings mentioned above were also done under conditions known to shut off all GABA-mediated inhibition. Altogether, these data strongly suggest that the depression of PPF in the mutants is due to lower neurotransmitter release in response to the second pulse of the stimulus pair. Remarkably, this decrease revealed during PPF does not appear to be associated with a general blunting of synaptic transmission, because a range of stimulus intensities elicits similar responses in heterozygotes, homozygotes, and controls. These results show that the partial or complete loss of αCaMKII does not shut off synaptic function.

Consistent with the observation that thiophosphorylated CaMKII increases synaptic release in the squid giant synapse, the loss of αCaMKII led to a decrease in synaptic release during PPF in the CA1 region of the hippocampus. This is evidence that αCaMKII normally enhances short-term increases in synaptic weights, perhaps by the phosphorylation of synapsin I, a negative regulator of synaptic transmission. Interestingly, the loss of synapsin I, the most abundant substrate for CaMKII, presynaptically leads to an enhancement in PPF.

Since the loss of αCaMKII could have also affected other short-lived enhancements of synaptic ef-
A) Paired-Pulse-Facilitation

**FIGURE 2** Paired-pulse facilitation is blunted in αCaMKII mutants, but augmentation is enhanced. (A) Percent potentiation (slope of the second response divided by the slope of the first response x 100) measured at interpulse intervals of 50, 100, 150, and 250 msec was significantly different between heterozygous mutants and wild-type controls (overall mean + S.E.M.) = 147 ± 4 (wild type), 129 ± 2 (heterozygotes); t = 4.745, df = 135, p < 0.001. (B) Average enhancement of synaptic responses produced by 10-burst theta stimulation for homozygotes (n = 4) and heterozygotes (n = 8) was significantly higher than wild-type controls (n = 8), both in the first second after tetanus (255 ± 2.5%, 217 ± 12%, and 147 ± 5%, respectively: F[2,17] = 18.01, p < .0001) and averaged over all 15 stimuli at 1 Hz (163 ± 5%, 160 ± 3%, and 128 ± 1%; F [2,17] = 11.23, p < .001).

B) Augmentation

ficacy, we studied augmentation, a potentiation of neurotransmitter release detected in the first 10–15 seconds following a tetanus. The tetanus used consisted of a total of ten high-frequency bursts of four stimuli delivered at the theta rhythm (5 Hz), a pattern of hippocampal electrophysiological activity predominant during exploration and during other behaviors correlated with learning in rodents. We avoided the potentially confounding superposition of LTP on measurements of augmentation by using a pharmacological blocker of LTP induction in CA1. Unexpectedly, the degree of augmentation induced by the tetanus in homozygous and heterozygous CA1 fields was significantly greater (p < .001) than in controls (Fig. 2B). As little as eight pulses delivered at the theta frequency were sufficient to reveal a difference (p < .001) between the responses of mutants and controls, even though with shorter tetani, the levels of augmentation in mutants and controls were smaller. The enhancement (p < 0.001) of augmentation in the mutants was also seen with longer tetani (80 pulses). This shows that the enhanced posttetanic responses of mutants are expressed throughout widely different presynaptic activation levels.

Even in the absence of GABA-mediated inhibition, augmentation in the heterozygotes was greater than that in the controls. To determine whether the enhancement in augmentation in the mutants was not due to postsynaptic changes in membrane potential, Bruno confirmed these results with whole-cell recordings under voltage clamp. Again, the size of responses immediately after the tetanus were greater in mutants than in controls. Contrary to the predictions of current models, the partial or complete loss of αCaMKII does not reduce all synaptic transmission. Instead, the absence of this kinase resulted in greater augmentation, implying that it normally depresses synaptic responses to tetanic stimulation.

Previous experiments showed that CaMKII activity can enhance neurotransmitter release in the squid giant synapse. Thus, it is not surprising that the partial or complete removal of αCaMKII from axonal terminals depressed PPF in the mutant neurons. However, we did not expect to find enhanced augmentation in these cells. This shows that immediately after tetanic stimulation, the αCaMKII does not promote synaptic release as it seems to do during PPF; instead, it inhibits synaptic responses. The high-calcium conditions induced by a tetanus sharply increase the autophosphorylation of this enzyme. Since the autophosphorylated kinase is an efficient trap for Ca++/calmodulin (Ca++/CaM) during and after high-frequency stimulation, this kinase could serve as a presynaptic calmodulin sink. This likely sequestering of Ca++/CaM by the autophosphorylated kinase could lead to the down-regulation of Ca++/CaM-dependent processes required for neurotransmitter release.
IMPAIRED LEARNING IN MICE HETEROZYGOUS FOR THE αCAMKII

Studies of the physiological basis of learning have focused on long-term synaptic plasticity. However, short-lived changes in synaptic strength might also have a role in learning. Consistent with this hypothesis, mice with a heterozygous targeted mutation of the αCaMKII have abnormal hippocampal short-term plasticity, and they are impaired in two distinct hippocampally dependent learning tasks: the spatial version of the water maze and contextual conditioning. Importantly, other aspects of hippocampal physiology, such as LTP and performance in hippocampally independent learning tasks, appeared much less affected in the mutant mice. Unlike the homozygous αCaMKII mutants, which have abnormal LTP, the heterozygotes seem to master the hippocampus-dependent tasks with additional training, suggesting that deficits in short-lived plasticity do not prevent learning, as those in long-term plasticity seem to do, but only slow it down.

Mice heterozygous for the αCaMKII have half of the kinase present in control littermates. Field and whole-cell recordings in the CA1 region of the hippocampus showed that these mutants have increased augmentation and decreased PPF, two forms of short-lived presynaptic plasticity (see above). Since αCaMKII homozygotes have a profound LTP impairment in the hippocampus, we also studied LTP in the heterozygotes. Our studies show that LTP in the heterozygotes, induced with a variety of tetanic parameters, is indistinguishable from that of control littermates (Table 1). In conclusion, the αCaMKII heterozygote mutants appear to have normal LTP but abnormal short-term plasticity.

To determine the impact of deficits of short-term plasticity in learning and memory, Diana Cioffi, an undergraduate student in the laboratory, and Rusiko Burchuladze started by studying the mice in the water maze tasks. In this test, animals are placed in a round pool, and they escape the water by learning to find a platform. In the visible platform version of this task, the location of the platform is marked, and the mice simply have to learn to swim toward it. Figure 3A shows that the performance of the heterozygotes in this version, which does not require hippocampal function, is indistinguishable from that of control littermates, suggesting that motivation to escape the water, vision, and motor coordination are normal in the heterozygotes.

Next, we tested a different group of mice in the "spatial" version of the water maze. In this test, the mice have to learn the position of a submerged platform relative to distal objects in the pool room. Strikingly, in this version requiring hippocampal function, the performance of the heterozygotes is inferior to that of controls (Fig. 3B). The heterozygotes take considerably longer to locate the submerged platform. This result was confirmed by a conventional probe trial, in which after 3 days of training, the platform was removed, and the mice were allowed to search for it for 60 seconds. The controls searched selectively for the absent platform, spending 53% of their time swimming in the quadrant of the pool where the platform had been during training, whereas the heterozygotes searched randomly in all four quadrants of the pool (Fig. 3C).

In a second probe trial after two more days of training (Fig. 3C), we found that the heterozygotes learned to search selectively for the absent platform. In contrast, the homozygous still did not search selectively for the platform, suggesting that they cannot learn the spatial position of the platform. Similar results were obtained using other assessments of search selectivity, such as number of platform-site crossings and the "Galagher measure," a platform-proximity measure. However, despite normal swimming speeds, the heterozygotes take longer than controls to reach the platform, showing that their trajectories are less direct than controls.
A) Wild-type controls (n = 10) and αCaMKII heterozygotes (n = 10) were trained to navigate to a visible platform. Analysis of variance of all trials revealed no significant difference between groups. (B) Performance of mice in the hidden platform version of the water maze. Wild-type controls (n = 12) and αCaMKII heterozygotes (n = 15) were trained to find a hidden platform located in a fixed position of the pool. (C) Probe trials without the platform given at the end of the third and fifth days of training, respectively. (T) Training; (AR) adjacent right; (AL) adjacent left; (OP) opposite quadrants.

FIGURE 3 Water maze tests. Even with hippocampal lesions, rodents can learn the spatial version of the water maze after extended training. However, unlike control subjects, hippocampally lesioned animals are profoundly impaired in relearning tests. These tests measure the ability of trained animals to quickly learn to find the hidden platform in a new position. Thus, it is possible that the hippocampal short-term plasticity deficits of the heterozygotes have the same impact on spatial learning as hippocampal lesions. To investigate this hypothesis, we tested the heterozygotes in a relearning experiment. Both heterozygotes and controls learned quickly to find the platform in a new location, a result confirmed with a probe trial: The searches of both heterozygotes and controls were selective for the platform’s new position. Together, the evidence presented demonstrates that the heterozygotes differ from the αCaMKII homozygotes, which have LTP deficits, and from animals with hippocampal lesions. Strikingly, heterozygotes learned slower than controls to find the hidden platform.

To extend these findings, Rusiko Burchuladze and Zachary Marowitz, an undergraduate in the laboratory, tested a new group of mice on another very different task known to require hippocampal function: contextual conditioning. In this test, ani-
FIGURE 4 Contextual conditioning in αCaMKII heterozygotes and wild-type mice. (A) Mice (n = 10 in each group) were given a single training trial consisting of an aversive stimulus delivered to their paws. Twenty-four hours after training, contextual conditioning was assessed by measuring how long the mice remained immobile in the same environment in which they were trained. After extended training (five training trials), the mice learned to recognize the environment in which they were trained as well as controls. Both mutants and controls remained immobile for the same amount of time.

mals were aversively conditioned to a specific environment or context. Figure 4A shows that after a single trial, controls are clearly conditioned since they spent 40 + 5% of a 5-minute testing interval without any perceptible movement (freezing), whereas the heterozygotes showed little freezing (6 + 4%). Omitting the sound during training, using another aversive stimulus, pre-exposing the mice to the conditioning environment for 15 minutes the day before training, or testing them 1 hour after training failed to reveal any contextual freezing in the heterozygotes. However, the heterozygotes did freeze (43 + 6%) when exposed to the sound, demonstrating that they can be conditioned and that conditioning can be studied in these mice by measuring freezing. Even conditioning to sound, which is known to be hippocampus-independent, seemed lower in heterozygotes. This could be due to their threefold higher levels of activity as reported by an open-field test. Alternatively, other brain regions, such as the amygdala, might also be affected by the loss of half of the αCaMKII in heterozygotes. Nevertheless, the heterozygotes were once again profoundly impaired in an hippocampus-dependent task, whereas in the version not requiring hippocampal function, they were not.

Next, we tested whether similarly to the water maze, extended training in this test could improve the performance of the heterozygotes. Figure 4B shows that with five trials, with 1-minute intertrial time, the heterozygotes showed contextual conditioning (48 + 6%). To investigate the specificity of this response, we trained another group of heterozygotes and controls in one cage and tested them on a different context (a different cage). In the novel context, both groups of mice showed little freezing, attesting to the specificity of the contextual freezing responses. Unlike heterozygotes, homozygotes did not show any contextual conditioning even after five trials, confirming the profound hippocampal impairment previously detected with water maze studies.

This work shows that despite abnormal short-term plasticity in the CA1 region of the hippocampus, mice heterozygous for the αCaMKII mutation appear to have normal LTP there. These results suggest a connection between learning and short-term plasticity. However, it is important to note that we have not yet checked LTP in other regions. Thus, it is still possible that deficits in LTP are underlying the slow learning of the mutants. To address this problem, we are currently testing LTP in other regions, and we are also testing another mutant mouse with apparently normal LTP but abnormal short-term plasticity.

CONCLUDING REMARKS

The studies described above are just the beginning of a research program designed to use modern genetic approaches to integrate information about molecules, cells, circuits, and behavior. It is easy to see how the suggestive richness of these results will continue to generate testable models about how physiology conditions learning. For example, the presence of this kinase presynaptically and its uncanny ability to be modified by interactions with Ca^{2+} could be at the heart of the surprising presynaptic responses observed. Either its putative role in freeing synaptic vesicles trapped in the cytoskeleton or its function as a calmodulin trap could be underlying crucial modulations of synaptic weights responsible for the slow rate of learning in the heterozygous mutants. Thus, indirectly, the intriguing biochemistry of the
αCaMKII might be an important component of the dynamic process by which information continuously fine tunes brain circuitry.

New developments in transgenic technology allow for subtler mutations, such as those that Karl Peter Giese is carrying out in our laboratory. Amino acid mutation in the various domains of the kinase will enable us to correlate directly biochemistry with physiology and behavior. Karl has set up the technique in the laboratory and he already has germ-line transmission of embryonic stem cells.

The growing number of brain mutants will be an important resource to further explore the issues raised by these experiments. The pharmacological, electrophysiological, neuroanatomical, and behavioral analyses of these mutants should prove to be an important catalyst for a much sought-after biological theory of brain function.

**SIGNAL TRANSDUCTION IN NEURONS**

G. Enikolopov  N. Peunova  P. Krasnov

We are trying to understand the mechanisms that link long-term changes in gene expression in a neuron with long-term changes in its synaptic transmission. We have developed new tools to dissect complex signaling pathways; our particular focus is on the role of protein kinases in regulatory cascades. This year, we began experiments to design targeting vectors to deliver chimeric protein kinase inhibitors to nerve terminals and growth cones.

Recently, we have initiated studies on how gene activity in neurons is modulated by gaseous messengers, such as nitric oxide (NO) and carbon monoxide (CO), which display properties similar to those of both para- and autocrine effectors and have important roles in the function of a neuron. This year, we have extended our studies to primary cortical cells, showing that NO is an integral part of the neuron's response to glutamate. We have also shown that induction of nitric oxide synthase (NOS) acts as a switch between mitogenic and cytostatic phases of neuronal differentiation. This observation suggests that a domain of adjacent cells might develop in coordination with a signal from a small group NO-producing neurons or even a single NO-producing neuron.

**NOS Acts as a Cytostatic Agent during Neuronal Differentiation**

N. Peunova, G. Enikolopov

Developmental mechanisms employ growth arrest to define roughly the size of the cellular population that is further committed to become a distinct domain of differentiated neurons. Arrest of cell division is a prerequisite for cells to enter a program of terminal differentiation. Differentiation of neuronal cells induced by nerve growth factor (NGF) involves a mitogenic phase followed by cytostasis and, later, by manifestation of the fully differentiated phenotype. We have found that (1) NOS activity is strongly induced during NGF-mediated differentiation of neuronal PC12 cells; (2) NO can act as an antimitogenic agent in these cells, and (3) inhibition of NOS, whereas not affecting the mitogenic component of NGF activity, reverses the antiproliferative cytostatic action of NGF and thereby prevents further development of the differentiated phenotype. The crucial phase of NOS action is cell-cycle-specific, and there is a critical time window for NO action within the first 2–3 days of NGF addition.

On the basis of our experimental evidence, we propose that NO can act as a growth-arrest agent for a population of proliferating neuronal precursor cells and that it serves as a necessary prerequisite for the full implementation of the differentiated phenotype.

Our data suggest a model for NGF action where, in the first stages, NGF activates a cascade of genes, eventually leading to mitogenesis and the induction of the NOS gene. In the second stage, the accumulated NOS enzyme produces enough NO to inhibit DNA synthesis and probably alerts further checkpoints, thereby slowing down the cell cycle. Finally, when the cell processes the cytostatic signal, it starts to implement the remainder of the differentiation pro-
processes such as neurite outgrowth, which can only occur once cell division has ceased.

Our data suggest that induction of NOS is an important step in the commitment of neuronal precursors during development and that NOS serves as a growth-arrest gene during differentiation of neuronal cells, switching the differentiation program from mitogenesis to cytostasis.

Does a similar NOS-mediated mechanism for cytostasis exist in other systems (including but not limited to the nervous system)? A positive answer to this question could explain the mechanism of action of other growth factors (and systems) during terminal differentiation in which the initial mitogenic response is replaced by a cytostatic phase. In the case of the brain, this could include not only embryonal brain development, but also regeneration and neuronal differentiation in the adult brain. It also suggests that the development of a domain of neighboring cells might be coordinated under the command of, in principle, a single activated NO-producing cell. Finally, because NO is involved in synaptic plasticity, such mechanisms could provide a link between the activity of a cell and the developmental fate of it and its nearest neighbors.

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Synergism between NO and Calcium Is Part of the Cortical Neurons’ Response to Glutamate

G. Enikolopov [in collaboration with H. Nawa, Cold Spring Harbor Laboratory]

We have recently found that NO can amplify weak calcium signals in the neuronal PC12 cell line. To test the relevance of these results for the brain neurons, we have conducted experiments with primary cultures of rat cortical neurons. Two complementary approaches were used: (1) We supplemented the action of various inducers (glutamate and BayK8644 in particular) with NO to see if their actions can synergize, and (2) we specifically inhibited the action of NOS to detect its input in neuronal signal transduction. Our major finding is that the synergism between NO and calcium we had described for PC12 cells is also present in rat cortical neurons. Very importantly, this synergism is an intrinsic part of the response of the cortical neurons to glutamate. This synergistic action is dependent on calcium influx through NMDA receptors. Calcium activates NOS and the produced NO molecules amplify calcium signals, thereby introducing an additional regulatory loop in the signaling network of the overall response to glutamate. Thus, glutamate induction of gene transcription involves the participation of the NOS system as an integral part of the response. This indicates that the phenomenon, which we have observed with cultured cells and an external source on NO, has direct parallels in brain neurons, where internally produced NO acts synergistically with NMDA channel-delivered calcium.

We have made several predictions based on our data with neuronal cells: (1) The effect of NO on signaling might be particularly important at very low levels of calcium action, at which this inducer acting alone would have negligible effect; these very weak signals, which would go unnoticed by the cell, might be amplified by NO, resulting in pronounced physiological changes for the cell. (2) Since NO diffuses freely and readily leaves and enters cells without need for a secretory system or a surface receptor, nearby synapses that receive very weak impulses simultaneously with exposure to NO might establish facilitated synaptic transmission. (3) NO and Ca++ must act within a very narrow time window for this enhancement to occur, suggesting that in the nervous system, this synergistic effect might be restricted to the recently active synapses, thereby coinciding with transient elevations of calcium levels. (4) Amplification of Ca++ action by NO, acting on signaling machinery either directly or through re-programming of gene activity, might be directly involved in NO-mediated steps in LTP and similar activity-dependent neuromodulation.

Important recent data confirm our hypothesis on the NO/Ca++ synergism in the brain. It was recently shown that hippocampal LTP can spread to the nearby synapses and that this spreading is totally dependent on the production of NO by the synapse with established LTP; it also was demonstrated that addition of NO to hippocampal synapses strengthens only those synapses that are already receiving nerve impulses. The described phenomena can be explained on a biochemical level by the mechanisms that we have suggested based on our experiments with PC12 cells and cortical neurons, and we plan experiments to test whether these explanations are correct.

We will continue these experiments to study the mechanisms of NO and calcium action in more detail and also to search for the other patterns of NO action.
We are particularly interested in whether different time coincidence requirements exist for different types of cells; e.g., are there types of neurons where noncoincident application of NO and Ca++ makes neurons refractive to the action of the second agent?

**Targeted Protein Kinase Inhibitors**

P. Krasnov, G. Enikolopov

Our earlier studies introduced pseudosubstrate-based recombinant inhibitors (RPKI) as tools to study signal transduction pathways. We are now extending this approach with a goal of directing chimeric inhibitory proteins to specialized structures and specific compartments of the cell. Our aim with respect to signal transduction in neurons is to manipulate the events in the terminals without affecting the processes in the nucleus and the soma. Potentially, this could be achieved by targeting recombinant inhibitors to the nerve terminals as parts of larger chimeric proteins containing domains of synaptic vesicle proteins (synaptotagmins, synaptophysins), of the GAP43 protein, or of other proteins that tend to accumulate in the nerve terminals and growth cones. As a first attempt to develop a targeting "protein vector," we tested the potential of synaptic vesicle proteins to be transported to the terminals after the transfection of constructs into cultured neuronal cells. We have achieved best results with constructs containing rat synaptotagmin II, fused to the influenza virus HA epitope tag (to permit visualization by immunofluorescence in situ and on Western blots) and an inhibitory pseudosubstrate peptide. We have shown that chimeric molecules accumulate in the growth cones of the neurites, with some fluorescence being present in the cell body and almost no signal in the nucleus. We are now testing whether, coordinate with the location, the activity of the inhibitors is also restricted to specific cellular compartments.

We will continue these experiments to (1) localize the sequences in the synaptotagmin that are sufficient for targeting the attached domains to the terminals and growth cones; (2) test whether the activity of the inhibitors is indeed restricted to the targeted compartments of the neuronal cells; and (3) test other components of synaptic vesicles and GAP43 for the possibility of targeting foreign polypeptides. We want to narrow down the targeting sequence, to test their potential as universal targeting vehicles (for RPKI as well as for any other potentially interfering protein domains) and to use them to introduce fine perturbation in the processes in the terminal without affecting the rest of the cell compartments.

**PUBLICATIONS**


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**GENETIC ANALYSIS OF CELLULAR MECHANISMS OF LEARNING AND MEMORY IN DROSOPHILA**

Y. Zhong N.J.D. Wright J. Shanley

The goal of our research is to understand the cellular mechanisms of learning and memory. We are taking an approach of electrophysiological analysis of *Drosophila* leaning and memory mutants. These mutants have been isolated on the basis of poor performance in learning.

Characterization of two such mutants *dunce* (*dnc*) and *rutabaga* (*rut*) has shown that the *dnc* gene encodes cAMP-specific phosphodiesterases, whereas the *rut* gene encodes an adenylyl cyclase and that expression of both genes is enhanced in a brain region called the mushroom body. Genetic or pharmacological ablation of mushroom bodies has been demonstrated to lead to diminished olfactory-associated learning. Therefore, our first mission was to identify neurotransmitters that activate the cAMP cascade involving Dnc and Rut proteins. We will then determine how neurotransmissions mediated by
the identified transmitters are affected in learning and memory mutants. In the past year, we have focused on the peptide function in mushroom body neurons of *Drosophila* learning and memory mutants. Neuropeptides comprise the largest single group of intercellular messengers in the central nervous system (CNS), and thus an understanding of their actions must be a major part in the understanding of neuronal communication. We have chosen to study three neuropeptides: pituitary adenylyl cyclase-activating peptide (PACAP), PACAP-related peptide (PRP), and proctolin primarily for three reasons: First, PACAP and proctolin are known, or are suggested, to stimulate cAMP synthesis. Second, all three peptides appear to act on *Drosophila* mushroom body neurons. Third, the memory mutant *amnisiac* gene may code for a PACAP-related peptide.

PACAP belongs to a VIP/secretin/glucagon neuropeptide family. Two bioactive forms of PACAP, PACAP38 and PACAP27, are derived from the precursor protein and their peptide sequences are identical in humans, rats, and sheep. PRP is also derived from the same precursor, but its function has not yet been identified. PACAP receptors are coupled to both adenylyl cyclase and phospholipase C. The receptors are widely distributed in vertebrate peripheral tissues and in the central brain, including the hypothalamus and hippocampus. PACAPs have been reported to regulate hormone or transmitter release, neurite outgrowth, and gene expression. The presence of PACAP and PRP-related neuropeptides in invertebrates has not yet been reported. Proctolin is one of the best-characterized neuropeptides in insects, which has been reported to modulate Ca++ currents in insect muscle and stomatogastric neurons. Proctolin-like immunoreactivity has also been observed in the *Drosophila* CNS and at neuromuscular junctions. The purified fraction from *Drosophila* extracts exhibits a proctolin-like bioactivity in *Drosophila* and grasshopper.

**FIGURE 1** PACAP38-like immunoreactivity in the *Drosophila* larval CNS and at the larval body-wall neuromuscular junctions. *(Left)* Dorsal view of immunoreactivity of vertebrate PACAP38 in the ganglion and brain lobes. Arrowheads point to a pair of stained neuronal soma. *(Inset)* Schematic drawing of the distribution of stained soma in the CNS. *(Right)* Immunoreactivity in the motor nerve terminals arborized onto larval body-wall muscle fibers 4, 6, 7, 12, and 13. Arrowheads point to stained varicosities.
Immunohistochemical Analysis of Neuropeptide Distribution

J. Shanley, Y. Zhong

One of the major strategies for identifying new neuropeptides is to utilize immunocross-reaction among the conserved peptide families in different organisms. Since the presence of PACAP-like neuropeptides in invertebrates is unknown, we have used vertebrate PACAP antisera to determine the distribution of the putative Drosophila PACAP. Using antisera against the vertebrate PACAP38, we were able to detect PACAP38-like immunoreactivity in the larval CNS and at larval body-wall neuromuscular junctions (Fig. 1). Figure 1 (left) shows the pattern of immunoreactivity in the ganglion and brain lobes. The number of the stained neurons in the ventral ganglion is rather large, roughly about 500. The categories of the stained neurons remain to be determined. However, the distribution and morphology of some of these neurons are very similar to those of previous identified neurosecretory cells (e.g., T1v-T3v, SE2, and SP1) that express other neuropeptide immunoreactivities, including FMRFamide-like immunoreactivity. However, the overall pattern of immunoreactivity is different from that of all previously examined peptides, such as FMRFamide, proctolin, and insulin-like immunoreactivity.

The PACAP38-like immunoreactivity was also found at neuromuscular junctions of most body-wall muscle fibers (about 30 in each hemisegment) in all segments. Representative examples of such immunoreactivity in muscle fibers 6, 7, 12, and 13 are shown in Figure 1 (right). Immunoreactivity of other peptides examined including proctolin and insulin was restricted to subsets of motor nerve terminals. Immunoreactivity was abolished when the antisera was incubated with PACAP38, suggesting the specificity of the immunoreactivity. However, PACAP27 antiserum did not stain Drosophila tissues.

Using an antiserum against vertebrate PRP, we have found PRP-like immunoreactivity in the CNS and neuromuscular junctions as well (data not shown), but the pattern is very different from that of PACAP-like immunoreactivity. Only a few cell bodies including neurosecretory-like cells were stained in the ventral ganglia. At the larval body-wall neuromuscular junctions, only one nerve terminal was stained in each hemisegment of abdominal segments 1–5.

Neuropeptide Functions at Neuromuscular Junctions

Y. Zhong

The larval body-wall neuromuscular junction is probably the only preparation suitable for quantitative analysis of synaptic transmission at identifiable synapses in Drosophila. We chose this preparation to carry out a rigorous analysis of the physiological function of PACAP. Results of this analysis will provide a necessary theoretical foundation for proper interpretation of results from our study of PACAP in the CNS.

To investigate the physiological significance of immunoreactivity, focal application of vertebrate PACAP38 to neuromuscular junctions was analyzed by intracellular and voltage-clamp recordings. Application of PACAP38 (4 μM) to the neuromuscular junctions induces a slow depolarization of 10–20 mV lasting for tens of seconds (Fig. 2). After preincubation with its antiserum, PACAP38 failed to depolarize the muscle membrane. In contrast, 10 μM VIP failed to induce any significant response. These data indicate the specificity of the PACAP38 response. In addition to the PACAP38-induced depolarization, two-electrode voltage-clamp analysis revealed a second type of muscle response: modulation of voltage-activated K+ currents, which occurred 5–8 minutes later after depolarization (Fig. 2). A dramatic enhancement (50–100-fold) of the K+ currents is observed several minutes later after perfused PACAP38 is removed. This enhancement of K+ current lasts for about 3–5 minutes, and then the amplitude of the K+ currents gradually decays to that of the control.

In addition, this novel PACAP effect could also be mimicked by higher-frequency (60 Hz) stimulation of motor axons. The stimulation evoked not only the early slow depolarization, but also a dramatically enhanced K+ current several minutes after stimulation. Taken together, these data suggest that PACAP38 and an endogenous neuropeptide released upon stimulation of motor axons induce the same novel muscle response.

When the vertebrate PRP was pressure ejected or bath perfused to the neuromuscular preparations, muscle membrane potential and K+ currents were not significantly affected. However, synaptic transmission measured by excitatory junctional currents (ejcs) was enhanced (data not shown). This enhancement of
FIGURE 2 A typical time course of PACAP38-induced responses in larval body-wall muscles recorded by voltage clamp. Muscle membrane is held at -80 mV. At 0 min (0 minute), a pulse of 4 μM PACAP38 (as indicated by the bar) is focally applied to the neuromuscular junction, which induces an inward current as the early response. This early inward current lasts for many seconds. The muscle K+ currents are also monitored before the application and each minute after the application. The command voltages are stepped from -80 to -50 and 0 mV, respectively. Only a leakage current is elicited by the voltage step to -50 mV, but voltage-dependent currents are activated by depolarization to 0 mV. The amplitude of voltage-activated K+ currents remains relatively stable during the period prior to the application and 6 min after the application. A dramatic increase, as the late response, is seen at 7 min (7') after the application, which peaks at 8' and returns to the control level at about 13'.

Characterization of K+ Currents in Identified Mushroom Body Neurons

N.J.D. Wright, Y. Zhong

The adult Drosophila or third instar larval mushroom body consists of about 2500 neurons and is located in the dorsal and posterior cortex of each brain lobe. Despite two decades of genetic, molecular, anatomical, and behavioral analyses of learning and memory mutants, it has not been possible to determine the functional defects of these mutants in the CNS, particularly in mushroom body neurons. A large part of the problem has been due to the difficulty in recording from the small-sized Drosophila brain and neurons composing it. As a first step, we developed a preparation that allows us to identify fluorescent-stained mushroom body neurons in acutely dissociated larval CNS cultures and to patch-clamp these neurons. A Drosophila enhancer-trap line 221 (isolated by R. Davis' laboratory, CSHL) expresses the lacZ reporter gene almost exclusively in the mushroom body. When the 221 larval CNS is dissociated in culture, individual lacZ-expressing mushroom body neurons can be identified by fluorescent staining. Electrophysiological and pharmacological characterization of K+ currents (Fig. 3) recorded from these identified neurons demonstrates that lacZ expression per se and the staining do not affect these cells. These K+ currents essentially can be grouped into two types based on the whole-cell K+ current observed. Type-1 current exhibits a slow inactivating component and is mainly sensitive to the drug TEA, whereas type-2 current exhibits a fast transient A current and a sustained component and is mainly blocked by the drug 4-AP. Both types of K+ currents can be modulated by cAMP. This suggests that the staining did not significantly affect the physiology of voltage-activated K+ currents.
Figure 3: Representative examples of voltage-activated whole-cell K⁺ currents recorded from acutely dissociated larval CNS cultures. The family of current traces were elicited by depolarizations to -60, -20, 20, 50 mV. (Left column) Recorded from cells not subject to hypotonic shock; (middle column) recorded from cells subjected to the hypotonic shock and loaded with fluorogenic substrate of β-galactosidase but unmarked due to the lack of expression of lacZ; (Right column) recorded from cells that are fluorescently labeled resulting from lacZ expression. The marked neurons are derived from the mushroom body.

Figure 4: Peptide-induced modulation of type-1 K⁺ current in mushroom body neurons. Controls were recorded prior to perfusion of proctolin (20 µM), PRP (20 µM), and PACAP38 (8 µM). The current traces under "Peptide" were recorded immediately after ceasing the perfusion, which is usually 30–60 sec. Traces under "Recovery" were recorded 2–4 min after ceasing the perfusion.

Modulation of K⁺ Currents Induced by Neuropeptides in Normal and Mutant Flies

N.J.D. Wright, Y. Zhong

Figure 4 presents responses to the application of PACAP38, PRP, and proctolin of type-1 current in stained mushroom body neurons. The control current was elicited by depolarization from the holding potential -80 mV to -50 and +20 mV. The current elicited by the -50 mV pulse represents leakage current, whereas the +20 mV pulse triggers voltage-activated K⁺ currents. After recording of control currents, peptides were perfused by pressure ejection for 30–60 seconds via a micropipette placed 50–100 µm away from the patched neuron. Leakage current was not affected by these peptides. In contrast, type-1 current was modulated by PACAP38, PRP, and proctolin but each in a different fashion (Fig. 4). PRP reduced the amplitude, whereas proctolin mainly altered kinetics (the inactivating component becomes faster). PACAP38 produced both a reduction in the amplitude and an alteration of inactivation kinetics (faster). Such peptide-induced responses were seen in all type-1 neurons examined, but to different degrees. One possible source of such interpatch variability may have resulted from variation of the concentration of peptides applied to the patched neuron, since the tip diameter of the perfusion micropipette and the distance of the micropipette from the patched neuron most likely varied from preparation to preparation.

The rut⁴ mutation completely abolishes Ca++/CaM-activated adenylyl cyclase activity and significantly but not completely disrupts olfactory learning and memory. We bred rut⁴ mutants, which also carried
the enhancer-driven lacZ gene from 221, thereby allowing the identification of rut\textsuperscript{1} mutant mushroom body neurons. When PACAP38 was perfused on these mutant cells, the response was abnormal (Fig. 5). As indicated in Figure 5, application of PACAP38 induced modulation of both amplitude and inactivation kinetics in normal mushroom body neurons. In contrast, all mutant neurons examined showed a reduction in the amplitude of type-1 K\textsuperscript{+} current, but the inactivation kinetics were not modulated by application of PACAP38. Thus, our approach promises to contribute to a better understanding of the electrophysiological defects in the CNS of learning and memory mutants.

**PUBLICATIONS**


**GENETICS OF HUMAN BEHAVIORAL DISORDERS**

H. Feilotter, S. Lincoln, R. Koskela

The Genetics of Manic Depressive Illness

H. Feilotter, S. Lincoln, R. Koskela [in collaboration with T Marr, Cold Spring Harbor Laboratory; J. Wilkowski, Banbury Center; R. DePaulo, S. Simpson, F. McMahon, M. McInnes, D. Meyers, C. Stine and J. Xu at Johns Hopkins University School of Medicine; and D. Botstein, C. Clark, C. Friddle, M. Gschwend, and D. Cox at Stanford University School of Medicine]

This year marked the beginning of a new research direction for the Laboratory on the genetics of human behavioral disorders. Because our involvement in the Dana Consortium began in the last months of 1993, this report will provide background about the project and a summary of ongoing work. The specific objective of this consortium is the identification of the gene or genes that are responsible for the disorder known as manic depression. Drawing from the diagnostic and genotyping expertise at Johns Hopkins and Stanford and the informatics expertise here at the Laboratory, the long-term goal of this work is to develop a novel informatics tool for use in studies of complicated human genetic disorders.

Manic depression, or bipolar affective disorder, is a prevalent disease, affecting roughly 1% of the population of America. It is also a complicated one, both from the point of view of the diagnostic uncertainties that accompany it and the potential complex genetics that may underlie it. Criteria for the
diagnosis of bipolar disorder are set out in the DSM-III-R handbook to facilitate a standardized approach among clinicians. However, the disorder is displayed as a startling variety of phenotypes, which may be influenced by the underlying genetics, the environment, or both. The continuum of phenotypes that may denote the same genetic lesion ranges from bipolar I disorder, characterized by full-blown manias and deep, lasting depressions, through to cyclothymia, characterized by less severe mood swings. The difficulty lies in determining who is suffering from the clinical manifestation of a specific genetic disorder and who may be displaying a disorder of a non-genetic nature.

There is evidence for a genetic component of bipolar disorder from both twin and family studies. However, these data have also demonstrated that penetrance of the genetic component is less than complete and have opened the possibility that the disease may be at least in part the result of complicated genetics. For example, the interaction of a number of mutant gene products might combine to produce the various phenotypes. Mutations might be present in more than one gene, each of which may be responsible for a subset of cases. Interactions of specific genetic predispositions with environmental factors might give rise to a variety of phenotypes. The obvious difficulties in a genetic approach to a disease that has no simple biochemical marker to flag its presence, nor a simple Mendelian pattern of transmission, have made studies on the genetics of manic depression very frustrating.

Genetic studies on human disease are initiated by attempting to find linkage between the disease phenotype and specific chromosomal regions that are tagged by polymorphic markers. These studies have recently been enormously improved by the construction of a relatively high-density genetic map of the human genome. Informative markers (i.e., those that display a high degree of polymorphism in the general population) are available at a resolution of about 1–2 cM over much of the genome. Rather than a methodical approach, researchers may look for candidate genes that are linked to the disorder, which might reasonably contribute to the observed phenotype when mutated. A variation on this strategy is the use of the candidate gene approach only after a general chromosomal region has been shown to be linked to the disease phenotype.

Our collaborative efforts toward understanding the genetics of manic depressive illness implement the same strategies. Following family selection, rigorous and repeated screenings of family members to maximize diagnostic accuracy have been carried out by the psychiatric group at Johns Hopkins Hospital. Only families that are segregating the illness in a unilinear pattern are chosen for study. Currently, 28 such families have been identified. Blood has been drawn from each willing family member, and immortalized lymphoblastoid cell lines have been created to provide a constant source of DNA for use in linkage analysis. Two hundred markers, spaced at roughly 20 cM across the genome, have been chosen for use by the genotyping group at Johns Hopkins to do linkage analysis. An additional 200 markers have been chosen by the genotyping group at Stanford for use on the same DNAs. The markers have been chosen such that approximately 10% of them are the same in both groups and the others interdigitate to create a 10 cM map across the genome.

The role of our group in the collaboration is to maximize the information gained from these studies and to facilitate data flow and data manipulation through the application of computing power. Tom Marr's laboratory has produced a new database known as Genome Topographer (GT). GT was designed to meet several specific needs of the biological research community and is being exploited and modified to be more useful for the psychiatric genetics community in this collaboration. A complete description of GT is given elsewhere in this Annual Report (see T. Marr in the Structure and Computation Section), but a brief outline is relevant here. The concept of GT stemmed from the realization that the vast amount of information currently being produced on the human and other genomes was being scattered among various databases and literature sources in forms that were largely incompatible with each other and often inaccessible to the average biologist. GT seeks to overcome this problem by integrating all genome information in a standardized format in a single easily accessible database. In addition to information on the human genome, GT stores data on all other model systems. It also stores biochemical and molecular data where these are known. The result is a huge information repository that allows a user to browse material and find relationships between pieces of data that might not have been obvious by using several different databases as sources. Researchers can simply browse the remote database to obtain information about specific regions of chromosomes, genes, contigs, probes, etc. Alternatively,
users can also enter their own data in local projects. These data can then be integrated into the publicly accessible large database, or they can bypass the public forum and make use of a number of the external applications or tools which GT also maintains. These tools allow manipulation of data using various programs such as sequence analysis or linkage analysis programs. Because of the size of the computers on which these programs are executed, the run time for such manipulations is much reduced, making possible complicated data analyses that laboratories with less computing power could not have done previously. Output from the external tools can then be used to search for new relationships in the remote database.

The current effort from our group is toward modification of GT to provide a system that will be useful in efforts to understand the genetic basis of manic depression. This is taking place in several ways that make use of the remote and local database setup, as well as the external tools. Using the remote database, we are currently entering information on available markers and genetic maps. Other efforts involve additions to the existing GT structure. These modifications are specifically designed to address some of the major problems encountered during genetic analysis of human disease. Therefore, initial emphasis is being put on the following areas: (1) the design of programs that allow researchers to input pedigree, genotyping, and diagnostic data in a standardized format, (2) the addition of currently available linkage analysis programs to the external tool selection in GT, (3) the writing of new linkage analysis tools that implement novel approaches to the linkage problem, (4) the writing of user interfaces for all the linkage analysis programs and for data input that are comprehensible to the biologists using the linkage programs, and (5) the construction of a survey database that incorporates published data which may be helpful to current research, including exclusion maps from previous linkage analyses.
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.

D. Barford

We have been pursuing our crystallographic investigations on protein phosphatases. Progress has been made in obtaining better quality crystals of the serine/threonine-specific protein phosphatase 1. Two protein tyrosine phosphatases, namely, protein tyrosine phosphatase 1B and the T-cell protein tyrosine phosphatase, have been crystallized, and their structures have been determined.

Protein phosphatases constitute a family of signal transducing enzymes that catalyze protein dephosphorylation and are important in regulating a variety of proteins. Two classes have been characterized on the basis of substrate specificity and primary structure. The serine/threonine protein phosphatases act specifically or preferentially on phosphoserine and phosphothreonine residues, whereas the structurally distinct protein tyrosine phosphatases specifically dephosphorylate phosphorysine residues. A subfamily of dual specificity phosphatases, characterized by p80cdc25 and MAP kinase phosphatase-1 has been characterized recently.

PROTEIN PHOSPHATASE 1 (PP1)

PP1 is a principal member of the serine/threonine class of protein phosphatases and is important in controlling a number of cellular processes including metabolism, division and differentiation, and learning and memory processes. The human isoform of PP1 has been crystallized. This year, better quality crystals have been obtained that diffract to 2.2 Å when exposed to synchrotron radiation. The space group and cell parameters have been characterized, and a complete native dataset was collected at beam-line X12C at the National Synchrotron Light Source, Brookhaven National Laboratory. A considerable advance was made following the acquisition of a cryo-
FIGURE 1 A ribbons diagram of human protein tyrosine phosphatase 1B showing the secondary structure and the position of the invariant and catalytic residues His-214, Cys-215, Arg-221, and Gln-262 and the tungstate ion.

system cooling device, allowing us to freeze and store crystals in liquid nitrogen and to collect crystallographic data at cryogenic temperature. Heavy atom screening is currently in progress.

HUMAN PROTEIN TYROSINE PHOSPHATASE 1B

This research was carried out in collaboration with A.J. Flint and N.K. Tonks (CSHL). The cDNA encoding the amino-terminal 321 residues of human PTP1B was cloned into the T7 polymerase expression vector, allowing the gene to be overexpressed in E. coli (A.J. Flint). A purification procedure was developed, allowing more than 50 mg of protein to be purified from a 4-liter culture. The protein is more than 99% homogeneous and has been crystallized.

The structure was determined to 2.8 Å from a single heavy atom derivative with use of anomalous scattering. All data were collected at beam-line X8C at the National Synchrotron Light Source, Brookhaven National Laboratory. The structure reveals a novel protein fold and the position of the substrate phosphate-binding site. The catalytic cysteine residue (C215) interacts directly with the phosphate group located at the catalytic site, consistent with its role as a nucleophile in the catalytic reaction. Other residues within the conserved PTP sequence signature motif form the phosphate-binding site. The catalytic cleft is the correct size to accommodate a phosphotyrosine-containing residue and is probably too deep for phosphoserine and phosphothreonine. The surface of the protein surrounding the catalytic site is predominantly electro-positive, which may explain the preference of PTP1B for dephosphorylating phosphotyrosine adjacent to acidic residues.

The structure reveals the position and roles of invariant residues shared among protein tyrosine phosphatases and explains previous biochemical and site-directed mutagenesis experiments performed by other investigators.

A ribbons diagram showing the secondary structure and the position of the invariant and catalytic residues His-214, Cys-215, Arg-221, and Gln-262 and the tungstate (phosphate analog) is shown in Figure 1.

HUMAN T-CELL PROTEIN TYROSINE PHOSPHATASE

This research was carried out in collaboration with N.K. Tonks (CSHL). The cDNA encoding the human
T-cell PTP was cloned into a T7 expression vector, and the overproduced protein was purified in a manner similar to that used for PTP1B. Small crystals were obtained in the presence of ammonium sulfate and data to a resolution of 3.0 Å were collected at beam-line X12C. The sequence of T-cell PTP shares 50% sequence identity with PTP1B and the structure of PTP1B was used as a model to determine the structure of T-cell PTP using the molecular replacement method. As expected, the structure is very similar to that of PTP1B. The deletions in the T-cell PTP sequence compared to the PTP1B sequence are located in loops connecting elements of secondary structure.

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COLD SPRING HARBOR MEETINGS
The academic program at Cold Spring Harbor Laboratory in 1993 included a series of advanced postgraduate laboratory and lecture courses in molecular genetics, structural biology, and neurobiology; conferences and workshops including the annual Symposium; and a summer research program for undergraduates. In an era of rapidly advancing scientific research, the courses and meetings serve multiple functions, both in teaching advanced state-of-the-art techniques and methodologies and in bringing together scientists in many areas to exchange their latest results.

More than 5000 scientists came to the Laboratory this year to attend or teach in the various courses and conferences. In 1993, 25 laboratory courses and 14 meetings were held, starting with a spring session of courses held early in April and extending through two sessions of fall courses ending in November. Several new laboratory and workshop courses were held this year: Early Development of *Xenopus laevis*, taught by Richard Harland and Hazel Sive; *Arabidopsis* Molecular Genetics, taught by JoAnne Chory, Joseph Ecker, and Athanasius Theologis; and Construction and Analysis of Two-dimensional Gel Protein Databases, taught by B. Robert Franza, James Garrels, Gerald Latter, and Scott Patterson. Two new Neurobiology lecture courses were also taught and were held at the Laboratory's Banbury Center: Human Functional Neuroimaging, taught by Francis Miezin, Steven Petersen, and Marcus Raichle; and Structure, Function, and Development of the Visual System, taught by Tobias Bonhoeffer and David Fitzpatrick. The entire program of courses and meetings is listed in detail on the pages that follow. Clearly, it is due to the large number of scientists who serve with great skill and enormous effort and goodwill that this very intensive program is run so successfully.

The course program is supported by grants from both private and government sources. This year, the grant to support the Advanced Bacterial Genetics course was renewed by the National Institute of General Medical Sciences. This grant, which entered its fourteenth year of funding, is crucial to the existence of the course, and the efforts of the instructors, Stan Maloy, Valley Steward, and Ron Taylor, in carrying on the tradition of the longest-running course at Cold Spring Harbor are greatly appreciated. Grants from the National Institutes of Health and the National Science Foundation support many of the molecular genetics courses, and a grant from the National Institute of Mental Health covers several neurobiology courses. More recently, awards from the Department of Energy and the Department of Agriculture have helped in the funding of the courses on Macromolecular Crystallography and Molecular Markers for Plant Breeding, respectively. The education grant from the Howard Hughes Medical Institute, now in its second year of renewal, continues to provide enormously important stable funding for the neurobiology program as well as support for new courses, a critically important area. The Laboratory is also fortunate to have an award from the Esther and Joseph A. Klingenstein Fund for the support of the neurobiology program as well as scholarship funds from the Grass Foundation for the support of students taking neurobiology courses. In addition, the Laboratory courses receive invaluable support from many companies that donate supplies and lend large amounts of equipment (see Educational Activities Section).

The Symposium organized by Bruce Alberts and Bruce Stillman was again
the highlight of the meetings this year. This year's topic, DNA and Chromosomes, was chosen not only to recognize the 40th anniversary of the discovery of the double helix, but also to celebrate the 25th anniversary of Jim Watson's tenure as Director of the Laboratory. Thirteen additional conferences were held on topics ranging from Neurobiology of Aplysia to Mechanisms of Eukaryotic Transcription. The latter meeting and those covering Genome Mapping and Sequencing, RNA Processing, Retroviruses, and Yeast Cell Biology, as well as the Symposium itself, had at least 400 participants each. Many of this year's meetings continue to be held in alternate years and thus will return in 1995. Others, including the conferences on Retroviruses and Genome Mapping and Sequencing, will be held again next year. As in past years, support from the Laboratory's Corporate Sponsor Program and from NIH, NSF, and the Department of Energy was important in helping scientists at all levels of their careers to attend the meetings. Contributors to the various meetings are listed in the pages that follow.

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 at the Laboratory during the summer. The program, headed by Winship Herr, allows students to do research in the laboratories of staff scientists.

The large numbers of courses and meetings proceed with skill and efficiency, thanks to the collaborative efforts of a large number of people at the Laboratory. The staff of the Meetings Office, including Barbara Ward, Micki McBride, Diane Tighe, Marge Stellabotte, Andrea Stephenson, and Nancy Weeks, coordinates with both skill and tact the visits of the thousands of scientists who come here to participate in the meetings and courses. A major change took place this year with the early retirement of Barbara Ward, the Registrar for the courses and head of the Meetings Office. Barbara presided over the office as the Academic Program grew to a large and almost year-round effort and always played a major part in the many aspects of managing the meetings and courses. David Stewart joined the Laboratory this year as the new head of the Meetings Office. He received his Ph.D. from the Institute of Biotechnology, University of Cambridge, and comes to the Laboratory from Affinity Chromatography, Ltd., where he was a Senior Scientist. David is the first scientist to head the Meetings Office and will be exploring an expansion of the Meetings Program. Micki McBride, who has worked in the Meetings Office for several years, has been promoted to Course Registrar. The audiovisual staff headed by Herb Parsons manages with great efficiency and good humor the thousands of slides and projections provided by the scientists at our meetings. The course instructors have come to depend on the skills of Cliff Sutkevich and his staff in setting up and maintaining all of the necessary equipment and supplies, as well as those of the Purchasing Office headed by Sande Chmelev and the new Purchasing Agent in charge of the courses, Edie Kapenberg. The many grants that support the academic program are ably coordinated by Mary Horton of the Grants Office.

Terri Grodzicker
Assistant Director for Academic Affairs
The 1993 Cold Spring Harbor Symposium on Quantitative Biology focused on DNA and Chromosomes in the 40th year since the discovery by James Watson and Francis Crick of the double helix of DNA. The central goal of the meeting was to bring together scientists who work on all aspects of DNA structure and function, including those working on gene expression and the influence of chromatin structure on this process. Other topics included chromosomal elements such as telomeres, centromeres, origins of DNA replication, locus controlling elements, and position effect elements. Chromosomal phenomena such as silencing, imprinting, recombination, DNA repair, and transcription were also discussed.

There were a total of 93 invited speakers, 152 posters, and approximately 450 participants, the maximum Cold Spring Harbor Laboratory could accept.

The impact of these studies on understanding human disease was also apparent. Alterations in DNA-repair processes frequently lead to higher rates of cancer, and the loss of coordination between DNA replication and DNA damage controls appeared to accelerate the rate of accumulation of genetic damage. For
example, in cells lacking the p53 tumor suppressor protein, DNA amplification is much more prevalent. New DNA mapping technologies, also reported at the meeting, have facilitated mapping of genes associated with inherited diseases. New advances in the human genome project were also highlighted at the meeting, including the sequence of chromosome XII from *Saccharomyces cerevisiae* and the T-cell receptor locus from humans.

PROGRAM

Welcoming Remarks: Bruce Stillman
Introduction
Chairperson: B. Alberts, University of California, San Francisco
Chromosome Replication
Chairperson: B. Brewer, University of Washington, Seattle
Silencing and Variegation
Chairperson: S.C.R. Elgin, Washington University
Methylation and Imprinting
Chairperson: R.J. Roberts, New England Biolabs, Beverly, Massachusetts
Chromosome Machinery
Chairperson: E. Blackburn, University of California, San Francisco
Transcription and Chromatin
Chairperson: H. Varmus, University of California, San Francisco
Chromosome Domains
Chairperson: J. Gall, Carnegie Institution, Baltimore
Centromeres, Telomeres, and Replicators
Chairperson: D. Gottschling, University of Chicago, Illinois
DNA and Chromatin Structure
Chairperson: J. Thomas, University of Cambridge, United Kingdom
Chromosome Organization and Stability
Chairperson: R. Kahmann, Universität München, Germany
Dorcas Cummings Lecture: "Mapping Genes and Genomes"
Speaker: Eric S. Lander, Whitehead Institute for Biomedical Research and Massachusetts Institute of Technology, Cambridge
Transcription Activation
Chairperson: T. Maniatis, Harvard University
Recombination and Repair
Chairperson: C. Laird, University of Washington, Seattle
Genome Structure
Chairperson: G. Bernardi, Institut Jacques Monod, Paris, France
Gene Regulation
Chairperson: S.M. Tilghman, Howard Hughes Medical Institute, and Dept. of Molecular Biology, Princeton University
Nuclear Structure
Chairperson: G. Felsenfeld, NIDDK, National Institutes of Health
Summary: H. Weintraub, Howard Hughes Medical Institute Laboratory, Fred Hutchinson Cancer Research Center, Seattle, Washington
MEETINGS

Neurobiology of Aplysia

April 21–April 25, 1993

ARRANGED BY

Wijnand Geraerts, Vrije Universiteit Amsterdam
Hersch Gerschenfeld, Ecole Normale Superieure
Philip Haydon, Iowa State University
Leonard Kaczmarek, Yale University Medical School
Eric Kandel, Howard Hughes Medical Institute, Columbia University College of Physicians & Surgeons
Irwin Levitan, Brandeis University
Richard Scheller, Stanford University
Micha Spira, Hebrew University
Felix Strumwasser, Uniformed Services, University of the Health Sciences

119 participants

The third international meeting on the Neurobiology of Aplysia was held at Cold Spring Harbor April 21–25, 1993. Since neurobiology of Aplysia is deeply entwined in the biology of other molluscs, the meeting has been progressively expanded to include representations from other important molluscan preparations such as the squid, Heliosoma, Hermisenda, and various land snails. Aplysia and related molluscs represent a distinctive niche in the cellular biological study of the nervous system, especially the molecular mechanisms of synaptic transmission, synaptic plasticity, the neural circuitry of behavior, and the modification of behavior by learning. This is due to the fact that the nerve cells of
these animals are large and identifiable and can be readily cultured, and their synapses can be visualized. As a result, a variety of imaging techniques make it possible to study the distribution of calcium influx or cAMP within specific neurons and even regions of neurons in a way that is not possible in other systems.

This meeting was funded in part by the National Institute of Neurological Disorders and Stroke, a division of the National Institutes of Health.

PROGRAM

Opening Remarks: Mechanisms of Exocytosis in the Squid Giant Synapse
Richard Scheller, Stanford University
George Augustine, Duke University

Molecular Imaging Approaches to Synapse Development and Function
Chairperson: H. Gerschenfeld, Ecole Normale Superieure

Ion Channels and Their Modulation
Chairperson: M. Spira, Hebrew University

The Expression and Processing of Peptides
Chairperson: Philip Haydon, Iowa State University

Peptides and Behavior
Chairperson: W.P. Geraerts, Vrije Universiteit Amsterdam

Rhythms and Behavior
Chairperson: L. Kaczmarek, Yale University Medical School

Learning and Short-term Memory
Chairperson: T.J. Carew, Yale University

Learning and Long-term Memory
Chairperson: R. Scheller, Stanford University

Closing Remarks: L. Kaczmarek, Yale University Medical School
The 1993 meeting on The Cytoskeleton and Cell Function covered all aspects of the cytoskeleton, including actin filaments, intermediate filaments, and microtubules. Talks focused on the function of the cytoskeleton in various biological processes, including development and differentiation, pathological aspects of contractile and cytoskeletal systems, cell communication, intracellular trafficking and organelle movement, and cell shape and morphogenesis. The fundamental importance of the cytoskeleton in cellular functions rendered the subject of the conference of interest to investigators from a wide number of disciplines including cell biologists, biochemists, molecular biologists, and developmental biologists. A broad range of experimental systems were discussed including yeast, Dictyostelium, flies, and vertebrates. A major theme of the meeting was the increasing use in cell biology of multidisciplinary approaches including powerful genetic and molecular tools. Another meeting on the cytoskeleton is scheduled for 1995.

This meeting was funded by contributions from the CSHL Corporate Sponsors Program, The March of Dimes Birth Defects Foundation, The U.S. Army Research Office, Pierce Chemical Company, and The Council for Tobacco Research.
PROGRAM

Actin Filament Assembly and Dynamics
Chairperson: P. Matsudaira, Massachusetts Institute of Technology

Cell Movement
Chairperson: S. Zigmond, University of Pennsylvania

Microtubule Assembly and Dynamics
Chairperson: D. Cleveland, Johns Hopkins University

Cell Division
Chairperson: R. McIntosh, University of Colorado

Intermediate Filaments
Chairperson: E. Fuchs, University of Chicago

Molecular Motors: Intracellular Trafficking
Chairperson: M. Mooseker, Yale University

Signal Transduction: Membrane-cytoskeleton Interactions
Chairperson: B. Geiger, Weizmann Institute

The Role of the Cytoskeleton in Development and Differentiation
Chairperson: E. Raff, Indiana University

Closing Remarks: David Helfman, Cold Spring Harbor Laboratory
The Regulation of Liver Gene Expression in Health and Disease meeting was devoted to various aspects of regulation of hepatic gene expression, liver development, and hepatic gene therapy. It was the fourth bi-annual international meeting in a series focused on the liver, which is the major organ that controls many metabolic processes and homeostasis under normal physiological state. The meeting attracted 264 scientists from Europe, Asia, and North America who are investigating various aspects of the cellular, molecular, and developmental biology of the liver in health and disease. It had three main themes: (1) liver gene expression and regulation; (2) liver architecture, cellular growth, and differentiation; and (3) liver diseases and gene therapy, including an update on the clinical trial for familial hypercholesterolemia by hepatic gene therapy.

This meeting was funded in part by the National Institute of Diabetes and Digestive and Kidney Diseases, a division of the National Institutes of Health, Boehringer Mannheim Corporation, Miles Inc., Pharmacia Biotech Inc., Amgen Inc., and Clontech Laboratories Inc.

PROGRAM

Liver-specific Gene Expression and Regulation
Chairperson: M. Yaniv, Institut Pasteur
Keynote Address: Regulation of Eukaryotic Transcription—General Factors, Activators, and Co-Factors
R.G. Roeder, Rockefeller University, New York
Genetic Control of Hepatic Gene Expression
Chairperson: M.C. Weiss, Institut Pasteur
Viral Hepatitis, Cirrhosis, and Hepatocellular Carcinoma
Chairperson: B. Knowles, Wistar Institute
Temporal Regulation of Liver Gene Expression by Hormones and Cytokines
Chairperson: R.H. Costa, University of Illinois College of Medicine
Differentiation and Embryonic Development
Chairperson: K. Zaret, Brown University
Liver Regeneration and Growth Control
Chairperson: G.J. Darlington, Baylor College of Medicine
Cell Biology, Extracellular Matrix and Gene Expression
Chairperson: J.E. Darnell, Jr., Rockefeller University
Gene Therapy
Chairperson: S.L.C. Woo, Howard Hughes Medical Institute, Baylor College of Medicine
Overview of Hepatic Gene Therapy
S.L.C. Woo, Howard Hughes Medical Institute, Baylor College of Medicine
Update of Clinical Trials for Familial Hypercholesterolemia
J.M. Wilson, Institute for Human Gene Therapy, University of Pennsylvania and the Wistar Institute, Philadelphia
The sixth annual Cold Spring Harbor Genome Mapping and Sequencing meeting was held between May 12th and 16th. Attended by some 400 participants, drawn largely from the United States, but with a strong showing from Europe and elsewhere, the 300 abstracts provided a rich and lively forum for discussion of recent advances and outstanding problems in the field. Sessions covered such topics as recent advances in identifying and studying human disease genes, large-scale physical mapping of genomes, meiotic mapping, megabase DNA sequencing, and cDNA clone isolation and mapping. In addition, there was a heavy representation of new technologies for mapping and sequencing, as well as an exciting session on how best to handle and interpret the wealth of new DNA sequence information and the complexity of data being generated by meiotic and physical mapping efforts. This year, more than ever, the meeting highlighted the vast contributions that studies of the genomes of ‘model organisms,’ including bacteria, yeast, worms, flies, and mice, have made to the rapid advances in biological knowledge, genome structure, and technologies for sequencing and mapping.

This meeting was funded in part by the National Center for Human Genome Research, a branch of the National Institutes of Health.
PROGRAM

Human Diseases
Chairperson:  D.R. Cox, Stanford University

Model Organisms
Chairperson:  R. Rothstein, Columbia University

Sequencing Methods
Chairperson:  G. Rubin, University of California, Berkeley

Informatics
Chairperson:  R. Waterston, Washington University School of Medicine

Mapping Methods
Chairperson:  B. Trask, University of Washington

cDNA Mapping and Other Methods
Chairperson:  E. Green, Washington University Medical School

Large Maps
Chairpersons:  R.M. Myers, Stanford University
              D.J. Porteous, MRC Human Genetics Unit, Edinburgh, United Kingdom

L. Stuve, R. Gould, M. Polymeropoulos, V. Smith
The 1993 RNA Processing meeting was oversubscribed, continuing the trend of the past several years, and thus attendance had to be limited. There were 100 talks and 265 poster presentations, reporting exciting developments in several areas. Of greatest potential for the field as a whole was the optimization of methods to obtain crystals of large RNA molecules, such as ribozymes, that diffract to high resolution. Insights on ribozyme higher-order structure have been obtained by cross-linking studies and by melting studies on ribozyme-substrate complexes.

A complex network of dynamic RNA-RNA, RNA-protein, and protein-protein interactions within the spliceosome has been studied in increasing detail. In particular, mutational studies of U6 snRNA have implicated the U6 snRNP in definition of the 5' splice site and in positioning this site near the catalytic center of the spliceosome. Subunit interactions in a heterotrimeric U2 snRNP-associated essential splicing factor were shown to be conserved between yeast and mammals. The RS domains present in several splicing factors and alternative splicing regulators were implicated in protein-protein interactions.

Studies of the cleavage and polyadenylation specificity factor showed that this factor recognizes not only the AAUAAA element, but also adjacent context sequences. Poly(A) polymerases from several species and their interactions with other 3'-end-processing components were analyzed. Feedback regulation of U1 snRNP A protein expression was shown to be achieved by U1-A binding to the 3'-UTR of its own pre-mRNA and interacting with poly(A) polymerase to inhibit polyadenylation. An apparent sequence of mRNA decay was defined, in which deadenylation leads to cleavage near the 5' cap, which in turn leads to 5' to 3'
exonucleolytic degradation. The basis for the nuclear retention of U6 snRNA was defined, and members of the yeast ras superfamily were implicated in mRNA export.

Selection and amplification approaches were employed to design new ribozymes, to identify specific targets for RNA-binding proteins, and to identify RNA sequences with affinity for small ligands. Small molecules that disrupt ribozyme function or specific protein-RNA interactions were reported. New protein motifs capable of binding RNA were also identified.

This meeting was funded in part by the National Science Foundation and the National Institute of General Medical Sciences, a branch of the National Institutes of Health.

**PROGRAM**

**Regulation of mRNA Splicing**  
Chairperson: D.C. Rio, University of California, Berkeley

**Ribozymes**  
Chairperson: J.M. Burke, University of Vermont, Burlington

**Mechanisms of mRNA Splicing**  
Chairperson: T.W. Nilsen, Case Western Reserve University School of Medicine

**3' End Processing and RNA Transport**  
Chairperson: W. Keller, University of Basel, Switzerland

**Ribonucleoprotein Particles**  
Chairperson: J. Beggs, University of Edinburgh, United Kingdom

**RNA Editing, Modification and Turnover**  
Chairperson: B.L. Bass, University of Utah

**Nuclear Architecture, RNA-Protein Interactions, and RNA Structure**  
Chairperson: J.D. Keene, Duke University Medical Center

A. Lambowitz, J. Keene, N. Pace
This year, the title of the meeting was changed from "RNA Tumor Viruses" to "Retroviruses." This change of title reflects a significant change in the content of the meeting and in the field itself. About ten to 15 years ago, a major focus of the meeting was oncogenes and the role of retroviruses in oncogenesis. Today, the primary focus of oncogene research is not in retroviruses. There are now separate meetings for investigators whose primary interest is oncogenes. The discovery of pathogenic human retroviruses has shifted our attention from the interaction of retroviruses and oncogenes to other disease mechanisms involving retroviruses, including AIDS.

Rather than try to single out particular accomplishments, it is probably more appropriate to point to the progress that has been made in the past 10 years in understanding pathogenic human viruses. We do not yet have cures for retroviral diseases; however, we have a broad and solid foundation on which to build. It is also important to say that these 10 years of progress rests on the foundation provided by previous and ongoing work in animal retroviruses. It is therefore entirely fitting that the Cold Spring Harbor meeting, once "RNA Tumor Viruses" now "Retroviruses," is the focus of the interchange of information between human retrovirus research and research in animal retroviruses, intimately related topics that are all too often treated as separate fields.

Contributions from Corporate Sponsors provided core support for this meeting.
Phylogeny and Receptors
Chairpersons: M. Sitbon, Institut Cochin de Génétique Moléculaire, Paris, France
N. Rosenberg, Tufts University School of Medicine

env
Chairpersons: J. Overbaugh, University of Washington
G. Franchini, National Cancer Institute, Bethesda

 gag
Chairpersons: A. Rein, NCI-Frederick Cancer Research Facility
S.P. Goff, Columbia University

Protease
Chairperson: H.-G. Kräusslich, German Cancer Research Center, Heidelberg, Germany

Reverse Transcription
Chairpersons: S.B. Sandmeyer, University of California, Irvine
M. Roth, University of Medicine and Dentistry of New Jersey/Robert Wood Johnson Medical School

Integration
Chairpersons: J. Coffin, Tufts University School of Medicine
D. Grandgenett, St. Louis University Medical Center

Vectors/Mutation/Recombination
Chairperson: M. Linial, Fred Hutchinson Cancer Research Center

Pathology
Chairpersons: K. Radke, University of California, Davis
L. Ratner, Washington University School of Medicine

Tat/Rev
Chairpersons: N. Hernandez, Cold Spring Harbor Laboratory
A.I. Dayton, NIAID, National Institutes of Health

RNA
Chairperson: C.M. Stoltzus, University of Iowa, Iowa City

Transcription
Chairpersons: J. Lenz, Albert Einstein College of Medicine
J. Dudley, University of Texas, Austin

Nontranscriptional Regulation
Chairpersons: A. Rabson, Center for Advanced Biotechnology and Medicine and University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School
F. Clavel, Institut Pasteur, Paris, France
Yeast Cell Biology
August 17–August 22, 1993

ARRANGED BY

Trisha Davis, University of Washington
Michael Douglas, University of North Carolina, Chapel Hill
Mark Rose, Princeton University

392 participants

The conference on Yeast Cell Biology was the fourth bi-annual international meeting devoted to major aspects of cell biology in yeast. Topics included the structure and function of the cytoskeleton, structure and biogenesis of organelles, secretion, endocytosis, and protein targeting. Recent advances in studying the cell cycle, signaling pathways, and developmental pathways were also described. More than 400 scientists attended; 287 abstracts were presented including 109 talks and 178 posters.

This meeting was funded in part by Labatt Breweries of Canada and Anheuser-Busch Companies, Inc.

PROGRAM

Cell Polarity and the Actin Cytoskeleton
Chairperson: J. Pringle, University of North Carolina, Chapel Hill

Developmental Pathways and Mating
Chairperson: S. Michaelis, Johns Hopkins University School of Medicine

M. Rose, T. Davis
Spindle Function and Microtubules  
Chairperson: C. Holm, Harvard University

Cell Cycle  
Chairperson: D. Botstein, Stanford University School of Medicine

Methodology  
Chairperson: J. Cooper, Washington University, St. Louis

Calmodulin, Ion Pumps and Cyclophilins  
Chairperson: E.W. Jones, Carnegie Mellon University

Secretion  
Chairperson: T. Stevens, University of Oregon, Eugene

Endocytosis, Protein Retention, and Sorting  
Chairperson: R. Fuller, Stanford University School of Medicine

Nuclear Structure, Targeting, and Telomeres  
Chairperson: M. Yaffe, University of California, San Diego

Organelle Biogenesis  
Chairperson: R. Butow, University of Texas Southwestern Medical Center

Signaling  
Chairperson: J. Thorner, University of California, Berkeley
Molecular Genetics of Bacteria and Phages
August 24–August 29, 1993

ARRANGED BY

Susan Gottesman, National Cancer Institute, National Institutes of Health
Peter Model, Rockefeller University
Jeffrey Roberts, Cornell University

194 participants

Attendance of the phage meeting stayed strong in 1993: 194 participants submitted 156 abstracts, gave 102 talks in 10 sessions, and explained 54 posters. The usual abundance of foreign scientists included a Russian whose travel was sponsored by International Science Foundation. The meeting was dedicated to the memory of Hatch Echols, and one session contained reminiscences and scientific talks from his family, students, and colleagues.

Some especially exciting reports described a novel mechanism of transcription antitermination regulated through direct tRNA-mRNA interaction, characterized a new cell-surface sorting signal and mechanism in S. aureus, and presented a remarkable technique for visualizing RNA polymerase sliding—and occasionally jumping—along a single DNA molecule. Other presentations detailed the role of DNA in transcriptional activation mediated by CRP protein and the a subunit of RNA polymerase and reported the identification of an excreted peptide in B. subtilis that regulates competence for DNA uptake by sensing cell density. An unexpected discovery—that mutation of thioredoxin reductase allows intracellular disulfide bond formation—has important implications for expressing active, disulfide-containing foreign proteins in E. coli.

The meeting served to emphasize the continued value of bacteria in providing an increasingly complete description of basic mechanisms of cell physiology, gene regulation, recombination, replication, and protein secretion.

This meeting will be held in Wisconsin in 1994, and the attendees look forward to its return to Cold Spring Harbor in 1995 to celebrate the 50th anniversary of the Cold Spring Harbor phage course.

Contributions from Corporate Sponsors provided core support for this meeting.

L. Silver, M. Russel, P. Model
PROGRAM

RNA Polymerase and Promoter Recognition  
Chairperson: C.A. Gross, University of Wisconsin, Madison

Transcriptional Regulation: Regulatory Strategies  
Chairperson: T.J. Silhavy, Princeton University

Remembrance of Hatch Echols/Mechanisms of Transcription  
Chairperson: S. Adhya, National Cancer Institute, National Institutes of Health

Transcription Elongation, Termination, and Antitermination  
Chairperson: C. L. Squires, Columbia University

Recombination and Transposition  
Chairperson: N. Kleckner, Harvard University

Life Stories of Phage  
Chairperson: M.M. Susskind, University of Southern California, Los Angeles

Structure and Folding  
Chairperson: S. Wickner, National Cancer Institute, National Institutes of Health

Translocation/Replication  
Chairperson: J. Beckwith, Harvard Medical School

Cell Surfaces and Transport  
Chairperson: D. Dubnau, Public Health Research Institute, New York

Translation and Posttranslation Events  
Chairperson: M. Springer, Institut de Biologie Physico-Chimique, Paris, France
Mechanisms of Eukaryotic Transcription

September 1–September 5, 1993

ARRANGED BY

Winship Herr, Cold Spring Harbor Laboratory
Robert Tjian, University of California, Berkeley
Keith Yamamoto, University of California, San Francisco

447 participants

The 1993 Cancer Cells meeting, Mechanisms of Eukaryotic Transcription, was the third biennial meeting devoted to mechanisms of transcriptional regulation in eukaryotes. It brought together scientists from all over the world that are best characterized by their diversity. They study organisms as diverse as yeast and humans and use biochemical, genetic, and cell biological approaches. Major advances in our understanding of how transcription is regulated were described, including the description of a startling DNA conformation induced by the TATA-box-binding protein TBP; a plethora of TBP-containing multiprotein complexes at least one of which is targeted to a non-TATA-box regulatory element; the association of TBP and RNA polymerase II in a large multisubunit holoenzyme that participates in transcription initiation; and connections between proteins involved in transcription and in DNA repair, DNA replication, and control of the cell cycle. The meeting was elegantly summarized by Richard Losick, who brought the diverse aspects of the meeting into coherent themes.

This meeting was funded in part by the National Institute of General Medical Sciences, the National Institute of Child Health and Human Development, and the National Cancer Institute, all branches of the National Institutes of Health; and the National Science Foundation.

W. Dynan, K. Yamamoto
PROGRAM

TBP, TAFs, and Cofactors  
Chairperson: C.A. Gross, University of Wisconsin, Madison

Basal Factors and RNA Polymerases  
Chairperson: A. Sentenac, Centre d’Etudes de Saclay, France

Activation  
Chairperson: D. Reinberg, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey

Repression  
Chairperson: A.D. Johnson, University of California, San Francisco

Structure and Function of Transcription Factors  
Chairperson: C. Wu, National Cancer Institute, National Institutes of Health

Regulation of Transcription Factor Activity  
Chairperson: S. Kustu, University of California, Berkeley

Chromatin Function and Regulation  
Chairperson: M. Grunstein, University of California, Los Angeles

Factor/Factor Interplay  
Chairperson: C. Prives, Columbia University, New York

Summary: R. Losick, Harvard University

R. Roeder, D. Reinberg
This year was the fourth bi-annual meeting on Eukaryotic DNA Replication that brought together investigators who work on all aspects of the replication of the genetic material from eukaryotes. As in past years, this meeting was an extraordinarily productive gathering of most of the scientists throughout the world interested in the replication of DNA in eukaryotes. Sessions included studies on the replication of viruses that infect mammalian cells, and this year saw a very strong program that included studies on the replication of the cells’ chromosomal DNA, including the proteins that replicate DNA. Other work discussed included the identification of the elements in the chromosomes that are responsible for maintaining chromosomal integrity, including replication origins and telomeres.

For the first time this year, the program also included research on DNA-repair, which has now become intimately tied in with the understanding of the replication of DNA due to the fact that many proteins are common to both processes of replication and repair. At the meeting, there were 234 abstracts and a total of 317 participants.

This meeting was funded in part by the National Institutes of General Medical Sciences, the National Institute of Child Health and Human Development and the National Cancer Institute, all branches of the National Institutes of Health, as well as funding from the National Science Foundation.
PROGRAM

Virus DNA Replication I
Chairperson: M.D. Challberg, National Institutes of Health

Chromosomal DNA Replication
Chairperson: B.K. Tye, Cornell University

Regulation of DNA Replication
Chairperson: C. Greider, Cold Spring Harbor Laboratory

Replication Proteins
Chairperson: D. Clayton, Stanford University School of Medicine

Origins of DNA Replication
Chairperson: T. Wang, Stanford University School of Medicine

DNA Repair/Telomeres
Chairperson: M. O'Donnell, Cornell University Medical College

The Replication Fork
Chairperson: T. Formosa, University of Utah School of Medicine

Virus DNA Replication II
Chairperson: E. Fanning, Institut für Biochemie der Universität München, Germany
The eleventh annual meeting on Modern Approaches to New Vaccines Including Prevention of AIDS, like its predecessors, provided a forum for the discussion of several advances in immunoprophylaxis. Of particular importance was the introduction of several keynote speakers to address the issues involved in vaccination against bacterial and parasitic diseases, areas that are frequently neglected.

The meeting in 1992 had included some papers on immunization by direct inoculation of the DNA coding for protective protein antigens. Sufficient expression of the proteins had been obtained to afford protection against challenge infection. The increased activity in this approach to immunization in the interim required that an entire session was needed for its discussion at this year’s meeting. Clearly, much remains to be worked out, not least the efficiency of the method compared with the more conventional approach. Nevertheless, the new approach is both exciting and fascinating.

A fundamental requirement in vaccination is the need to identify the immune responses that afford protection against natural infection. These are not the same for all diseases, but it is recognized that most infections take place via mucosal surfaces. Consequently, it is gratifying that methods to achieve mucosal immunity are being vigorously pursued and were described in several papers.
The meeting continues to attract many contributions on vaccination against AIDS. This task is beset with major problems such as antigenic variation and integration of the viral genome into the host cell. Nevertheless, an understanding of the requirements for successful vaccination is gradually being reached.

Contributions from the Corporate Sponsors provided core support and in addition a generous gift from Lederle-Praxis and Pharmacia LKB Biotechnology allowed the organizers to invite several keynote speakers.

**PROGRAM**

**AIDS I: HIV—Vaccine**  
*Chairperson: H. Ginsberg, Columbia University College of Physicians & Surgeons*

**Bacteriology Symposium I**  
*Chairperson: R. Austrian, University of Pennsylvania School of Medicine, Philadelphia*

**Virology I**  
*Chairperson: F. Brown, USDA Plum Island Animal Disease Center*

**Gene Vaccination**  
*Chairperson: R. Chanock, NIAID, National Institutes of Health*

**AIDS II: SIV**  
*Chairperson: P. Nara, National Cancer Institute, National Institutes of Health*

**Bacteriology and Parasitology II**  
*Chairperson: J. Robbins, National Institute of Child Health and Human Development, National Institutes of Health*

**Immunology**  
*Chairperson: J. Berzofsky, National Cancer Institute, National Institutes of Health*

**Virology II**  
*Chairperson: A. R. Neurath, Lindsley F. Kimball Research Institute, New York Blood Center, New York*

**AIDS III: HIV—Pathogenesis and Immunology**  
*Chairperson: E. Norrby, Karolinska Institute Stockholm, Sweden*

**Summary:**  
E. Norrby, Karolinska Institute
Molecular and Cellular Biology of Plasminogen Activation

September 28–October 3, 1993

ARRANGED BY

Francesco Blasi, Dipartimento di Genetica, Università di Milano, and DIBIT, H.S. Raffaele, Milan, Italy
Keld Dano, Finsen Laboratory, Rigshospitalet, Copenhagen, Denmark
James Quigley, State University of New York School of Medicine, Stony Brook
Daniel Rifkin, New York University School of Medicine, New York
Sidney Strickland, State University of New York School of Medicine, Stony Brook

227 participants

The fourth international meeting on the Molecular and Cellular Biology of Plasminogen Activation was held at Cold Spring Harbor Laboratory September 28, 1993; 227 people from Europe, North America, and Asia attended this meeting. The varied functions of this enzymatic system in both normal and pathological processes ranging from clot lysis to neural development were considered. The span of presentations went from physical-chemical analysis of structure to phenotypic analysis of "knock out" mice for urokinase and tissue-type plasminogen activators. The primary foci of the presentations were (1) regulation of plasminogen activation, (2) role of plasminogen activation in normal cellular and tissue physiology, and (3) role of plasminogen activation in disease states.

This meeting was funded in part by American Diagnostica Inc.; National Institutes of Health, National Institute of Child Health and Human Development; Boehringer Mannheim Italia; Boehringer Mannheim GMBH; Miles Inc. Pharmaceutical Division; Abbott Laboratories Pharmaceutical Products Division; and the Genetics Institute.

K. Karito, D. Belin, H. Chapman
PROGRAM

Urokinase Receptor: Structure/Function
Chairpersons: E. Reich, State University of New York, Stony Brook
G.S. Lazarus, University of Pennsylvania, Philadelphia

Plasminogen Activator Inhibitors
Chairpersons: R. Miskin, Weizmann Institute of Science, Israel
D.C. Rijken, Gaubius Laboratory, The Netherlands

Cancer
Chairpersons: P. Verde, International Institute of Genetics and Biophysics, CNR, Naples, Italy
T.D. Gelehrter, University of Michigan, Ann Arbor

Mechanisms of Regulation: Promoters
Chairpersons: D.B. Rifkin, New York University Medical Center
R.L. Medcalf, Central Hematology Laboratory, Lausanne, Switzerland

Proteolytic Cascades: Other Reactions
Chairpersons: M. Ploug, Finsen Laboratory, State University Hospital, Copenhagen, Denmark
W.-D. Schleuning, Schering Research Laboratories, Berlin, Germany

Mechanisms of Internalization of PA and PA Inhibitors
Chairpersons: D.J. Loskutoff, Scripps Research Institute
K. Dane, Finsen Laboratory, Rigshospitalet, Copenhagen, Denmark

Plasminogen Activator in Nontumor Systems
Chairpersons: J.P. Quigley, State University of New York, Stony Brook
E.L. Wilson, New York University Medical Center

Plasminogen Activator in Other Diseases
Chairpersons: F. Blasi, University of Milan, Italy, DIBIT, H.S. Raffaele, and University of Copenhagen, Denmark
T. Ny, Umeå University, Sweden

Transgenic Mice as Genetic Systems for PA Studies
Chairpersons: A. Vaheri, University of Helsinki, Finland
M. Johnsen, University of Copenhagen, Denmark

Transgenics (Continued)
Chairpersons: A. Vaheri, University of Helsinki, Finland
M. Johnsen, University of Copenhagen, Denmark

Discussion: Points of Contention
Chairpersons: D. Belin, University of Geneva Medical School, Switzerland
L. Ossowski, Mount Sinai School of Medicine, New York

Structural Studies
Chairpersons: S. Strickland, State University of New York, Stony Brook
J. Henkin, Abbott Laboratories, Illinois
270 participants

The fifth meeting of the Neurobiology of Drosophila attracted some 270 participants from the United States, Europe, and Asia. A wide range of topics were covered in the areas of development, neural function, and behavior. There were seven formal lecture sessions and three excellent poster sessions. Whereas in previous meetings, the rapid progress in dissecting developmental processes and the isolation and characterization of ion chemicals dominated the highlights, this year was noteworthy with exciting progress reported on the molecular and genetic dissection of synaptic transmission. The convergence of these studies with secretion in yeast and mammalian systems is a very important development in the field. This year marked the occasion of the 2nd Elkins Lecture to honor an outstanding Ph.D. thesis in the Neurobiology of Drosophila. This year’s speaker was Dr. Ilaria Rebay who described her studies on the Notch protein completed in Dr. Spyro Artavanis-Tsakonas’ laboratory at Yale University.

This meeting was funded in part by the National Science Foundation and the National Institute of Neurological Disorders and Stroke, a branch of the National Institutes of Health.
J. Posakony

PROGRAM

Introductory Comments

Neurogenesis
Chairperson: J. Posakony, University of California, San Diego

Axon Guidance, Connectivity, and Synaptogenesis
Chairperson: C.S. Goodman, Howard Hughes Medical Institute and University of California, Berkeley

Neurotransmitter Function, Metabolism, and Receptors
Chairperson: P.H. Taghert, Washington University Medical School, St. Louis, Missouri

Elkins Lecture
Excitability and Synaptic Transmission
Chairperson: T.L. Schwarz, Stanford University Medical Center

Sensory System Development
Chairperson: E. Hafen, Zoologisches Institut, Universität Zürich, Switzerland

Sight, Smell, and Touch
Chairperson: J. O'Tousa, University of Notre Dame

Brain Structure and Behavior
Chairperson: M. Heisenberg, Lehrstuhl für Genetik, Theodor-Boveri-Institut, Würzburg, Germany

L. Zipursky, C. Goodman
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.

Construction and Analysis of Two-Dimensional Gel Protein Databases

April 13–April 20

INSTRUCTORS

Franza Jr., B. Robert, Ph.D., Cold Spring Harbor Laboratory
Garrels, James I., Ph.D., Cold Spring Harbor Laboratory
Latter, Gerald I., Ph.D., Cold Spring Harbor Laboratory
Patterson, Scott D., Ph.D., Cold Spring Harbor Laboratory

This course presented the practical aspects of constructing and analyzing a protein database through quantitative analysis of two-dimensional gels. The instructors lectured on methods of two-dimensional gel electrophoresis, strategies and methodologies for database construction, analysis of protein databases, and methods for characterization and identification of proteins in two-dimensional gels. In the hands-on part of the course, students built and analyzed a protein
database from existing two-dimensional gel images using the Quest II software. Topics in database construction included data acquisition, protein quantitation, and matching of proteins across sets of gels. Topics in database analysis included retrieval of quantitative data, searches for common expression profiles, and analysis to determine relatedness of protein samples. Guest speakers and instructors covered applications in cell regulation, medical applications, protein identifications in two-dimensional gels, and connections to genome sequencing projects. This course was intended for those who have prior experience with two-dimensional gels.

PARTICIPANTS
Amess, R., Ph.D., University of Oxford, England
Corbett, J., B.A., Harefield Hospital, Harefield, England
Dean, D., M.S., Pfizer Central Research, Groton, Connecticut
Kanitz, M., Ph.D., National Institute for Occupational Safety and Health, Cincinnati, Ohio
Madden, B., Ph.D., University College, Dublin, Ireland
Martzen, M., Ph.D., University of Rochester, New York
Pleissner, K.-P., Ph.D., Deutsches Herzzentrum, Berlin, Germany
Smith, D., Ph.D., University of California, San Francisco
Tanzer, L., B.S., Lilly Research Laboratories, Indianapolis, Indiana
Volker, U., Ph.D., University Greifswald, Germany

SEMINARS
Aebersold, R., University of British Columbia. Protein isolation and microanalysis at the primary structure level.
Celis, J., University of Aarhus. Comprehensive two-dimensional gel protein databases and cDNA cloning; toward linking protein and DNA information.
Hanash, S., University of Michigan. Lymphoid protein database.
Hochstrasser, D., University Hospital Geneva. Clinical applications of 2D-PAGE.
Latham, K., Temple University. Construction and applications of a mouse embryo database.
McLaughlin, C., University of California, Irvine. Construction of the yeast database.

Cloning and Analysis of Large DNA Molecules

April 13–April 26

INSTRUCTORS
Abderrahim, Hadi, M.D., Ph.D., Cell Genesys, Inc., Foster City, California
Birren, Bruce, Ph.D., California Institute of Technology, Pasadena
Klaholz, Sue, M.D., Ph.D., Cell Genesys, Inc., Foster City, California
Vollrath, Doug, M.D., Ph.D., Whitehead Institute, Cambridge, Massachusetts

ASSISTANTS
Mendez, Michael, Cell Genesys, Inc., Foster City, California
Van Bokklen, Gil, Stanford University, California
Wang, David, California Institute of Technology, Pasadena

This course covered the theory and practice of manipulating and cloning high-molecular-weight DNA. The course focused on the use of yeast artificial chromosome (YAC), bacterial artificial chromosome (BAC), and bacteriophage P1 cloning systems for library construction and techniques of pulsed-field gel electrophoresis (PFGE). Lectures and laboratory work included an introduction to
yeast genetics, the isolation and manipulation of high-molecular-weight DNA from a variety of sources, and preparative and analytical PFGE. YAC, BAC, and P1 clones were produced and characterized by a number of approaches, including hybridization, recovery of YAC ends, physical mapping, and PCR-based methods. A variety of size standards for PFGE were prepared, and their separations on many different types of PFGE gel boxes were compared. Lectures by outside speakers on topics of current interest supplemented the laboratory work.

PARTICIPANTS
Baez-Camargo, M., B.S., Cinvestav-IPN, Mexico City, Mexico
Chen, H., Ph.D., Harvard Medical School, Boston, Massachusetts
Dewar, K., M.S., University Laval, Ste-Foy, Canada
Funke, R., M.S., University of Tennessee, Knoxville
Gardner, J., Ph.D., Fox Chase Cancer Center, Philadelphia, Pennsylvania
Iraqi, F., Ph.D., USDA, East Lansing, Michigan
Janaswami, P., Ph.D., Jackson Laboratory, Bar Harbor, Maine
Kaur, G., Ph.D., New Jersey Medical School, Newark

SEMINARS
Beach, D., Cold Spring Harbor Laboratory. Genome mapping in fission yeast.
Ecker, J., University of Pennsylvania. Progress toward a complete map of the Arabidopsis genome.
Foote, S., Whitehead Institute. Cloning human chromosomes in YACs: Yesterday the Y, tomorrow the genome.
Gemmill, R., Eleanor Roosevelt Institute. Isolation and mapping of YACs on human chromosome 3.
Lai, E., University of North Carolina. Pulsed-field gel techniques for large DNA.
Shepherd, N., DuPont Merck Pharmaceutical. The P1 cloning system.
Shizuya, H., California Institute of Technology. Large fragment cloning with the BAC system.
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 and 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 instrumen-
tal 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
Baserga, S., Ph.D., Yale University, New Haven Connecticut
Bockus, B., Ph.D., Tufts Medical School, Boston, Massachusetts
Cohn, M., Ph.D., University of California, San Francisco
DePinho, R., M.D., Albert Einstein College of Medicine, New York, New York
Greenberg, M., Ph.D., Wayne State University, Detroit, Michigan
Jin, P., Ph.D., Harvard Medical School, Boston, Massachusetts
Jordan, S., Ph.D., Trinity College, Dublin, Ireland
Joy, J., Ph.D., St. Elizabeth's Hospital, Washington, D.C.

SEMINARS
Burgess, R., University of Wisconsin, Madison. Protein purification: Basic methods.
Guidotti, G., Harvard University. Membrane proteins.
Pace, C.N., Texas A&M University. Thermodynamics of protein folding.
Paterson, Y., University of Pennsylvania. Preparation of antipeptide antibodies.

Lazard, D., Ph.D., Institut Cochin, Paris, France
Martinez del Rio, C., Ph.D., Princeton, University, New Jersey
Miller, M., B.S., University of California, Irvine
Myer, V., M.S., Yale University, New Haven, Connecticut
Rincon-Limas, D., M.S., Baylor College of Medicine, Houston, Texas
Sandmeyer, S., Ph.D., University of California, Irvine
Tetzloff, S., M.S., University of New Mexico, Albuquerque
Zack, D., Ph.D., Johns Hopkins University, Baltimore, Maryland

Patterson, S., Cold Spring Harbor Laboratory. Two-dimensional gel electrophoresis.
Reinberg, D., University of Medicine and Dentistry of New Jersey. Transcription by RNA polymerase II.
Rose, G., Washington University School of Medicine. Fundamentals of protein structure.
Rothman, J., Memorial Sloan Kettering Cancer Center. Protein trafficking.
Stillman, B., Cold Spring Harbor Laboratory. DNA replication in eukaryotes.
Tonks, N., Cold Spring Harbor Laboratory. Protein phosphorylation.

Early Development of Xenopus laevis

April 14–April 22

INSTRUCTORS
Harland, Richard, Ph.D., University of California, Berkeley
Sive, Hazel, Ph.D., Whitehead Institute, Cambridge, Massachusetts

ASSISTANTS
Doniach, Tabitha, University of California, San Francisco
Wilson, Paul, Harvard University, Cambridge, Massachusetts

This course provided extensive laboratory exposure to the biology and manipulation of embryos from the frog, *Xenopus laevis*. The course was particularly suited for investigators who have a current training in molecular biology and knowledge
of developmental biology but had no experience with *Xenopus*. Intensive laboratory sessions were supplemented by daily lectures from experts in both experimental and molecular embryology. Six main areas were covered: (1) care and handling of adults and embryos, (2) stages of embryonic development and anatomy, (3) whole-mount in situ hybridization and immunocytochemistry, (4) microinjection of eggs and oocytes, (5) micromanipulation of embryos, and (6) induction assays.

PARTICIPANTS
Agus, N., M.S., Albert Einstein College of Medicine, New York, New York
Cecchini, M., B.A., University of Colorado, Denver
Dompenciel, R., Ph.D., USUHS, Bethesda, Maryland
Howell, M., Ph.D., ICRF, London, England
Kondo, M., M.S., University of Tokyo, Japan
Lohmann, E., M.S., University of Essen, Germany
Mantell, L., Ph.D., Cold Spring Harbor Laboratory, New York
Martin, K., B.S., Duke University, Durham, North Carolina
Mead, P., Ph.D., University of Auckland, New Zealand
Pierceall, W., Ph.D., Yale University, New Haven, Connecticut
Shuldiner, A., M.D., Johns Hopkins University, Baltimore, Maryland
Seidman, S., M.S., Hebrew University, Jerusalem, Israel
Snider, L., Ph.D., Fred Hutchinson Cancer Center, Seattle, Washington
Suzuki, K., Ph.D., Frederick Cancer Research Center, Frederick, Maryland
Vignali, R., Ph.D., Universita Degli Studi di Pisa, Italy
Zuber, M., Ph.D., Colorado State University, Fort Collins

SEMINARS
Gerhart, J., University of California, Berkeley. Axis formation in *Xenopus laevis*.
Harland, R., University of California, Berkeley. Expression cloning of new cytokines, patterning of the mesoderm, and neural induction.
Harris, B., University of California, San Diego. Development of the *Xenopus* nervous system.
Keller, R., University of California, Berkeley. Gastrulation.
Sive, H., Whitehead Institute. Anteroposterior patterning of the *Xenopus* embryo.
Wickens, M., University of Wisconsin, Madison. Translational control and oogenesis.
In recent years, advances in neuroimaging have opened exciting new avenues in the study of the human brain. In particular, there is growing interest in the area of functional neuroimaging of indirect measures of neuronal activity related to different task states. Since this is a relatively recent development, there is considerable discussion on the ways in which neuroimaging can be applied to basic neurobiological problems. This lecture course was designed to survey questions at several levels, including a general overview of the performance characteristics of the different technologies, focusing on positron emission tomography (PET) and functional magnetic resonance imaging (MRI); the way data are collected and reconstructed into various image formats; assessment of image quality and significance; applications of the different technologies to studies in the areas of vision, attention, language, etc., in normal populations; and applications of functional neuroimaging to abnormal populations. These subjects were presented in lecture and discussion, as well as with hands-on manipulation of images on computer workstations.

Since the course was designed as a survey with ample time for discussion of problems and applications, both scientists with a direct interest in neuroimaging approaches and systems and behavioral neuroscientists with an interest in understanding neuroimaging in relation to their current approaches were encouraged to apply.
Advanced Bacterial Genetics

June 11–July 1

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
Brown, C., University of Tennessee, Memphis
Lin, J., Cornell University, Ithaca, New York
Ostrovsky de Spicer, P., University of Illinois, Urbana-Champaign

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 included the isolation, characterization, and mapping of mutations; use of transposable genetic elements as mutagens, linked selectable markers, and portable regions of homology; construction and analysis of operon and gene fusions; use of bacteriophage in genetic analysis; molecular cloning and restriction endonuclease mapping; allele...
exchange; Southern blot analysis; polymerase chain reaction; and site-specific mutagenesis. The course consisted of a series of experiments that employed these techniques in the genetic analysis of diverse bacterial and bacteriophage species. Lectures and discussions concentrated on the application of genetic analysis to contemporary questions in bacterial physiology, diversity, and pathogenesis.

PARTICIPANTS
Anderson, P., B.A., University of California, Los Angeles
Andrews-Cramer, K., Ph.D., Washington University, St. Louis
Armitage, L., B.A., University of Texas, Houston
Cipriani, K., B.S., Purdue University, Lafayette, Indiana
Collazo-Custodio, C., B.S., SUNY at Stony Brook, New York
Flarh, K., M.S., University of Goteborg, Sweden
Gil, A., M.S., Kings College, London, England
Hammar, M., M.S., Washington University, St. Louis
Hansson, K., M.S., Uppsala University, Sweden
Mackenzie, R., Ph.D., University of Texas, Houston
Marini, P., Ph.D., Universidad Nacional de Rosario, Argentina
Ramakrishnan, L., Ph.D., Stanford University, California
Toro, C., M.S., Universidad Catolica, Santiago, Chile
Tsolis, R., B.S., Oregon Health Sciences University, Portland
Vanet, A. M.S., Institut de Biologie, Paris, France
Webber, C., B.S., University of Virginia, Charlottesville

SEMINARS
Grossman, A., Massachusetts Institute of Technology. Genetics of signal transduction and development in Bacillus subtilis.
Herskowitz, I., University of California, San Francisco. The lysis-lysogeny decision of bacteriophage λ.
Miller, V., University of California, Los Angeles. Isolation and characterization of invasion-defective mutants of Salmonella enteritidis.
Trun, N., National Cancer Institute. Genetics of altered ploidy in E. coli.
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 pre-implantation 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, retroviral infection of embryos, microinjection of cell lineage tracers, in situ hybridization, and immunohistochemistry. Guest lecturers discussed current research in the field.
Molecular Approaches to Ion Channel Expression and Function

June 11–July 1

INSTRUCTORS
Enyeart, Jack, Ph.D., Ohio State University, Columbus
Goldin, Al, M.D., Ph.D., University of California, Irvine
Papazian, Diane, Ph.D., University of California, Los Angeles
Ruben, Peter, Ph.D., University of Hawaii, Honolulu

This intensive laboratory/lecture course was designed to introduce students to the combined use of molecular biological techniques and electrophysiological analysis for the study of neurotransmitter receptors and voltage-gated ion chan-
nels. The course covered expression of cloned channels and receptors in *Xenopus* oocytes, including preparation of RNA transcripts in vitro, microinjection in oocytes, in vitro mutagenesis, and characterization of channels and receptors in oocytes using two-microelectrode voltage clamping and patch clamping. Additional topics included using *Baculovirus* to express transiently voltage-gated ion channels in insect cells and using patch clamp techniques to characterize channels and receptors in cultured cells. Lectures covered the theory and analysis of ionic currents.

**PARTICIPANTS**

Altan, N., B.A., Rockefeller University, New York, New York  
Campo, M., Ph.D., University Extremadura, Caceres, Spain  
Conley, P., Ph.D., COR Therapeutics, South San Francisco, California  
Cross, K., B.S., University of Southampton, England  
Koch, B., Ph.D., Syntex Discovery Research, Palo Alto, California  
Kuziemko, G., B.S., University of California, Berkeley

Laukkanen, M.-L., M.S., Technical Research Centre, Espoo, Finland  
Lautermilch, N., B.S., University of California, San Diego  
Lieberman, M., B.S., University of Vermont, Burlington  
Magoski, N., B.S., University of Calgary, Canada  
Schulteis, C., B.S., University of California, Los Angeles  
Witchel, H., Ph.D., University of Bristol, England

**SEMINARS**

Adelman, J., Vollum Institute. A novel mechanism for ion channel activation.  
Andersen, O., Cornell University. Kinetic analysis of ion movement through channels.  
Bezanilla, F., University of California, Los Angeles. Voltage-dependent channels.  
Enyeart, J., Ohio State University. A novel potassium current that regulates steroid hormone secretion.  
Goldin, A., University of California, Irvine. Molecular basis of sodium channel inactivation.  
Kallen, R., University of Pennsylvania. Striated muscle voltage-sensitive sodium channel in health and disease.  
Lindstrom, J., University of Pennsylvania. Structure and function of the nicotinic acetylcholine receptor.  

Mandel, G., SUNY at Stony Brook. Regulation of a neuron-specific sodium channel.  
Papazian, D., University of California, Los Angeles. Biochemistry and activation of potassium channels.  
Ruben, P., University of Hawaii. Sodium channels: Macro-patches, mutations, mode switches, and more.  
Sigworth, F., Yale University. Yet more stuff on Shaker potassium channels.  
Trimmer, J., SUNY at Stony Brook. Potassium channel expression in mammalian cells.  
White, M., Medical College of Pennsylvania. Insights on the acetylcholine receptor.
INSTRUCTORS

Tobias Bonhoeffer, Ph.D., Max Planck Institute, Martinsried, Germany
David Fitzpatrick, Ph.D., Duke University Medical Center, Durham, North Carolina

This lecture/discussion course explored the functional organization and development of the visual system as revealed by the use of a variety of anatomical, physiological, and behavioral methods. It was designed for graduate students and more advanced researchers who wish to gain a basic understanding of the biological basis for vision and to share in the excitement of the latest developments in this field. Topics included phototransduction and neural processing in the retina; gating functions of the lateral geniculate nucleus; functional architecture of striate cortex; cellular basis of cortical receptive field properties; the anatomy, physiology, and perceptual significance of parallel pathways; functional parcellation of extrastriate cortex; sensory-motor integration in the superior colliculus; and role of patterned neuronal activity in the development of central visual pathways.

PARTICIPANTS

Alonso, J., Ph.D., Rockefeller University, New York, New York
Feller, M., Ph.D., AT&T Bell Laboratory, Murray Hill, New Jersey
Freiwold, W., M.S., Max Planck Institut, Frankfurt, Germany
Gibson, J., B.A., Baylor College of Medicine, Houston, Texas
Gustincich, S., Ph.D., C.I.B., Trieste, Italy
Jaworski, D., Ph.D., Yale Medical School, New Haven, Connecticut
Mejia-Monasterio, N., M.S., Boston University, Massachusetts
Arabidopsis Molecular Genetics

July 5–July 25

INSTRUCTORS

Chory, Joanne, Ph.D., Salk Institute, San Diego, California
Ecker, Joseph, Ph.D., University of Pennsylvania, Philadelphia
Theologis, Athanasios, Ph.D., University of California, Berkeley

ASSISTANTS

Abel, Steffen, University of California, Berkeley
Bell, Callum, University of Pennsylvania, Philadelphia
Poole, Dan, Salk Institute, San Diego, California

This course provided an intensive overview of current topics and techniques in Arabidopsis biology, with an emphasis on molecular genetics. It also introduced approaches used in yeast that have the potential to be utilized for the advancement of Arabidopsis molecular genetics. It was designed for scientists with experience in molecular techniques who are working or wish to work with Arabidopsis.
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 Arabidopsis genetics and development; transient gene expression assays in protoplasts; gene transplacement and complementation of yeast mutants for cloning Arabidopsis genes; transformation by Agrobacterium; in situ detection of RNA; pulsed-field gel electrophoresis and analysis of yeast artificial chromosomes containing the Arabidopsis genome; use of PCR-based markers for genetic mapping of mutations; and a tutorial on access and use of the Arabidopsis resource databases available on the internet.

PARTICIPANTS
Batchelder, C., Ph.D., John Innes Centre, Norfolk, England
Belostotsky, D., Ph.D., University of Georgia, Athens
Bruggemann, E., Ph.D., National Institutes of Health, Bethesda, Maryland
Grater, T., B.S., Ciba Seeds, Research Triangle Park, North Carolina
Johnson, K., Ph.D., Dartmouth Medical School, Hanover, New Hampshire
Kakutani, T., Ph.D., Washington University, St. Louis, Missouri
Kniessl, M., B.S., Penn State University, University Park
Levy, Y., B.S., Brandeis University, Waltham, Massachusetts
Oliveira, R., B.S., New York University, New York

SEMINARS
Baker, B., Plant Gene Expression Center. Ac-Ds insertional mutagenesis in Arabidopsis.
Bender, J., Whitehead Institute. Yeast complementation.
Bell, C., University of Pennsylvania. Physical mapping of the Arabidopsis genome.

Pilgrim, M., B.A., Dartmouth College, Hanover, New Hampshire
Ronemus, M., B.A., Yale University, New Haven, Connecticut
Shah, J., Ph.D., Waksman Institute, Piscataway, New Jersey
Siddiqi, I., Ph.D., Centre for Cellular & Molecular Biology, Hyderabad, India
Snowden, K., M.S., University of Auckland, New Zealand
Trentmann, S., Ph.D., Michigan State University, East Lansing
Zhong, H.-H., B.S., Dartmouth College, Hanover, New Hampshire

Bowler, C., Rockefeller University. Phytochrome signal transduction pathways.
Callis, J., University of California, Davis. Proteolysis and the ubiquitin pathway in higher plants.
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 procedures), plaque screening techniques (probe hybridization, antibody interaction), DNA sequencing, PCR amplification, RNA amplification, and DNA-mediated gene transfer. A portion of the laboratory work was devoted to characterization and analysis of nucleic acids from single live neurons and glia. 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
Amato, A., M.S., University College London, England
Connolly, J., Ph.D., University of Strathclyde, Glasgow, Scotland
Cooke, H., Ph.D., Ohio State University, Columbus
Corfas, G., Ph.D., Harvard Medical School, Boston, Massachusetts
Galli, T., Ph.D., Yale University, New Haven, Connecticut
Hunter, K., Ph.D., Rockefeller University, New York, New York
Levin, M., M.D., Cornell Medical Center, New York, New York
Lu, Q., Ph.D., Emory University, Atlanta, Georgia
Macagno, E., Ph.D., Columbia University, New York, New York
Metcalf, A., M.S., University of Texas, Dallas
Murphy, T., Ph.D., Johns Hopkins University, Baltimore, Maryland
Olschowka, J., Ph.D., University of Rochester, New York
Oyesiku, N., M.D., Emory University, Atlanta, Georgia
Rehder, V. Ph.D., Colorado State University, Fort Collins
Streit, A., Ph.D., University of Oxford, England
Witchel, H., Ph.D., University of Bristol, England

SEMINARS
Edwards, R., University of California, Los Angeles.
Molecular cloning by functional expression.
Ganetzky, B., University of Wisconsin-Madison. Genetic analysis of ion channels in Drosophila: From mutants to molecules and from molecules to mutants.
Julius, D., University of California, San Francisco. Molecular biology of serotonin and ATP receptors.
Lemke, G., Salk Institute. Molecular genetics of myelination.
Mandel, G., SUNY at Stony Brook. Regulation of a neuron-specific Na** channel gene.
Morgan, J., Roche Institute of Molecular Biology. Molecular biology of development and plasticity in the nervous system.
Poo, M., Columbia University. Reconstitution of transmitter secretion in nonneuronal cells.
Reed, R., Johns Hopkins University. The molecular mechanisms of diversity in odorant recognition.
Neurobiology of Drosophila

July 5–July 25

INSTRUCTORS
Bieber, Alan, Ph.D., Purdue University, West Lafayette, Indiana
Hardie, Roger, Ph.D., University of Cambridge, United Kingdom
Taghert, Paul, Ph.D., Washington University, St. Louis, Missouri

ASSISTANTS
Hall, Stephen, Purdue University, West Lafayette, Indiana
Silber, Judy, Washington University, St. Louis, Missouri

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, em-
bryonic and postembryonic development of the CNS and PNS, axonal pathfind-
ing, development of the nervous system, mesoderm and muscle development,
eye and optic lobe development, olfaction, learning, and the neural control of
flight.

PARTICIPANTS
Bates, D., Ph.D., University of Glasgow, Scotland
Brewster, R., M.S., University of Michigan, Ann Arbor
Ericsson, C., Ph.D., Karolinska Institutet, Stockholm, Sweden
Kania, A., B.S., Baylor College of Medicine, Houston, Texas
Li, Q., M.S., Dalhousie University, Halifax, Canada
Lin, F., Ph.D., Salk Institute, La Jolla, California

SEMINARS
Bieber, A., Purdue University. Axon pathfinding.
Crews, S., University of California, Los Angeles. Neurogenesis I.
Davis, R., Cold Spring Harbor Laboratory. Learning and memory I.
Doe, C., University of Illinois. Neurogenesis II.
Ffrench-Constant, R., University of Wisconsin. Ligand-gated receptors.
Ganetzky, B., University of Wisconsin. Ion channel studies.
Hardie, R., Cambridge University. (1) Visual systems. (2) Introductory electrophysiology.
Hartenstein, V., University of California, Los Angeles. Embryogenesis early neurogenesis.
Isacoff, E., University of California, Berkeley. K+ channel structure and function.

The Biology of Memory: From Molecules to Behavior

July 9–July 22

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

Ajima, A., Ph.D., Frontier Research Program, Saitama, Japan
Borroni, A., Ph.D., Max Planck Institute, Frankfurt, Germany
Burwell, R., Ph.D., Salk Institute, San Diego, California
Gerlai, R., Ph.D., Mt. Sinai Hospital, Toronto, Canada
Gewirtz, J., M.S., Yale University, New Haven, Connecticut
Kim, J., Ph.D., University of Southern California, Los Angeles
Kornau, H.-C., B.S., Zentrum fur Molekulare Biologie, Heidelberg, Germany
Krupa, D., Ph.D., University of Southern California, Los Angeles
Lopez-Garcia, J., M.S., Columbia University, New York, New York
Rajachandran, L., Ph.D., Neurogen Corporation, Branford, Connecticut

SEMINARS

Byrne, J., University of Texas, Houston. (1) Overview of membranes and synaptic transmission I. (2) Overview of membranes and synaptic transmission II. (3) Learning in Aplysia III: Classical conditioning.
Crow, T., University of Texas, Houston. Cellular mechanisms of associative learning in Hemisenda.
Davis, M., Yale University. Neural mechanisms of fear conditioning.
Ramus, S., M.S., University of California, San Diego
Rogan, M., B.A., New York University, New York
Roy, R., Ph.D., Laval University, Quebec, Canada
Sheffield, L., M.S., University of Kansas, Kansas City
Stanhope, K., Ph.D., Wyeth Research Ltd., Berkshire, England
Sun, W., B.S., University of California, San Diego
Van Praag, H., Ph.D., Robert Wood Johnson Medical School, Piscataway, New Jersey
Wang, Y., B.S., University of Miami, Florida
Wicks, S., B.S., University of British Columbia, Vancouver, Canada
Wood, E., Ph.D., University of Oregon, Eugene
Zhang, F., M.S., University of Texas, Houston

Davis, R., Cold Spring Harbor Laboratory. 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: Genetic analysis of ion channels and membrane excitability in Drosophila.
Greenough, W., University of Illinois. Morphological correlates of learning and experience.
Holland, P., Duke University. Introduction to learning theory.
Kaas, J., Vanderbilt University. Cortical plasticity and learning.
Kandel, E., Columbia University. (1) Introduction to the cellular study of learning. (2) Learning in Aplysia I: Habituation. (3) Learning in Aplysia II: Sensitization. (4) Long-term potentiation II.
Nicoll, R., University of California, San Francisco. Long-term potentiation I.
Nottebohm, F., Rockefeller University. Bird-song learning.

Pfaffinger, P., Baylor College of Medicine. (1) Cloning of genes important to learning I. (2) Cloning of genes important to learning II.
Rankin, C., University of British Columbia. Genes and the behavior of C. elegans.
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.
Thompson, R., University of Southern California. Classical conditioning of the nictitating membrane response.

Developmental Neurobiology

July 24–August 6

INSTRUCTORS
Greg Lemke, Ph.D., The Salk Institute, San Diego, California
Dennis O'Leary, Ph.D., The Salk Institute, San Diego, California

The aim of this lecture course was to discuss established principles and recent advances in developmental neurobiology. Major topics considered were proliferation, migration, and aggregation of neurons; determination and differentiation of neural cells; trophic interactions in neural development; patterns, gradients, and compartments; genetic programs for development; the guidance of axons to targets; and the formation of synaptic connections. These topics were considered within the context of the development of both invertebrate and vertebrate neural systems. Students had a background in neurobiology or molecular biology.
PARTICIPANTS
Chen, M., Ph.D., Cambridge Neuroscience, Massachusetts
Dietz, G., B.S., Rockefeller University, New York, New York
Fernandes, J., M.S., Tata Institute of India, Bombay
Habecker, B., Ph.D., Case Western Reserve University, Cleveland, Ohio
Heller, S., B.S., Max Planck Institute, Frankfurt, Germany
Heyman, I., M.S., Guy’s Hospital, London, England
Hutson, L., B.A., University of Washington, Seattle
Jensen, A., Ph.D., University College London, England
Lawrence, A., B.S., University College London, England
McCullumsmith, C., B.S., University of Michigan, Ann Arbor
Orr Urtreger, A., Ph.D., Baylor College of Medicine, Houston, Texas

SEMINARS
Bronner-Fraser, M., University of California, Irvine. Neural crest differentiation and specification.
Chao, M., Cornell University. Neurotrophin receptors (molecular biology).
Fraser, S., California Institute of Technology. Retino-tectal development.
Goodman, C., University of California, Berkeley. Molecular genetics of pathfinding in simple systems.
Jessell, T., Columbia University. Axial specification and dorsal-ventral patterning of the vertebrate neuroepithelium.
Kintner, C., Salk Institute. Neural induction and early patterning of the vertebrate nervous system.
Kuwada, J., University of Michigan. Axonal development and scaffolding in Zebrafish.

Yeast Genetics
July 27–August 16

INSTRUCTORS
Kaiser, Chris, Ph.D., Massachusetts Institute of Technology, Cambridge
Michaelis, Susan, Ph.D., Johns Hopkins University, Baltimore, Maryland
Mitchell, Aaron, Ph.D., Columbia University, New York, New York

ASSISTANTS
Baker, Cary, Johns Hopkins University, Baltimore, Maryland
Hammer, Ruth, Massachusetts Institute of Technology, Cambridge
Smith, Harold, Columbia University, New York, New York

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 elec-
trophoretic 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
Arkowitz, R., Ph.D., University of California, Los Angeles
Ben Yaacov, R., M.S., Tel Aviv University, Israel
Caron, J., Ph.D., University of Connecticut, Farmington
Franssen, J.-H., M.D., Erasmus University, Rotterdam, The
Netherlands
Frydman, J., Ph.D., Memorial Sloan-Kettering Cancer Cen-
ter, New York, New York
Ghannoum, M., Ph.D., Harbor-UCLA Medical Center, Tor-
rance, California
Griffin, L., B.S., University of Massachusetts, Worcester
Izumi, T., Ph.D., University of Colorado, Denver
Laybourn, P., Ph.D., Colorado State University, Fort Collins
Leidich, S., B.S., University of Illinois, Urbana
McMaster, C., Ph.D., Duke University Medical Center, Dur-
ham, North Carolina
Mukherjee, M., B.S., Johns Hopkins University, Baltimore,
Maryland
Okorokova, A., M.S., Federal University of Rio de Janeiro,
Brazil
Payvar, F., Ph.D., St. Louis University, Missouri
Reinero, A. Ph.D., American Cyanamid Company, Princeton,
New Jersey
Zaman, S., Ph.D., National University of Singapore

SEMINARS
Adams, A., University of Arizona. Genetic analysis of the ac-
tin cytoskeleton.
Carlson, M., Columbia University. Transcriptional control in
response to glucose.
Elledge, S., Baylor College of Medicine. Heterologous ex-
pression cloning.
Fink, G., Whitehead Institute. Control of pseudohyphal
growth.
Futcher, B., Cold Spring Harbor Laboratory. Role of cyclins
in cell cycle control.
Herskovitz, I., University of California, San Francisco. Yeast
mating type.
Hieter, P., Johns Hopkins University. Molecular genetics of
the yeast kinetochore.
Kaiser, C., Massachusetts Institute of Technology. Genetic
analysis of early steps in secretion.
This course focused on both the cloning and characterization of eukaryotic genes to probe their structure, function, and expression. As a model system, students examined cis- and trans-acting components involved in the regulation of eukaryotic gene expression. Eukaryotic transcription factors were expressed in E. coli and purified by affinity chromatography. Mutations were generated in the DNA-binding domain of these factors by oligonucleotide-directed and random mutagenesis procedures and characterized by DNA sequencing and DNA-binding assays. Using both the wild-type and mutant proteins, students learned the techniques and theory for detecting and characterizing the interaction between regulatory DNA sequences and trans-acting protein factors. In addition, transfection was used to introduce cloned DNA molecules that had been manipulated in vitro into mammalian tissue culture cells. The regulated expression of these transfected DNAs were analyzed by nuclease protection and enzymatic assays. Finally, expression libraries from various organisms were prepared and screened with recognition site probes for specific DNA-binding proteins as a means of learning the theoretical and practical aspects of constructing cDNA libraries. Guest lecturers discussed current 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
Amfo, K., M.S., Vrije University, Brussels, Belgium
Filler, S., M.D., Harbor-UCLA Medical Center, Torrance, California
Frostesjo, L., B.S., University of Umea, Sweden
Gadbut, A., Ph.D., Brigham & Women’s Hospital, Boston, Massachusetts
Gallo, R., Ph.D., Children's Hospital, Boston, Massachusetts
Gamberi, C., Ph.D., University of Verona, Italy
Hansen, R., B.S., Loma Linda University, California
Hardin, M., B.S., University of South Carolina, Columbia
Harris-Kerr, C., Ph.D., Medical College of Virginia, Richmond
Lummis, S., Ph.D., University of Cambridge, England
Paulsen, R., Ph.D., Norwegian Defence Research Establishment
Pedersen, H., B.S., Maribo Seeds, Copenhagen, Denmark
Ragland, M., Ph.D., Monsanto Company, St. Louis, Missouri
Raventos, D., B.S., Conseil Superior d'Investigaciones Científicas, Barcelona, Spain
Skouv, J., Ph.D., Danish Cancer Society, Copenhagen
Soto, J., M.S., University of Santiago de Compostela, Spain

SEMINARS

Burdis, K., University of California, Davis. Structure and function of double sex proteins of Drosophila.
De Lange, T., Rockefeller University. Remaking vertebrate chromosome ends in vivo and in vitro.
Desplan, C., Rockefeller University. Molecular determinants of homeoprotein specificity.
Elledge, S., Baylor College of Medicine. Genetics of cell cycle checkpoints in yeast and the two-hybrid system.
Gilman, M., Cold Spring Harbor Laboratory. Mechanics and specificity of signal transduction to the nucleus.
Kingston, R., Massachusetts General Hospital. Gene activators and transcription of nucleosomal templates.
Reinberg, D., Robert Wood Johnson Medical School. Regulation of initiation of transcription by RNA polymerase II.
Yamamoto, K., University of California, San Francisco. Combinatorial regulation at a composite response element.

Imaging Structure and Function in the Nervous System

July 27—August 16

INSTRUCTORS

Augustine, George, Ph.D., Duke University, Durham, North Carolina
Lichtman, Jeff, Ph.D., Washington University, St. Louis, Missouri

ASSISTANTS

Balice-Gordon, Rita, Washington University, St. Louis, Missouri
Dalva, Matt, Duke University, Durham, North Carolina
Parsons, Tom, Max Planck Institute, Heidelberg, Germany
Recent advances in optical, video, and computer-assisted microscopy, coupled with the development of powerful new fluorescent probes, present unique opportunities for visualizing the structure and function of individual neurons and neuronal assemblies. This intensive 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; the use of caged compounds to study intracellular and synaptic signaling, voltage-sensitive dyes. 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, cultured cells, and even living animals.

PARTICIPANTS
Baier, H., B.S., Max Planck Institut, Tubingen, Germany
Hohn, A., Ph.D., University of California, Berkeley
Hsu, M., M.S., Rutgers University, Newark, New Jersey
Kirkpatrick, K., B.S., Montreal General Hospital, Canada
Kuner, T., University of Heidelberg, Germany
Maycox, P., Ph.D., Salk Institute, La Jolla, California
Phenna, S., B.S., University of Southampton, England
Ray, K., M.S., Tata Institute, Bombay, India
Sekino, Y., Ph.D., Tokyo Metropolitan Institute, Japan
Simerly, R., Ph.D., Oregon Regional Primate Center, Beaverton
Von Gersdorff, H., Ph.D., SUNY at Stony Brook, New York
Webb, B., M.S., University of Florida, Gainesville

SEMINARS
Betz, W., University of Colorado. Fluorescence measurements of synaptic vesicle cycling.
Connor, J., Roche Institute of Molecular Biology. Ca imaging in dendritic spines.
Fraser, S., California Institute of Technology. MRI imaging.
Grinvald, A., Weizmann Institute of Science. Voltage-sensitive dyes and intrinsic optical signals.
Inoue, T., Universal Imaging Corporation. Image processing.
Lewis, R., Stanford University. Types of calcium indicator dyes.
Nerbonne, J., Washington University. Caged compounds.
Ross, W., New York Medical College. Ca measurements from central neurons in slices.
Smith, S., Stanford University. Limits in confocal microscopy.
Tsien, R.W., University of California, San Diego. Design and function of indicator dyes.
Webb, W., Cornell University. 2-Photon excitation microscopy; fluorescence recovery after photobleaching.

Neurobiology of Human Neurological Disease

August 10–August 16

INSTRUCTORS

Choi, Dennis, M.D., Ph.D., Washington University, St. Louis, Missouri
Mobley, William, M.D., Ph.D., University of California, San Francisco

This lecture course focused on selected arenas where recent advances in neuroscience have improved our understanding of a disease of the human nervous system. Substantial time was allotted to discussions led by the invited lecturers, so that in-depth delineation of specific approaches, methods, and results would be possible. An underlying general theme of the course was the exploration of the highly dynamic interface between basic neuroscience and clinical medicine. What can we learn about certain disease mechanisms by studying molecules, cells, and animals, and what must we learn by studying patients? What experimental and clinical problems hinder the development of novel therapeutic approaches? What can the study of neurological or psychiatric diseases reveal about the nature of the normal nervous system? Applicants with either neuroscience or clinical backgrounds were encouraged to apply.
PARTICIPANTS
Bermingham-McDonogh, O., Ph.D., Cambridge Neuroscience, Massachusetts
Gordon, M., M.D., SUNY Downstate Medical Center, Brooklyn, New York
Green, S., D.V.M., University of California, Davis
Hochberg, K., B.S., Emory University, Atlanta, Georgia
Koh, J., M.D., Ph.D., Washington University, St. Louis, Missouri
Kowallis, G., M.D., St. Vincent’s Hospital, New York, New York
Miller, P., B.S., Rutgers University, Newark, New Jersey

SEMINARS
Chalfie, M., Columbia University. Genetically determined neurodegeneration.
Choi, D., Washington University. Excitotoxicity.
Ciaranello, R., Stanford University. Neural development and psychiatric diseases.
Diamond, I., University of California, San Francisco. Alcohol and the nervous system.
Fischbeck, K., University of Pennsylvania. Molecular genetics of neuromuscular diseases.
Landis, D., Case Western Reserve University. Role of glia in CNS injury.
Mazziotta, J., University of California, Los Angeles. (1) PET neuroimaging. (2) Functional anatomy and neurological disease.
McNamara, J., Duke University. Epilepsy.
Mobley, W., University of California, San Francisco. Neuronal growth factors.
Monyer, H., University of Heidelberg. Glutamate receptors in health and disease.
Prusiner, S., University of California, San Francisco. Prion diseases of the nervous system.
Pierce, L., University of Utah. Disorders of excitable membranes.
Selkoe, D., Brigham & Women’s Hospital. Alzheimer’s disease.

Macromolecular Crystallography

October 13–October 26

INSTRUCTORS
Furey, William, Ph.D., V.A. Medical Center, Pittsburgh, Pennsylvania
Gilliland, Gary, Ph.D., Center for Advanced Research in Biotechnology, Rockville, Maryland
McPherson, Alexander, Ph.D., University of California, Riverside
Pflugrath, James, Ph.D., Cold Spring Harbor Laboratory, New York

ASSISTANT
Ji, Xinhau, Center for Advanced Research in Biotechnology

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 crystallization, membrane protein crystallization, crystal characterization, data collection, cryocrystallography, data reduction, anomalous dispersion, phase determination, molecular replacement and averaging, electron density interpretation, structure
refinement, molecular graphics, molecular dynamics, and multidimensional nuclear magnetic resonance. Participants learned through extensive hands-on experiments and informal discussions and lectures on current applications of these and related procedures given by outside speakers.

PARTICIPANTS
Danley, D., M.S., Pfizer Central Research
Eger, B., Ph.D., University of Toronto
Fethiere, J., Ph.D., University of Alberta
Francklyn, C., Ph.D., University of Vermont
Hruza, A., B.A., Shering Plough Research Institute
Jewell, D., Ph.D., Scripps Research Institute
Jessen, S., Ph.D., Rutgers University
Kuyper, L., Ph.D., Burroughs-Wellcome Company
Parmentier Lesage, F., Ph.D., University of Montreal
Perrakis, A., B.S., European Molecular Biology Lab
Schaper, S., B.S., Max Planck Institute, Germany
Sridhar, V., Ph.D., National Institutes of Health
Vaughn, D., B.S., California Institute of Technology
Vasudevan, S., M.S., Princess Margaret Hospital, United Kingdom
Verboven, C., B.S., Catholic University Leuven, Belgium
Yuvaniyama, J., B.S., University of Michigan

SEMINARS
Aggarwal, A., Columbia University. The Application of MAD to protein phasing.
Brunger, A., Yale University. Models of thermal motion and bulk solvent for macromolecular crystal structures.
Burley, S., Rockefeller University, HHMI. Co-crystal structure of TBP recognizing the minor groove of a TATA element.
Clark, K., The Rockefeller University. Crystallography and flash-freezing.
Clore, G.M., National Institutes of Health. Determination of high-resolution structures of proteins by three- and four-dimensional NMR.
Furey, W., V.A. Medical Center. Structure of the super-antigen staphylococcal enterotoxin B.
Gilliland, G., Center for Advanced Research in Biotechnology. (1) Crystallographic studies of enzymatic and substrate specificity of Mu-class glutathione S-transferase. (2) Ribonuclease A revisited: Crystallographic evidence for solvent modulated pyrimidine specificity and the high-resolution anisotropic refinement at 1.04 Å resolution.
Jurnak, F., University of California, Riverside. Structure and function of pectate lyases, the secreted plant virulence factors that fold into a new type of domain motif.
Kjeldgaard, M., Aarhus University. EF-Tu: A molecular flip-flop device.
Kossiakoff, A., Genentech, Inc. Structure of the growth hormone bound to its receptor: Implications for receptor recognition and activation.
McPherson, A., University of California, Riverside. (1) Crystallization of macromolecules. (2) The structure of pancreatic α-amylase and its complexes with polysaccharides.

Otwinowski, Z., Yale University. Scaling and merging of data.

Pflugrath, J., Cold Spring Harbor Laboratory. Area detector data collection.

Poljak, R., Center for Advanced Research in Biotechnology. Structure and thermodynamics of an antigen-antibody reaction.

Quirocho, F., Baylor College of Medicine, HHMI. Protein plasticity in molecular recognition and signal transduction.

Sweet, R., Brookhaven National Laboratory. (1) X-ray sources and optics. (2) Laue crystallography: A great way to get data REALLY fast. Some results from dynamic studies of catalysis.

Tronrud, D., University of Oregon. Macromolecular refinement.

Steigemann, W., Max Planck Institute, Germany. Protein: A program system for the crystal structure analysis of macromolecules.


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**Advanced In Situ Hybridization and Immunocytochemistry**

**October 13–October 26**

**INSTRUCTORS**

**Hough, Paul**, Ph.D., Brookhaven National Laboratory, Upton, New York

**Jacobson, Ken**, Ph.D., University of North Carolina, Chapel Hill

**Spector, David**, Ph.D., Cold Spring Harbor Laboratory, New York

**Trask, Barbara**, Ph.D., University of Washington School of Medicine, Seattle

**ASSISTANTS**

**Derby, Robert**, Cold Spring Harbor Laboratory, New York

**Lipfert, Jennifer**, University of North Carolina, Chapel Hill

**Oliver, Tim**, University of North Carolina, Chapel Hill

This course focused on specialized techniques and concepts in microscopy related to localizing nucleic acid sequences and proteins in cells and preparing isolated nucleic acids for microscopic examination. The course was designed for the molecular biologist who is in need of microscopic cell biological approaches and for the cell biologist who is not familiar with the practical application of the advanced techniques presented in the course. The course emphasized the use of the latest techniques and methods in epifluorescence microscopy, confocal laser scanning microscopy, and electron microscopy to localize nucleic acids and proteins in mammalian cells. Among the methods presented were preparation of tagged nucleic acid probes, fixation methods, detection of multiple nucleic acids in a single cell, chromosome spreads, use of a variety of reporter molecules and non-antibody fluorescent tags, direct and indirect antibody labeling, and detection of multiple proteins in a single cell. In addition, electron microscopic techniques to observe isolated nucleic acids and proteins were presented. For each technique, several experimental protocols were presented allowing the students to assess the relative merits of each and to relate them to their own research. The students were encouraged to bring specific probes to the course that could be used in addition to those provided by the instructors. The laboratory portion of the course was supplemented by invited lecturers who gave up-to-the-minute reports on current research using the techniques presented in the course.
PARTICIPANTS
Aratake, H., Ph.D., Harvard Medical School
Bassnett, S., Ph.D., Uniformed Services University
Becker-Andre, M., Ph.D., Glaxo Institute, Geneva
Blencowe, B., Ph.D., Massachusetts Institute of Technology
Carver, E., B.S., Oak Ridge National Laboratory
Denison, K., B.A., Los Alamos National Laboratory
Fuentes, L., Ph.D., Agriculture Canada Research Station
Glencorse, T., Ph.D., University of Alberta
Kaskel, F., Ph.D., SUNY at Stony Brook, New York
Kutchera, W., M.D., University of Utah
Lauer, P., B.S., University of California, Berkeley
Ma, N., Ph.D., Collaborative Research Incorporated
Niblock-Foxx, M., B.A., Bowman Gray School of Medicine
Schroeter, S., Ph.D., Emory University
Southard Smith, M., Ph.D., University of Michigan
Yaworsky, P., B.S., Mayo Clinic, Scottsdale

SEMINARS
Block, S., Rowland Institute for Science. Using optical tweezers to study biological motors.
Brinkley, W., Baylor College of Medicine. Ultrastructural organization of the kinetochore.
Haaf, T., Yale University. Localizing DNA sequences at the fluorescence level.
Hough, P., Brookhaven National Laboratory. Image production in the electron microscope and a brief overview of different applications.
Jacobson, K., University of North Carolina. Basic introduction to light microscopy and fluorescence microscopy.
Murray, J., University of Pennsylvania. Use of confocal microscopy and deconvolution techniques.

Oshiro, M. Hamamatsu Photonic Systems, N.J. Video microscopy.
Singer, R., University of Massachusetts. Cytoplasmic organization of mRNA.
Spector, D., Cold Spring Harbor Laboratory. (1) An integrated microscopic approach to examining nuclear organization. (2) Immunocytochemistry.
Trask, B., University of Washington School of Medicine. Mapping chromosomes and interphase nuclei by in situ hybridization.
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.
Molecular Genetics, Cell Biology, and Cell Cycle of Fission Yeast

November 2–November 15

INSTRUCTORS
Chappell, Tom, Ph.D., Duke University, Durham, North Carolina
McLeod, Maureen, Ph.D., State University of New York, Brooklyn
Young, Paul, Queens University, Canada

ASSISTANTS
Edwards, Kevin, Duke University, Durham, North Carolina
Schettino, Annette, State University of New York, Brooklyn

Recent advances in cell cycle research, largely due to studies on the fission yeast *Schizosaccharomyces pombe*, have prompted much interest in this organism. Although cell cycle studies continue to flourish, *S. pombe* is increasingly being chosen as a model organism for investigations into other aspects of cell biology and genetics using the powerful molecular and genetical techniques available. The content of the course reflected all these areas of interest and provided participants with the skills necessary to pursue their own investigations. Topics covered included mutagenesis and analysis of mutants, transformation and gene transplacement techniques, isolation of nuclei, preparation of nuclear DNA, plasmid recovery from yeast into bacteria, cell cycle methods, cytology, and immunological techniques. In addition to hands-on experience, participants had the opportunity to learn through informal group discussions and formal lectures given by prominent *S. pombe* researchers drawn from the expanding international community.
PARTICIPANTS
Atkins, D., Ph.D., R.W. Johnson Pharmaceutical Research Institute
Birse, C., Ph.D., University of Oxford, United Kingdom
Bonneville, J.-M., Ph.D., CNRS/ISV, France
Bureik, M., Ph.D., Universitat des Saarlandes, Germany
Causton, H., Ph.D., University of Uppsala, Sweden
Clyne, R., B.A., Johns Hopkins University
Erpel, T., M.S., EMBL, Germany
Hildebrandt, V., Ph.D., University of California, Los Angeles
Jensen, I., B.S., University of Illinois, Urbana
Kanipes, M., B.S., Carnegie Mellon Mellon Institute
Morse, D., Ph.D., University of Utah
Ocampos, M., M.S., University of Cambridge, United Kingdom
Ortiz, J., Ph.D., Universidad de Cantagria, Spain
Scocca, J., Ph.D., Johns Hopkins School of Medicine
Tonkin, E., Ph.D., University of Wales, United Kingdom
Zhang, M., Ph.D., Cold Spring Harbor Laboratory

SEMINARS
Beach, D., Cold Spring Harbor Laboratory. Cell cycle.
Dunphy, W., California Institute of Technology. Biochemical characterizations of cell cycle control.
Enoch, T., Harvard University Medical School. Why don't cells divide until DNA replication is complete?
Hoffman, C., Boston College. Trials and tribulations of cloning gift* genes in S. pombe.
Klar, A., NCI Frederick Cancer Facility. Cellular differentiation and the fission yeast mating-type interconversion.
Marks, J., Queens University, Canada. Integration of mitosis and cytokinesis: The role of cal 16.
Potashkin, J., University of Health Sciences, Chicago. Pre-RNA splicing.
Singer, C., Singer Instrument Company, Inc. S. pombe tetrad dissection.
Wigler, M., Cold Spring Harbor Laboratory. Studies of RAS in yeast.

Monoclonal Antibodies from Combinatorial Libraries

November 2–November 15

INSTRUCTORS
Barbas, Carlos, Ph.D., Scripps Research Institute
Burton, Dennis, Ph.D., Scripps Research Institute

ASSISTANTS
Bastidas, Raiza, Scripps Research Institute
Briones, Amelia, Scripps Research Institute
Pilkington, Glenn, American Bio-Technologies
Recent advances in the generation and selection of antibodies from combinatorial libraries allow for the rapid production of antibodies from immune and nonimmune sources. This laboratory/lecture course focused on the construction of combinatorial antibody libraries expressed on the surface of phage and selection of desired antibodies from pre-existing libraries by panning. Students learned the theoretical and practical aspects of constructing combinatorial libraries from immune and nonimmune sources as well as the construction of synthetic antibody libraries. Production, purification, and characterization of Fab fragments expressed in E. coli were also covered. The lecture series, presented by a number of invited speakers focused on the biology of filamentous phage and the utility of surface expression libraries, expression of antibodies in E. coli and mammalian cells, antibody structure and function, antibody diversity, catalytic antibodies, and recent results on the use of antibodies in therapy.

PARTICIPANTS
Kanjilal, S., Ph.D., Dartmouth Medical School
Karrasch, M., Ph.D., Hoffman-La Roche
Kufer, P., M.D., Institut fur Immunologie, Germany
Lidell, E., Ph.D., University of Wales, United Kingdom
Linney, E.A., Ph.D., Duke University
Mueller, E., Ph.D., University of Bern, Switzerland
Hiroyoshi, O., Ph.D., Science University of Tokyo, Japan
Rosell Vives, E., Ph.D., MERCK-IGODA, S.A., Spain

SEMINARS
Barbas, C., Scripps Research Institute. Antibodies from synthetic libraries.
Burton, D., Scripps Research Institute. Antibodies from immune libraries.
Clackson, T., Genentech, Inc. Phage display of proteins.
Dimmock, N., University of Warwick. Neutralization of animal viruses by antibody.
Scarselli, E., M.D., I.R.B.M., Rome, Italy
Schlager, J., Ph.D., U. S. Army Medical Research Institute
Steinberger, P., M.S., University of Vienna, Austria
Valdivia, M., Ph.D., University of Cadiz, Spain
Vieira, J.G., Ph.D., Escola Paulista de Medicina, Spain
Walker, J., B.S., Bristol, United Kingdom
Wang, Y., M.D., Beijing Institute for Cancer Research, China
Yokoyama, I., Ph.D., Public Health Research Institute, New York

Model, P., Rockefeller University. Phage biology.
Sanz, I., University of Texas Health Science Center. Generation and features of antibody diversity.
Wilson, I., Scripps Research Institute. Structural basis of antigen recognition by antibody.
Molecular Markers for Plant Breeding

November 2–November 15

INSTRUCTORS
Burr, Ben, Ph.D., Brookhaven National Laboratory, Upton, New York
Helentjaris, Tim, Ph.D., University of Arizona, Tucson
Tingey, Scott, Ph.D., DuPont Experimental Station, Wilmington, Delaware

ASSISTANTS
Doerge, Rebecca, Cornell University, Ithaca, New York
Krespan, William, DuPont Experimental Station, Wilmington, Delaware
Stevenson, Becky, University of Arizona, Tucson

This course was designed to train scientists in the use of molecular markers in plant breeding. It was taught through both a theoretical and practical approach to the subject, including lectures and extensive laboratory work. Participants learned a variety of techniques that can be used to approach problems such as single gene introgression, analysis of genetic diversity, gene mapping, and quantitative trait analysis. These techniques included DNA preparation, non-radiosotopic Southern analysis, PCR, RAPDs, and DNA sequencing. The course included training in computational analysis of both RFLP and RAPD data and taught methods from a variety of plant and animal systems with an emphasis on potential future strategies. Course participants were expected to have knowledge of, but not necessarily practical experience with, the basic principles of molecular biology and genetics.
PARTICIPANTS
Bohac, J., Ph.D, USDA, ARS, South Carolina
Carson, S., Ph.D., New Zealand Forest Research Institute, New Zealand
Di Stilio, V., B.S., University of Massachusetts, Amherst
Garcia Garcia, P., Ph.D., Universidad de Leon, Spain
Hookstra, G., Linkage Genetics, Utah
Kindinger, B., Ph.D., USDA, ARS, Oklahoma
Kump, B., Center for Plant Biotechnology & Breeding, Slovenia
Legg, E., M.S., Monterey Laboratories, California
Oliveira, A., M.S., Purdue University
Orjeda, G., M.S., Genetic Resources, Peru
Sutherland, K., M.S., Agriculture Canada, Canada
Van Toai, T., Ph.D., USDA, ARS, Ohio
Wilcox, P., North Carolina State University
Woeste, K., Ph.D., University of Massachusetts, Amherst

SEMINARS
Beavis, W., Pioneer Hi-Bred International. Comparative mapping with molecular markers.
Beckmann, J., Centre d'Etude du Polymorphism Humain, France. Utilization of microsatellites as molecular markers.
Dudley, J., University of Illinois, Urbana. Application of molecular markers to plant breeding.
Gayle, M., John Innes Center, Cambridge Laboratory, United Kingdom. Synteny and polyploidy considerations in plant genomic studies.
Gibson, S., Michigan State University. Use of physical mapping methods in plant genomic studies.
Michelmore, R., University of California, Davis. Future directions with the application of molecular markers.
Rafalski, A., DuPont Experimental Station. New technologies and the application of molecular markers.
Sederoff, R., North Carolina State University. Application of molecular markers to perennial crops.
Weir, B., North Carolina State University. Use of molecular markers in analyzing populations.

Essential Computational Genomics for Biologists
November 3–November 12

INSTRUCTORS
Branscomb, Elbert, Ph.D., Lawrence Livermore Laboratory
Goodman, Nat, Ph.D., Massachusetts Institute of Technology
Marr, Tom, Ph.D., Cold Spring Harbor Laboratory

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 is 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
Acland, G., B.S., Cornell University
Bottu, G., Ph.D., Universite Libre de Bruxelles, Belgium
Burk, R., M.D., Albert Einstein College of Medicine
Chiapello, H., M.D., I.N.R.A., France
Cozzani, F., B.S., C.R.I.B.I., Italy
De Vicente, C., Ph.D., I.R.T.A., Spain
Ellis, L., Ph.D., Texas A&M University
Ismailov, I., Ph.D., University of Alabama, Birmingham
Knecht, M., Ph.D., Biocenter Basel, Switzerland
Leung, M., B.S., Tulane University Medical Center
Pouliot, Y., Ph.D., Genethon, France
Saxon, M., Ph.D., Mount Sinai School of Medicine
Shechter, I., Ph.D., University of Colorado
Snoddy, J., Ph.D., Department of Energy, Washington D.C.
Stassen, A., M.S., The Netherlands Cancer Institute, Netherlands
Warwar, V., M.S., University of Nebraska
Worley, K., B.S., Baylor College of Medicine

SEMINARS
Branscomb, E., Lawrence Livermore Laboratory. (1) An integrated relational database to support genome mapping. (2) Physical mapping of genomes.
Chang, W., Cold Spring Harbor Laboratory. Physical mapping of genomes.
Cinkowsky, M., Los Alamos National Laboratory. Relational database to support genome mapping and analysis.
Davison, D., University of Houston. Unix and biological resources on the INTERNET and various data servers.
Fasman, K., Johns Hopkins University. The genome databases.
Goodman, N., Massachusetts Institute of Technology. Database technologies: Relational, object oriented, etc.
Green, P., Washington University Medical School. (1) Basic statistics and probability. (2) The Worm sequencing project: Gene finding.
Lincoln, S., Whitehead Institute. Genetic Linkage, MapMaker/QTL.
Marr, T., Cold Spring Harbor Laboratory. Physical mapping of genomes.
Pearson, W., University of Virginia. (1) Protein evolution. (2) Sequence analysis, FASTA, Smith-Waterman.
Schuler, G., National Institutes of Health. MACAW/BLAST and other NCBI tools.
Smith, R., Dana-Farber Cancer Institute. PIMA, PAUP, sequence clustering.
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.

1993

January

Roberto Malinow, University of Iowa. Long-term synaptic potentiation: Cellular mechanisms of learning and memory. (Host: Alcino Silva)

Hank Sadkowski, Cold Spring Harbor Laboratory (Gilman Laboratory). Cell-free activation of a DNA-binding protein by epidermal growth factor.

Gian Luigi Russo, Cold Spring Harbor Laboratory (Marshak Laboratory). Interaction between casein kinase II and p34^cdc2 in HeLa cell division cycle.

Mu-Ming Poo, Columbia University. Plasticity of developing synapse and molecular mechanisms. (Host: Yi Zhong)

Paul Bingham, SUNY Stony Brook. Nuclear organization and regulated pre-mRNA processing in Drosophila. (Host: David Helfman)

Roberto Mariani, Cold Spring Harbor Laboratory (Skowronski Laboratory). CD4 down-regulation by nef alleles isolated from HIV-1-infected individuals.

February

Eric Olson, Baylor College of Medicine. Regulatory networks controlling myogenin. (Host: David Helfman)

Maria Zapp, University of Massachusetts Medical Center. Small molecules that selectively block binding of the HIV-1 Rev protein to its viral RNA site. (Host: Nouria Hernandez)

Gideon Dreyfuss, HHMI/University of Pennsylvania School of Medicine. Ribonucleoprotein structures along the pathway of mRNA formation. (Host: Adrian Krainer)

March

Eileen White, Center for Advanced Biotechnology and Medicine, Piscataway, New Jersey. Regulation of programmed cell death (apoptosis) by the products of oncogenes and tumor suppressor genes. (Host: Bruce Stillman)


Peter Yaciuk, Cold Spring Harbor Laboratory (Moran Laboratory). Characterization of the E1A-associated 300-kD host cell protein and its associated cell growth regulating activity.

Susan Lobo, Cold Spring Harbor Laboratory (Hernandez Laboratory). The role of the TATA-binding protein in RNA polymerase III transcription.

April

Pat Hearing, SUNY Stony Brook. Viral-cellular protein interactions involved in activation of adenovirus transcription. (Host: Winship Herr)

Mike Tyers, Cold Spring Harbor Laboratory (Futcher Laboratory). How S. cerevisiae cell cycles are started, stopped, and coupled.

Barbara Meyer, University of California, Berkeley. Molecular themes in C. elegans sex determination and dosage compensation. (Host: Hong Ma)

Andrew Tomlinson, Columbia University. Organization of the proximodistal axis of Drosophila appendages: The combined action of wingless and dpp. (Host: Rob Martienssen)

Robert Mihalek, Cold Spring Harbor Laboratory (Tully Laboratory). Progress toward cloning latheo, a new gene involved in learning in Drosophila.

October

Tony Pawson, Samuel Lunenfeld Research Center, Mt. Sinai Hospital, Toronto. SH2 and SH3 domains control protein interactions in signal transduction. (Host: Dafna Bar-Sagi)
October (continued)
Linda Van Aelst, Cold Spring Harbor Laboratory (Wigler Laboratory). Ras function and map kinase cascade.
Tim Mitchison, University of California, San Francisco. Cytoskeletal dynamics in vivo and in vitro. (Host: Dave Casso)
Xiaodong Cheng, Cold Spring Harbor Laboratory. How does DNA become methylated? The crystal structure of Hhal methylase in complex with DNA.
Stanley Korsmeyer, HHMI/Washington University. Bcl-2/Bax: A rheostat that regulates cell death. (Host: Carol Greider)
Cindy Sadowski, Cold Spring Harbor Laboratory (Hernandez Laboratory). Targeting TBP to the PSE, a non-TATA box cis-regulatory element present in snRNA promoters.

November
William Stetler-Stevenson, Extracellular Matrix Pathology Section, National Cancer Institute. Tumor invasion and metastasis: Role of matrix metalloproteinases and their inhibitors. (Host: David Casso)
Carol Greider, Cold Spring Harbor Laboratory. Telomerase mechanism, reconstitution, and potential role in cellular immortalization.

Laura Manuelidis. Yale University, Section of Neuropathology. Elements of chromosome structure. (Host: David Spector)
Anthony Rossomando, Cold Spring Harbor Laboratory (Marshak Laboratory). Negative regulation of map kinase kinase (MKK).

December
Roland Kannar, University of California, Berkeley. Biochemical and genetic analysis of pre-mRNA splicing factors in Drosophila. (Host: Carol Greider)
Hong Sun, Cold Spring Harbor Laboratory. MKP-1, a phosphatase that dephosphorylates and inactivates MAP kinases in vivo.
Sung-Hou Kim, University of California, Berkeley. The structure and its functional implications of CDK2, a cell cycle kinase. (Host: James Pflugrath)
Howard Eichenbaum, SUNY Stony Brook. Rhyme and rhythm in the hippocampus: Linking neural coding and neural plasticity. (Host: Alcino Silva)
Dafna Bar-Sagi, Cold Spring Harbor Laboratory. The Ras signaling complex.
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, 397 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, research workers, and centers for study.

The following students, selected from more than 170 applicants, took part in the program, which was supported by Bio-Rad Laboratories, Burroughs-Wellcome Fund, C. Bliss Memorial Fund, The Garfield Internship, Hanson Industries, Libby Internship, National Science Foundation, Philips Petroleum Foundation., Inc., William Shakespeare Internship, Frederica von Stade Internship, and Zeneca.

Diane Alonso, Scripps College
Advisor: Xiaodong Cheng
Sponsor: National Science Foundation
Determining the role of the glutamine residue in the binding and sequence recognition by the Hhal methyltransferase.

Nadine Bewry, Tennessee State University
Advisor: David Helfman
Sponsor: National Science Foundation
Role of the polypyrimidine-tract-binding protein (PTB) in the regulation of alternative splicing in the rat β-tropomyosin gene.

John (Ewan) Birney, Oxford University
Advisor: Adrian Krainer
Sponsor: Olney/Garfield Internship
mRNA splicing in mammalian cells.

Keith Brennan, Cambridge University
Advisor: Michael Gilman
Sponsor: Hanson Industries
Activation specificity of SRF and Phox1.
Julie Carruthers, University of California, Santa Cruz
Advisor: Hong Ma
Sponsor: Burroughs-Wellcome Fund
Assay for function of the AGAMOUS gene of Arabidopsis thaliana in yeast using a fusion construct of the AGAMOUS DNA-binding domain and the GAL4 activation domain.

Andrea Castillo, Albertson College
Advisor: Robert Martienssen
Sponsor: National Science Foundation
A simple method for cloning mutator suppressible mutants in maize using PCR.

Howard Chang, Harvard University
Advisor: Kim Arndt
Sponsor: Burroughs-Wellcome Fund
Genetic dissection of the signaling pathways that activate G1 cyclin expression.

Nupur Ghoshal, Iowa State University
Advisor: Robert McCombie
Sponsor: National Science Foundation
Applications of automated fluorescence sequencing in a large-scale random and directed sequencing project.

Stephanie Knabe, Pomona College
Advisor: Tim Tully
Sponsor: Burroughs-Wellcome Fund
Effects of dunce mutations on habituation of a jump reflex in Drosophila melanogaster.

Frank Lee, Duke University
Advisor: Alcino Silva
Sponsor: National Science Foundation
Study of spatial learning and synaptic plasticity in von Recklinghausen's neurofibromatosis type 1 (NF1) mutant mice and α-Ca++-calmodulin-dependent kinase II (α-CaMKII)/NF1 double-mutant mice.

Eric Liao, Stanford University
Advisor: Yi Zhong
Sponsor: National Science Foundation
Immunocytochemical mapping of PACAP-like neuropeptide distribution in the third instar larval and adult CNS and PNS of Drosophila melanogaster.

Laurie Littlepage, University of Texas, Austin
Advisor: Bruce Futcher
Sponsors: Bliss Internship; Bio-Rad Laboratories, Inc.; Phillips Petroleum Foundation, Inc.
Use of the two-hybrid screen to find proteins associated with Cln2, Cln3, and other cell cycle proteins.

Michele Pierre-Louis, Brown University
Advisor: Winship Herr
Sponsor: National Science Foundation
Protein-protein interactions between virion protein-16 and the Oct-1 transcription factor as a study in the role of a DNA-binding domain in regulating transcription.

Marko Piirsoo, Tartu University
Advisor: Arne Stenlund
Sponsor: Libby Internship
Studies on the functions of M protein in BPV-1 replication.

Marta Rosario, University of Glasgow
Advisor: Michael Wigler
Sponsor: Shakespeare Internship
Detection of specific protein-protein interactions in the mammalian Ras signal transduction pathway using the two-hybrid system.

Carolyn Ruddell, University of Liverpool
Advisor: David Beach
Sponsor: von Stade Internship
Identification of novel proteins that physically interact with cell cycle regulators, using the two-hybrid screen in Saccharomyces cerevisiae.

Wendy Schaub, Beloit College
Advisor: Jeff Kuret
Sponsor: National Science Foundation
Determining the functional role of members of the casein kinase 1 family of proteins.

Patricia Sung, University of Texas, Austin
Advisor: Venkatesan Sundaresan
Sponsor: Burroughs-Wellcome Fund
Use of inverse polymerase chain reaction for amplifying Arabidopsis genomic sequences flanking transposed Ds elements.

Fiona Thistlethwaite, Cambridge University
Advisor: Bruce Stillman
Sponsor: Zeneca
Subcloning and expression of Mcm2.
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, younger students can engage in introductory programs such as Nature Bugs and Nature Detectives I and II, and older students can enroll in more advanced programs such as Marine Biology and Nature Photography.

During the summer of 1992, a total of 420 students participated in 29 courses within the program; 17 of these courses were filled to capacity. The classes were held outdoors, weather permitting, at the Uplands Farm Preserve, the headquarters of the Long Island Chapter of the Nature Conservancy. The Laboratory has equipped and maintains classroom and laboratory facilities as well as a darkroom at Uplands Farm. This facility is used as a base for the students' 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 three 2-week sessions, the Adventure Education course meets on two Saturdays for trips. The students go on a 10-mile bicycle 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

REGISTRAR
Catherine Schratwieser, M.A., Dowling College

INSTRUCTORS
Kimberly Hamilton, B.S., Colgate University
Michael Manfredonia, Teaching Candidate, Marist College
Linda Payoski, B.A., Science Teacher, Uniondale High School
Marjorie Pizza, B.A., Science Teacher, Bayville School District
Donna Stokes, Teaching Candidate
Sarah Whiteside, Photography Undergraduate

COURSES
Nature Bugs
Nature Detectives I and II
Advanced Nature Study
Ecology Explorers
Frogs, Flippers, and Fins
Pebble Pups
Rock Hounds
Bird Study
Freshwater Life
Seashore Life
Marine Biology
Nature Photography
Adventure Education
The Laboratory would like to acknowledge the generosity of the following companies who loaned equipment and reagents to the various courses:

Adams & List
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Newport
Nikon, Inc.
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Banbury Center continues to be one of the premier sites in the world for small, workshop-style meetings. In 1993, there were 16 meetings at the Center, attended by 506 participants. In addition, five neurobiology courses were held over the summer months, and the Center was used for eight other meetings. As usual, the topics of the scientific meetings were diverse, ranging from highly technical subjects in molecular biology to Lyme disease to the genetics of manic-depressive illness.

Participants

We made some refinements to our database at the beginning of 1993, so for the first time, we can provide information about the geographical distribution of our visitors. Participants from the United States came from 31 states. California supplied the largest number (65), followed closely by New York (63), Massachusetts (44), and Maryland (40). These four states alone accounted for 42% of visitors to Banbury Center in 1993. Ninety participants (18%) came from 14 foreign countries. Most came from the United Kingdom, and it is striking that Japanese participants ranked joint second with those from France. This is due in part to the very strong support Japanese companies are providing to the Cold Spring Harbor Laboratory Corporate Sponsor Program. We would like to increase the numbers of scientists from abroad, especially from countries more distant than Europe; however, travel expenses weigh very heavily on the Center's finances.

Molecular Biology

One feature of Banbury Center meetings that contributes to their value and success is that they are interdisciplinary. We make a special effort to bring together scientists working on related topics from different points of view and in different organisms. The advantages of this approach were exemplified in the meeting Epigenetic Mechanisms of Gene Regulation. Originally intended to discuss some recent fascinating research in plants, it became clear that to do the subject justice, the meeting would have to include research on other organisms. In the end, maize, petunia, tobacco, yeast, fungi, fruit flies, mice, and human beings were included! It was a pleasure to have Amar Klar returning to his old haunts.

Once the amino acids of a protein have been put together in accordance with the instructions in its gene, the protein has still to assume its final three-dimensional shape. For a long while it was thought that the newly synthesized amino acid chain did this spontaneously, with no additional help. However, it became clear that other proteins were needed and these were called chaperones. Subsequent research has shown that chaperones are involved in many if not all stages of the life of a protein and have a key role in cellular metabolism. The Molecular Chaperones meeting discussed what is known of the roles of cellular chaperones and of how they function. How, for example, does a chaperone recognize its target protein? And having done so, how does it interact with the
protein? This was one of three 1993 Banbury Center meetings organized by Cold Spring Harbor Laboratory alumni. In this case it was Mary-Jane Gething who did the hard work of choosing the participants and preparing the program.

The **Apoptotic Cell Death: Functions and Mechanisms** meeting was notable on three counts. First, apoptosis is being recognized as a key phenomenon in an ever-increasing number of biological systems. It occurs as a programmed event during development in the nervous and immune systems and in response to agents such as radiation and cytotoxic drugs. Second, a number of genes, including genes involved in cancers, have been discovered that induce or suppress apoptosis, and this should speed understanding of the cellular mechanisms involved. Third, it is a striking example of the way in which an area of research can suddenly become a very "hot" topic. This was evident in the presence of a BBC film crew shooting material for a forthcoming program on cell death! The timing of the meeting was again just right, and we were able to attract all the top researchers in the field, including Andrew Wylie, who was the first to describe the phenomenon as long ago as 1972.

One of the great challenges in biology is to learn how the transcription of genes is regulated. An ever-increasing number of DNA sequences are being found that, when bound by specific proteins, control expression of genes. One of the best characterized of these elements is the κB element that is associated with a large number of viral and cellular genes. The Banbury Center meeting **κB-binding Proteins: Their Role in Development and Growth Control** was designed to examine this element, especially its control by Rel-related proteins.

The **In Vitro Selection from Combinatorial Libraries** meeting examined the latest strategies for producing molecules with new properties. This is very important for those searching for new drugs, but unfortunately it is difficult to do so systematically, in part because we do not know enough about the relationship between structure and function to make predictions from first principles. An alternative strategy makes use of combinatorial libraries of molecules together with selection of those forms that have enhanced function. And recently a mutational step has been included so that the process has parallels with Darwinian evolution. This was a fascinating meeting in the interplay of theoretical treatments of
generating molecular diversity and the experimental results of those trying to produce molecules.

Just a few years ago, the idea that nitric oxide could act as a messenger molecule would have seemed preposterous. But now it is clear that nitric oxide is a key intermediate in a variety of physiological processes, including mediating blood vessel relaxation and functioning as a novel form of neurotransmitter. It may even be involved in the development of long-term memory. There remain many interesting and perplexing features of nitric oxide and this meeting, Nitric Oxide: Molecular Mechanisms of Synthesis and Action, examined some of them, especially the regulation of nitric oxide synthase, the enzyme that makes nitric oxide.

Angiogenesis is the process by which new blood vessels are formed. This is a crucial part not only of normal development, but also of tumor growth. The Mechanisms of Developmental and Tumor Angiogenesis meeting reviewed the latest findings on how angiogenesis is controlled. A major emphasis of the meeting was on the evolving knowledge of proteins that influence normal and pathological angiogenesis. These include a variety of stimulatory growth factors as well as inhibitory factors such as thrombospondin. In addition, cell surface and extracellular matrix molecules have a role in determining how endothelial cells proliferate and form new blood vessels. This was the second of the Cold Spring Harbor Laboratory alumni meetings, and in this case, Doug Hanahan was one of the principal organizers.

**Developments in Human Genetics**

Diagnostic tests using DNA probes are proving to be extremely powerful because of their specificity, accuracy, and speed. The real impact of DNA-based diagnosis will come only when the tests can be used in routine clinical chemistry, microbiology, and pathology laboratories. This meeting, The Future of DNA-based Diagnosis, funded by Helix Partners Inc., was held to review some of the new research strategies being developed to move these mutation detection systems into routine large-scale application. Thus, a number of talks discussed the use of robot systems to increase the reliability and speed of tests, and there was a critical evaluation of advances in DNA sequencing by hybridization. It was a particular pleasure to have Ed Southern presenting his work in this area.

DNA-based diagnostics are all very well, but the ultimate goal of clinical geneticists is to treat individuals afflicted with genetic disorders. This goal has been achieved for the immunodeficiency caused by adenosine deaminase, where gene therapy is being used successfully. Duchenne muscular dystrophy (DMD) is a genetic disorder, affecting boys, in which the muscles degenerate. There is no treatment or cure and the boys die in their late teens or early twenties. We explored in this meeting, Gene Reactivation as a Therapeutic Strategy for Duchenne and Becker Muscular Dystrophies, a novel approach to treating DMD in which a muscle protein that is normally turned off in adult life might be turned on again and so take the place of the mutant protein that fails to work in DMD. The meeting included the world’s authorities on the molecular biology of DMD and scientists who have been working on a similar strategy for treating the thalassemias. In part as a result of these deliberations, the Laboratory has entered into a collaboration with Oncogene Science, Inc., to search for drugs that could switch on the second protein.
The monies from the William Stamps Farish Fund are being used for the support of Banbury Center meetings on complex, polygenic disorders and the 1993 meeting in the series was the Polygenic Basis of Cancer. For many years, epidemiological data had suggested that cancers were the result of mutations in a number of genes, a prediction confirmed by the molecular analysis of mutations in oncogenes. We decided that the time was ripe to bring together scientists working on a wide variety of cancers to see what common themes are emerging as regards the mechanisms underlying the origin and selection of multiple genetic alterations in primary human tumors. A session of special interest was that discussing how the products of tumor suppressor genes were related to control of the cell cycle. The meeting was notable also for beginning on the day of the worst snow storm of the 1992–1993 winter. One participant made three attempts to come here from Boston before succeeding and arriving at the end of the first day!

Lyme Disease

Banbury Center was the site of a very successful meeting on Lyme disease in 1991. Since that time, the pace of research on all aspects of the causative agent, a spirochaete called Borrelia burgdorferi, has accelerated, and the time was right to review what has and has not been achieved. For this reason, the meeting, Molecular and Immunologic Aspects of Lyme Disease, covered a wide range of topics, from molecular biology and immunology, through pathogenesis, detection, and diagnosis, to vaccine development. Unfortunately, many issues, especially in relation to diagnosis and treatment, remain unresolved.

Science Policy Meeting

One of the keys to the success of the Banbury Center is that we have the flexibility to put on meetings at very short notice. This is important for scientific meetings but even more so for meetings discussing science policy: We are able to respond very rapidly to new developments. An example of such a meeting was that on Industrial Sponsorship of Research in Academic Institutions, which was prompted by the controversy raised by the research agreement between the Scripps Research Institute and Sandoz. However, this controversy was not the focus of the meeting which reviewed and discussed the different types of arrangements being made between academic institutions and companies and the merits and demerits of these agreements. The meeting was notable in having representatives of academic institutions, large and small companies, congressional staff (including staff from the Congressional committee that is pursuing this matter and from the Vice President’s office), the NSF Inspector-General, a deputy commissioner from the FDA, and eminent scientists. It is always hard to measure the success of such a meeting, but it was clear that the participants welcomed the opportunity to discuss the issues in a nonadversarial situation.

Baring Brothers/Dillon Read/Cold Spring Harbor Laboratory Meeting

This was the eighth in the series of Banbury Center meetings for senior executives of pharmaceutical, biotechnology, and venture capital companies. They are
extraordinary meetings and this year's meeting, The Human Genome Project In-
cluding Its Commercial Application, was no exception. We were fortunate to
have as speakers eminent scientists who not only are at the forefront of their dis-
cipline, but are wonderful speakers as well. The meeting began with a talk by
Francis Collins on the revolution that molecular human genetics is bringing about
in clinical practice and finished with a vision of the future with a presentation by
Kenneth Culver on gene therapy. This is the last of the present series to be
funded by the London merchant bank Baring Brothers & Co. and Dillon Read, its
American partner. It was especially pleasing that our collaboration ended on
such a high note.

Charles A. Dana Foundation Project on Manic Depressive Illness

The Charles A. Dana Foundation made a very substantial grant to Cold Spring
Harbor Laboratory as part of a new Dana initiative on research on the genetic
basis of manic depressive illness. Our partners in the project are Johns Hopkins
Medical School and Stanford University Medical School. Part of the Cold Spring
Harbor Laboratory effort is to promote research through having meetings. The
first of these, The Genetics of Manic Depressive Illness, held in December,
brought together psychiatrists and geneticists who are working on this difficult
problem to review current progress, to examine what makes this research so dif-
ficult, to suggest new strategies for genetic analysis, and to look to the future. It
was very helpful in orientating the Cold Spring Harbor Laboratory database pro-
ject and we, hope, promote closer cooperation between all the groups pursu-
ing this research.
Human Genetics and Genome Analysis Workshops

We held the last of the current series of Genetics Workshops for Nonscientists, funded by the Department of Energy, in February. These workshops, styled after the Sloan Foundation workshops but dealing exclusively with human genetics and doing so in more detail, have been very successful. A total of 92 participants from 28 states took part in the four workshops. They included teachers, Congressional staff, theologians, bioethicists, journalists, lawyers, and patient advocates. The workshops provided an opportunity for individuals with a special interest in human genetics to learn about the science of genetics and its application to human beings and what the consequences might be of our increasing knowledge of human genetics. Fortunately, the Department of Energy agrees with our assessment of the workshops and has decided to fund a further two workshops. We are going to hold the first of these for a section of society that needs to be brought up to speed on human genetics, namely, primary care physicians. Our first workshop will be for the directors of continuing medical education in local hospitals. We hope that they will be so enthused by modern human genetics that they will return to their colleagues and encourage them to take the same course.

Other Meetings

The Center has been used by local groups throughout the year. The Village of Lloyd Harbor held two seminars for residents in February and March. In May, the Cold Spring Harbor School District came here, and in August, West Side School held a faculty meeting here. The Board of Huntington Hospital held their annual review at Banbury in September, and the Lloyd Harbor Conservation Board hosted the North Shore Environmental Conference here in November.

Funding

Unfortunately, the costs of travel and board and lodging continue to rise. Fortunately, the generosity of the members of the Cold Spring Harbor Laboratory Corporate Sponsor Program, and other companies and foundations, enables us to continue our exciting program at Banbury Center. There is no doubt in my mind that this program would not survive if we had to rely on federal funding; the funding is not there and I would be buried under a mountain of grant applications. The Corporate Sponsors provided support for six meetings in 1993: Nitric Oxide: Molecular Mechanisms of Synthesis and Action; Mechanisms of Developmental and Tumor Angiogenesis; κB-binding Proteins: Their Role in Development and Growth Control; Apoptotic Cell Death: Functions and Mechanisms; Molecular Chaperones; and Epigenetic Mechanisms of Gene Regulation. An average of six scientists from Corporate Sponsor companies attended each of these meetings. Smith-Kline Beecham Pharmaceuticals very kindly allowed us to use funds remaining from an earlier meeting for the 1993 meeting In Vitro Selection from Combinatorial Libraries. Companies were especially generous in their support of the meeting Molecular and Immunologic Aspects of Lyme Disease. Abbott Laboratories, Allen & Hanburys, Connaught Laboratories, MedImmune Inc., and Pfizer Laboratories all helped to make this a successful meeting. Helix Partners Inc. sponsored The Future of DNA-based Diagnosis, a meeting of special interest in that we designed it to cover basic research and potential applications.
Foundations continue to be supportive of Banbury Center meetings. The William Stamps Farish Fund provided support for the second of the series of meetings on complex human genetics, and the Charles A. Dana Foundation funded the first in the series of meetings related to the genetics of manic depressive illness. Last year, I wrote a special tribute to the Alfred P. Sloan Foundation, which had, after 12 years, reluctantly declined to provide us with further support. However, the good that foundations do lives on after they are gone, and in this case, we had sufficient funds left to hold one last meeting, that on Industrial Sponsorship of Research in Academic Institutions. It was among the most interesting of the Sloan meetings that we have held. The Association Francaise contre Les Myopathies and the Muscular Dystrophy Association both contributed to the meeting, Gene Reactivation as a Therapeutic Strategy for Duchenne and Becker Muscular Dystrophies. Finally, the Office of Health and Environmental Research of the Department of Energy funded The Workshop on Human Genetics and Genome Analysis, a Genetic Workshop for Nonscientists. These have been very successful and we look forward to the new series of workshops funded by D.O.E.
Looking Forward to 1994

The 1994 Banbury Center program looks to be as interesting and varied as in previous years. We will continue to have meetings on topics that are at the cutting edge of research as well as meetings that deal with wider issues of research. I am looking forward especially to continuing the meetings related to manic depressive illness sponsored by the Charles A. Dana Foundation. The program includes public education, and this year we will be holding a workshop for journalists and Congressional staff. A second project of great promise is being supported by the Robert Wood Johnson Foundation. Gordon Hargraves (Development Office) and I persuaded the Foundation that human genetics should be a part of its health care program. Banbury Center has held many meetings on research aspects of human molecular genetics and now we are going to look at the provision of genetic services and the role of genetics in the modern health care system.

Finally, my thanks to Bea Toliver and Ellie Sidorenko in my office and to Katya Davey in Robertson House. They, as usual, have kept the whole operation running smoothly. Danny Miller and Andy Sauer have kept the Banbury Center grounds looking wonderful, one of the features of the Center that makes it unique.

Jan A. Witkowski
The Future of DNA-based Diagnosis

January 10–January 13

FUNDED BY
Helix Partners

ARRANGED BY
C.T. Caskey, Howard Hughes Medical Institute, Baylor College of Medicine, Houston, Texas
J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York

SESSION 1: Technical Advances I
Chairperson: E.M. Southern, University of Oxford, United Kingdom

E.S. Winn-Dean, Applied Biosystems, Inc., Foster City, California: Applications of DNA probe ligation detection technology.
F.W. Studier, Brookhaven National Laboratory, Upton, New York: DNA sequencing by priming with strings of contiguous hexamers.
J. Quint, Beckman University, Fullerton, California: Robotic automation of dot blots.
M. Bywater-Ekegard, Pharmacia LKB Biotechnology AB, Uppsala, Sweden: Sequence-based diagnostics: A standardized system for the handling of multiple clinical samples.

SESSION 2: Technical Advances II
Chairperson: C.T. Caskey, Howard Hughes Medical Institute, Baylor College of Medicine, Houston, Texas

M.N. Kronick, Applied Biosystems, Inc., Foster City, California: Application of four-color fluorescent sequencing technology to new methods of mutation screening.
S.P.A. Fodor, Affymax, Palo Alto, California: Oligonucleotide arrays and parallel hybridization analysis.
E.M. Southern, University of Oxford, United Kingdom: Analysis of sequence differences by hybridization to array of oligonucleotides.
R. Crkvenjakov, Argonne National Laboratory, Illinois: Sequencing by hybridization (SHB) format I and DNA diagnostics: Present and future applications.
M. Eggers, Houston Advanced Research Center, The Woodlands, Texas: Microfabricated detection devices for automated diagnosis by hybridization (DbH).
SESSION 3: Implementation: Consequences of Different Disorders I

Chairperson: A.G. Motulsky, University of Washington, Seattle

A. Cao, Universita Studi Cagliari, Sardinia, Italy: Application of DNA technology for screening and prenatal diagnosis of β-thalassemia in Sardinia.

D.R. Witt, Kaiser Permanente, San Jose, California: The status of population screening for cystic fibrosis.

B. Weber, Howard Hughes Medical Institute, University of Michigan, Ann Arbor: Breast cancer DNA-based diagnosis: Moving from research to practice.

R.A. Gibbs, Baylor College of Medicine, Houston, Texas: Diagnostic DNA sequencing for genetics and infectious diseases.

C.T. Caskey, Howard Hughes Medical Institute, Baylor College of Medicine, Houston, Texas: Screening for premutation parents.

SESSION 4: Implementation: Consequences of Different Disorders II

Chairperson: C.T. Caskey, Howard Hughes Medical Institute, Baylor College of Medicine, Houston, Texas

A.G. Motulsky, University of Washington, Seattle: Late onset diseases.

S.T. Reeders, Howard Hughes Medical Institute, Yale University, New Haven, Connecticut: Adult polycystic kidney disease: Need for a test in a common late-onset disease.

D.W. Yandell, Massachusetts Eye & Ear Infirmary, Boston: DNA-based diagnostic testing for cancer predisposition: Experience to date with p53 and RB.

S.H. Friend, Massachusetts General Hospital Cancer Center, Charlestown: Functional assays of tumor suppressor genes.

D.C. Ward, Yale University School of Medicine, New Haven, Connecticut: Fluorescence in situ hybridization (FISH) as a tool for DNA-based diagnostics.

SESSION 5: Implementation: Economic Factors

Chairperson: J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York


E.R.B. McCabe, Baylor College of Medicine, Houston, Texas: Regulation, certification, and public health.

P.G. Debenham, Cellmark Diagnostics, Abingdon, Oxon, United Kingdom: Development and experience with test trials of CF kits in Europe.

T.M. Tsakeris, FDA Office of Biotechnology, Rockville, Maryland: The FDA and DNA diagnostic kits.

T.J. White, Roche Molecular Systems, Inc., Alameda, California: Impact of FDA actions on diagnostic testing.

E. Southern
Workshop on Human Genetics and Genome Analysis

February 4–February 7

FUNDED BY

Ethical, Legal, and Social Issues Program of the Department of Energy Human Genome Initiative (Office of Health & Environmental Research)

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

M. Saxton, Massachusetts Office on Disability, Boston: Genetics and cultural attitudes to disability.
J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York: Modern view of the gene.

SESSION 2

M. Bloom, DNA Learning Center, Cold Spring Harbor Laboratory, New York: Cloning genes.
D. Micklos, DNA Learning Center, Cold Spring Harbor Laboratory, New York: Using restriction enzymes and constructing chromosome maps.

SESSION 3

J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York: RFLPs and PCR: What they are; what they do.
P. Ward, Institute of Molecular Genetics, Baylor College of Medicine, Houston, Texas: DNA-based diagnosis for human genetic diseases.
N. Holtzman, Johns Hopkins Medical Institute, Baltimore, Maryland: Map or maze: The future of human genetics.

SESSION 4

M. Bloom, DNA Learning Center, Cold Spring Harbor Laboratory, New York: Laboratory: Human DNA fingerprinting by polymerase chain reaction.
M. Bloom, DNA Learning Center, Cold Spring Harbor Laboratory, New York: Laboratory: Inserting DNA into bacteria and making gene libraries.

SESSION 5

M. Bloom, DNA Learning Center, Cold Spring Harbor Laboratory, New York: Laboratory results: Inserting DNA into bacteria and human DNA fingerprinting.
B. Weber, Howard Hughes Medical Institute, University of Michigan, Ann Arbor: Breast cancer genetics: Moving from research to practice.
K. Culver, National Institutes of Health, Bethesda, Maryland: The first human gene therapy trials.
SESSION 1: Rate-limiting Mutations, Penetrance of Inherited Mutations, Cell Senescence, and Other Things We Do Not Understand About Carcinogenesis

J.C. Barrett, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina: Genetic basis for cell senescence, aging, and cancer.

SESSION 2: Multiple Genetic Alterations in Human Cancers

J.D. Minna, University of Texas, Dallas: Lung cancer requires multiple genetic lesions to become clinically evident.


W. Isaacs, Johns Hopkins Hospital, Baltimore, Maryland: Molecular and cellular biology of prostate cancer.

J. Yokota, National Cancer Center Research Institute, Tokyo, Japan: Biological significance of multiple genetic alterations in human cancer.

E.J. Stanbridge, University of California, Irvine, School of Medicine: Suppression of tumorigenicity by single chromosome transfer: Why are multiple genetic alterations selected for in human cancers?

SESSION 3: Role and Mechanisms of Tumor Suppressor Gene Inactivation in Human Cancers I

C.C. Harris, National Cancer Institute, Bethesda, Maryland: Mutational spectra of tumor suppressor genes: Hypothesis-generating clues to cancer etiology and mechanism.

S.H. Friend, Massachusetts General Hospital Cancer Center, Charlestown: Why Rb and p53 germ-line mutations are rate-limiting in only certain tissues.

Y. Nakamura, Cancer Institute, Tokyo, Japan: Role of APC inactivation in human cancer.

J. Groden, Howard Hughes Medical Institute, University of Utah Medical Center, Salt Lake City: Relationship between APC germ-line mutations and the polyposis phenotype: Addressing APC function.
SESSION 4: Analysis of Tumor Suppressor Gene Function

D.W. Yandell, Massachusetts Eye & Ear Infirmary, Boston: A comprehensive analysis of mutations in retinoblastoma: Does the two-hit model fit?


A. Bradley, Baylor College of Medicine, Houston, Texas: Mutational analysis of tumor suppressor genes in mice.


D. Haber, Massachusetts General Hospital, Charlestown: Mutational analysis and functional studies of WT-1.

F.J. Rauscher, The Wistar Institute, Philadelphia, Pennsylvania: Regulation of transcription by tumor suppressor gene products: Can a common set of target genes regulated by WT1, p53, and Rb be identified whose deregulation accompanies cell transformation?

SESSION 5: Genetic Instability and Alterations in Cell Cycle Control and Checkpoints in Cancer

T.A. Weinert, University of Arizona, Tucson: Speculation on compromised checkpoints and genetic instability.

D. Beach, Cold Spring Harbor Laboratory, New York: Cyclin D and cancer.

G.M. Wahl, The Salk Institute, San Diego, California: Altered G₁ control mediated by tumor suppressor genes: A prelude to chromosome instability.


M.B. Kastan, Johns Hopkins Hospital, Baltimore, Maryland: Consequences of abnormalities in p53 and other controls of a mammalian G₁ checkpoint.

Epigenetic Mechanisms of Gene Regulation

March 7–March 10

FUNDED BY

Cold Spring Harbor Laboratory Corporate Sponsor Program

ARRANGED BY

R. Jorgensen, University of California, Davis
A. Klar, National Cancer Institute–Frederick Cancer Research and Development Center, Maryland
R. Martienssen, Cold Spring Harbor Laboratory, New York

SESSION 1: Methylation

Chairperson: R. Martienssen, Cold Spring Harbor Laboratory, New York

R. Holliday, CSIRO Laboratory for Molecular Biology, North Ryde, Australia: Epigenetic inheritance based on DNA methylation.

T.H. Bestor, Harvard Medical School, Boston, Massachusetts: Regulation of DNA modification during mammalian development.

E. Richards, Washington University, St. Louis, Missouri: Plant DNA methylation mutants.

SESSION 2: Repeated Genes and Gene Silencing I

Chairperson: V.E.A. Russo, Max-Planck-Institute for Molecular Genetics, Berlin, Germany

M. Matzke, Austrian Academy of Sciences, Salzburg, Austria: Interactions between unlinked, partially homologous transgene loci leading to inactivation and methylation of transgenes in tobacco.

E. Selker, University of Edinburgh, United Kingdom: DNA methylation and gene inactivation in neurospora.

SESSION 3: Repeated Genes and Gene Silencing II
Chairperson: M. Matzke, Austrian Academy of Sciences, Salzburg, Austria


R. Fray, University of Nottingham, Loughborough, United Kingdom: Are sense inhibition and antisense down-regulation manifestations of the same phenomenon?

D. Inze, Laboratorium Voor Genetica, Gent, Belgium: Post-transcriptional co-suppression of endogenous and transgene β-glucanase gene expression.

SESSION 4: Nuclear Organization and Chromatin Structure I
Chairperson: S.C.R. Elgin, Washington University, St. Louis, Missouri

J.S.P. Heslop-Harrison, John Innes Center, Norwich, United Kingdom: Nuclear domains, gene expression, and transmission of expression patterns.

S. Henikoff, Howard Hughes Medical Institute, Fred Hutchinson Cancer Research Center, Seattle, Washington: Variegation phenomena involving somatic pairing in Drosophila.
SESSION 5: Nuclear Organization and Chromatin Structure II  
Chairperson: V. Pirrotta, University of Geneva, Switzerland

B. Stillman, Cold Spring Harbor Laboratory, New York: Possible epigenetic inheritance of the origin of recognition complex.  
S.C.R. Elgin, Washington University, St. Louis, Missouri: Heterochromatin protein 1, a known participant in position-effect variegation in Drosophila, is a highly conserved chromosomal protein.  

SESSION 6: Trans-acting Regulators  
Chairperson: A. Klar, National Cancer Institute–Frederick Cancer Research and Development Center, Maryland

N.V. Fedoroff, Carnegie Institution of Washington, Baltimore, Maryland: Molecular dissection of the Spm transposable element's epigenetic control system in transgenic tobacco.  
V. Pirrotta, University of Geneva, Switzerland: Zest and polycomb group genes: Relationship between transvection, PEV, and maintenance of gene repression.

SESSION 7: Paramutation  
Chairperson: E.R. Signer, Massachusetts Institute of Technology, Cambridge

J. Kermicle, University of Wisconsin, Madison: Gene duplication and maize R-locus paramutation.  
V.L. Chandler, University of Oregon, Eugene: Paramutation at the and loci in maize: A heritable alteration in the gene expression of one allele caused by the presence of another allele.  

SESSION 8: Imprinting  
Chairperson: J. Kermicle, University of Wisconsin, Madison

A. Surani, University of Cambridge, United Kingdom: Mechanism of gene regulation by chromosomal imprinting in mice.  
D.P. Barlow, Research Institute of Molecular Pathology, Vienna, Austria: Methylation is the imprinting signal at the mouse Igf2r locus.  
A. Klar, National Cancer Institute-Frederick Cancer Research and Development Center, Maryland: Regulation of cell-type switching and silencing the mating type genes in fission yeast.

SESSION 9: Epigenetic Patterns in Development  
Chairperson: V.L. Chandler, University of Oregon, Eugene

V.E.A. Russo, Max-Planck-Institute for Molecular Genetics, Berlin, Germany: Methylation and reversible inactivation of a foreign gene in N. crassa.  
K. Cone, University of Missouri, Columbia: Molecular genetic analysis of an anthocyanin regulatory gene of maize that leads to variegated pigmentation.  
R. Martienssen, Cold Spring Harbor Laboratory, New York: Developmental patterns of Mu transposon activity in maize.  
M. Donoghue, Washington University Medical School, St. Louis, Missouri: Methylation as an imprint of rostrocaudal position in mice.  
F. Meins, Jr., The Friedrich Miescher Institute, Basel, Switzerland: Developmental and environmental regulation of homologous gene expression in plants transformed with plant-defense-related genes.  
R. Jorgensen, University of California, Davis: Novel flower color patterns can be elicited by pigment transgenes and reprogrammed by transmissible developmental imprints.
Molecular and Immunologic Aspects of Lyme Disease

March 28–March 31

FUNDED BY
Allen & Hansburys, Pfizer Labs, Abbott Laboratories, Connaught Laboratories, Inc., and MedImmune Inc.

ARRANGED BY
S.E. Schutzer, UMDNJ-New Jersey Medical School, Newark
J.J. Dunn, Brookhaven National Laboratory, Upton, New York

SESSION 1: Molecular Biology
Chairperson: J.J. Dunn, Brookhaven National Laboratory, Upton, New York

W.M. Huang, University of Utah Medical Center, Salt Lake City: The chromosome and gene organization of *B. burgdorferi*.

P. Rosa, National Institute of Allergy and Infectious Diseases, Hamilton, Montana: Molecular mechanisms of outer surface protein variation.

I. Schwartz, New York Medical College, Valhalla: Ribosomal genes of *B. burgdorferi*.

A. Sadziene, University of Texas Health Science Center, San Antonio: Resistant variants and application to immunity and pathogenesis.

J.L. Benach, State University of New York, Stony Brook: Point mutations in OspB result in escape variants and different monoclonal antibody reactivity.

D.E. Dykhuizen, State University of New York, Stony Brook: Genetic diversity and clonality.

SESSION 2: Immunology
Chairperson: P.K. Coyle, State University of New York, Stony Brook

R.J. Dattwyler, State University of New York, Stony Brook: Influence of antimicrobials on the immune response.

S.E. Schutzer, UMDNJ–New Jersey Medical School, Newark: Early antibody response to OspA.

F.S. Kantor and L.K. Bockenstedt, Yale University School of Medicine, New Haven, Connecticut: Mapping T-cell epitopes of OspA N40.

S.E. Malawista, Yale University School of Medicine, New Haven, Connecticut: The fate of *B. burgdorferi* in mouse macrophages: Destruction, survival, recovery.

SESSION 3: Vaccine
Chairperson: R.A. Flavell, Yale University School of Medicine, New Haven, Connecticut

L.E. Erdile, Connaught Laboratories, Inc., Swiftwater, Pennsylvania: Protective immune responses to OspA.

E. Fikrig, Yale University, New Haven, Connecticut: Immunization against tick-borne Lyme borreliosis.


J. Radolf, University of Texas Southwestern Medical Center, Dallas: New insights into the molecular architecture of *B. burgdorferi* relevant to Lyme disease pathogenesis and vaccine development.

SESSION 4: Animal Models
Chairperson: S.W. Barthold, Yale University School of Medicine, New Haven, Connecticut

J.L. Goodman, University of Minnesota, Minneapolis: Guinea pig model.

J.N. Miller, University of California, Los Angeles, School of Medicine: Erythema migrans-rabbit model.

J.J. Weis, University of Utah, Salt Lake City: Quantitation of spirochete number in severely and mildly arthritic congenic mice and mapping of an arthritis susceptibility locus.

M.T. Phillip, Tulane Regional Primate Research Center, Covington, Louisiana: Early Lyme disease in the rhesus monkey.
SESSION 5: Pathogenesis
Chairpersons: S.E. Schutzer, UMDNJ-New Jersey Medical School, Newark
F.S. Kantor, Yale University School of Medicine, New Haven, Connecticut
A. Spielman, Harvard School of Public Health, Boston, Massachusetts: Mode of transmission of the Lyme disease spirochete.
A.C. Steere, New England Medical Center, Boston, Massachusetts: Pathogenetic factors in chronic Lyme arthritis.
A. Spielman, Harvard School of Public Health, Boston, Massachusetts: A role for outer surface proteins in Lyme arthritis.
J. Leung, New England Medical Center Hospital, Boston, Massachusetts: Adhesion of B. burgdorferi in pathogenesis.
A.R. Pachner, Georgetown University Hospital, Washington, D.C: Parallels between human and animal CNS B. burgdorferi infection.
L.H. Sigal, UMDNJ-Robert Wood Johnson Medical School, New Brunswick, New Jersey: Molecular mimicry in neurologic B. burgdorferi infection.
P.K. Coyle, State University of New York, Stony Brook: Evidence for early and persistent infection in neurologic Lyme disease.

SESSION 6: Detection and Diagnostics
Chairperson: B.J. Luft, State University of New York, Stony Brook
D.H. Persing, Mayo Clinic, Rochester, Minnesota: Molecular diagnosis and monitoring of Lyme disease.
L.W. Mayer, Centers for Disease Control and Prevention, Fort Collins, Colorado: Design of new diagnostic tests for Lyme disease.
C.A. Norton-Hughes, University of Minnesota Medical School, Minneapolis: Scanning and quantification of Western blot staining.

DISCUSSION AND FUTURE DIRECTIONS
Chairperson: R.L. Quackenbush, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland
SESSION 1: Chaperones: Cooperation and Interactions

Chairperson: M.-J.H. Gething, Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas

S. Wickner, National Cancer Institute, National Institutes of Health, Bethesda, Maryland: The function of DnaJ, DnaK, and GrpE in the monomerization of P1 RepA protein.
M. Zylicz, University of Gdansk, Poland: Different mechanisms for reactivation of heat-treated RNAP by two E. coli chaperone systems.
M.G. Douglas, University of North Carolina, Chapel Hill: Specificity and mechanism for regulation of polypeptide hsp70 by Ydj1p.

SESSION 2: Other Chaperones/Protein Folding 'Catalysts'

Chairperson: L.M. Gierasch, University of Texas Southwestern Medical Center, Dallas

D.B. Williams, University of Toronto, Ontario, Canada: Participation of the chaperone-like molecule, p88 (calnexin) in the biogenesis of class I MHC molecules.
C.S. Zuker, Howard Hughes Medical Institute, University of California, San Diego, La Jolla: The in vivo role of the cyclophilin homolog, ninaA, in rhodopsin biogenesis.
A.A. Gatenby, DuPont Company, Wilmington, Delaware: Novel chaperones involved in the biosynthesis of cytosolic photoreceptors and plastids.
SESSION 3: Chaperone: Protein Interactions
Chairperson: F.U. Hart!, Sloan-Kettering Institute, New York, New York

L.M. Gierasch, University of Texas Southwestern Medical Center, Dallas: Chaperone/substrate recognition.


M.-J.H. Gething, Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas: Peptide binding specificity of BiP.

L.E. Hightower, University of Connecticut, Storrs: Identification of hsc70-binding peptides selected from phage display libraries.

J.F. Dice, Tufts University School of Medicine, Boston, Massachusetts: A role for hsc70 in lysosomal degradation of intracellular proteins.

SESSION 4: Chaperones: Structure/Function Studies I
Chairperson: A. Horwich, Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, Connecticut

D. MacKay, Stanford University School of Medicine, California: Structure and mechanism of a 70-kD heat shock cognate protein.

J.G. Fink, University of California, Santa Cruz: Molecular mechanism of hsp70: Substrate protein dissociation precedes ATP hydrolysis.

SESSION 5: Chaperones: Structure/Function Studies II
Chairperson: L. Hendershot, St. Jude Children’s Research Institute, Memphis, Tennessee

R. McMacken, Johns Hopkins University, Baltimore, Maryland: Mechanistic studies of the ATPase activity of DnaK and its modulation by peptides, DnaJ and GrpE.


R.I. Morimoto, Northwestern University, Evanston, Illinois: Role of the carboxyl domain of hsp70 in substrate recognition and oligomerization.

L. Hendershot, St. Jude Children's Research Institute, Memphis, Tennessee: Characterization of the protein binding and dimerization domain of BiP.

A. Horwich, Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, Connecticut: Functional analyses of mutant forms of the GroEL chaperone, in vivo and in vitro.

G. Flynn, University of Oregon, Eugene: GroEL and protein assembly.
Gene Reactivation as a Therapeutic Strategy for Duchenne and Becker Muscular Dystrophies

April 25–April 27

FUNDED BY
Association Francaise contre Les Myopathies and the Muscular Dystrophy Association

ARRANGED BY
K.P. Campbell, Howard Hughes Medical Institute, The University of Iowa College of Medicine, Iowa City
K.E. Davies, University of Oxford, United Kingdom
J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York

SESSION 1: Factors in DMD Gene Therapy
Chairperson: L. Charash, Muscular Dystrophy Association Medical Advisory Committee, Hicksville, New York
T. Partridge, Charing Cross & Westminster Medical School, London, United Kingdom: Myogenesis and myogenic stem cells.
A.H.M. Burghes, Ohio State University, Columbus: Dystrophin production when it is not expected: Revertants in frame Duchennes and exon 3–7 deletions.
M. Perricaudet, Institut Gustave Roussy, CNRS, Villejuif, France: Adenovirus-mediated gene transfer to muscle fibers.
I. Danko, University of Wisconsin, Madison: Direct injection of genes into muscle.
R.H. Brown, Jr., Massachusetts General Hospital East, Charlestown: Approaches to cell therapy in muscular dystrophy.

SESSION 2: Regulation of Dystrophin and Dystrophin-related Protein Gene Expression
Chairperson: D.A. Fischman, Cornell University Medical College, New York, New York
D. Helfman, Cold Spring Harbor Laboratory, New York: Alternative RNA processing in the control of gene expression in muscle and nonmuscle cells.
K.P. Campbell, Howard Hughes Medical Institute, The University of Iowa College of Medicine, Iowa City: Dystrophin and utrophin: Functional and comparative aspects.
K.E. Davies, University of Oxford, United Kingdom: Utrophin regulation in skeletal muscle.
J.S. Chamberlain, University of Michigan Medical School, Ann Arbor: Dystrophin expression in wild-type, mdx, and transgenic mice.

G. Foulks, K. Davies
SESSION 3: Reactivation of Genes as a Therapeutic Strategy
Chairperson: S. Hauschka, University of Washington, Seattle

M.D. Schneider, Baylor College of Medicine, Houston, Texas: Activation of a generic fetal program in cardiac myocytes by peptide growth factors.
P.A. Jones, University of Southern California, Los Angeles: Gene activation by 5-azacytidine.
F. Grosveld, National Institute for Medical Research, London, United Kingdom: Control of globin gene expression.
G.D. Ginder, University of Minnesota Medical School, Minneapolis: Pharmacologic activation of embryonic globin genes in adult erythroid cells.
G.J. Dover, Johns Hopkins University School of Medicine, Baltimore, Maryland: Reactivation of a fetal gene.
S. Perrine, Children’s Hospital, Oakland, California: Activating fetal globin genes with arginine butyrate.

Industrial Sponsorship of Research in Academic Institutions

May 23-May 25

FUNDED BY
The Alfred P. Sloan Foundation

ARRANGED BY
J.D. Watson, Cold Spring Harbor Laboratory, New York
J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York

SESSION 1: Development of Technology Transfer and Biotechnology Policy
Chairperson: J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York

S. Brenner, The Scripps Research Institute, La Jolla, California: Biotechnology transfer in the United Kingdom.
E. Beuller, The Scripps Research Institute, La Jolla, California: Lessons from clinical science.
SESSION 2: Current Models for Large-scale Industrial-Academic Interactions I
Chairperson: D. Brown, Carnegie Institution of Washington, Baltimore, Maryland

D. Korn, Stanford University School of Medicine, California: Stanford University Medical School.
J.I. Gordon, Washington University School of Medicine, St. Louis, Missouri: Washington University School of Medicine.

G.R. Galluppi, Monsanto Company, St. Louis, Missouri: Monsanto Company.

SESSION 3: Current Models for Large-scale Industrial-Academic Interactions II
Chairperson: B. Stillman, Cold Spring Harbor Laboratory, New York

R.A. Lerner, The Scripps Research Institute, La Jolla, California: Scripps Research Laboratory.
G.G. Dellenbaugh, Robert Wood Johnson Pharmaceutical Research Institute, Raritan, New Jersey: Johnson & Johnson.
D.M. Livingston, Dana-Farber Cancer Institute, Cambridge, Massachusetts: Dana-Farber Cancer Institute.
T.O. Poehler, Johns Hopkins University, Baltimore, Maryland: Johns Hopkins University.
R.B. Shelton, University of California, Davis: University of California.
SESSION 4: Perspectives by Interested Parties I: Funding Bodies

**Chairperson: J. Halpern,** University of Chicago, Illinois

G.J. Galasso, National Institutes of Health, Bethesda, Maryland: National Institutes of Health.
C.A. Alexander, Howard Hughes Medical Institute, Chevy Chase, Maryland: Howard Hughes Medical Institute.

SESSION 5: Perspectives by Interested Parties II: Scientists

**Chairperson: R.C. Herdman,** Office of Technology Assessment, Washington, D.C.

**Discussants:**
F. Grinnell, Southwestern Medical School, Dallas, Texas
D. Nathans, Johns Hopkins Medical School, Baltimore
D. Brown, Carnegie Institute, Washington, D.C.

SESSION 6: Perspectives by Interested Parties III: Biotechnology Companies

**Chairperson: R.C. Herdman,** Office of Technology Assessment, Washington, D.C.

**Discussant:**
B.S. Conta, Regeneron Pharmaceuticals, Inc., Tarrytown, New York

SESSION 7: Perspectives by Interested Parties IV: Washington

**Chairperson: J.A. Witkowski,** Banbury Center, Cold Spring Harbor Laboratory, New York

**Discussant:**
S. Jenning, House Subcommittee on Regulation, Business Opportunities, and Technology, Washington, D.C.

**Panel Discussion:**
K. Hudson, Office of the Assistant Secretary of Health, Washington, D.C.
C. Scheman, Food and Drug Administration, Rockville, Maryland
SESSION 1: Endonucleases and Other Nuclear Events during Apoptosis
Chairperson: A.H. Wyllie, University Medical School, Edinburgh, United Kingdom

W.C. Earnshaw, Johns Hopkins University School of Medicine, Baltimore, Maryland: Nuclear events of apoptosis in a cell-free mitotic extract.
D. Ucker, Medical Biology Institute, La Jolla, California: Physiological cell death is an abortive mitosis.

SESSION 2: Cell Cycle Control
Chairperson: A.H. Wyllie, University Medical School, Edinburgh, United Kingdom

R.T. Schimke, Stanford University, California: A nested cell cycle progression as a signal for apoptosis: Potential mechanisms.
P. Neiman, Fred Hutchinson Cancer Research Center, Seattle, Washington: Apoptotic cell death during normal and neoplastic B-cell development in the bursa of fabrius.

SESSION 3: p53
Chairperson: G. Evan, Imperial Cancer Research Fund, London, United Kingdom

M.B. Kastan, Johns Hopkins Hospital, Baltimore, Maryland: Activation of a p53-dependent pathway by irradiation.
M. Oren, The Weizmann Institute, Rehovot, Israel: Role of p53 in apoptosis: Relevance to survival factor dependence.
A.H. Wyllie, University Medical School, Edinburgh, United Kingdom: Regulation of apoptosis by p53 in vitro and in vivo.
E. White, Rutgers University, Piscataway, New Jersey: Regulation of apoptosis by the transforming gene products of adenovirus.

SESSION 4: bcl-2 and Related Genes
Chairperson: M.C. Raff, M.R.C. Laboratory for Molecular Cell Biology, London, United Kingdom

S.J. Korsmeyer, Howard Hughes Medical Institute, Washington University School of Medicine, St. Louis, Missouri: Regulation of programmed cell death: An endogenous rheostat of bcl-2/Bax.
D. Loh, Howard Hughes Medical Institute, Washington University School of Medicine, St. Louis, Missouri: T-cell development and cell death.
C.B. Thompson, Howard Hughes Medical Institute, University of Chicago, Illinois: bcl-x, a bcl-2-related gene that functioned as a dominant regulator of apoptotic cell death.
M. Hengartner, Massachusetts Institute of Technology, Cambridge: Molecular genetics of programmed cell death in C. elegans.
SESSION 5: Cooperative Gene Effects in Lymphoid Cells
Chairperson: M.C. Raff, M.R.C. Laboratory for Molecular Cell Biology, London, United Kingdom

S. Cory, The Walter and Eliza Hall Institute of Medical Research, Victoria, Australia: bcl-2 in lymphopoiesis and lymphomagenesis.

A.B. Rickinson, The University of Birmingham Medical School, United Kingdom: Epstein-Barr virus genes protecting B cells from apoptosis.

SESSION 6: Social Controls
Chairperson: S.J. Korsmeyer, Howard Hughes Medical Institute, Washington University School of Medicine, St. Louis, Missouri


J. Savill, Royal Postgraduate Medical School, London, United Kingdom: Phagocyte recognition and clearance of cells undergoing apoptosis.

SESSION 7: Apoptotic Signals in T-lymphocyte Interactions
Chairperson: S.J. Korsmeyer, Howard Hughes Medical Institute, Washington University School of Medicine, St. Louis, Missouri

S. Nagata, Osaka Bioscience Institute, Japan: Fas antigen-mediated cell death and death of animals.

J.H. Russell, Washington University School of Medicine, St. Louis, Missouri: fas/gld pathway couples TCR/CD3 stimulation to T-cell suicide in mature T cells.

P. Golstein, Centre d'Immunologie de Marseille-Luminy, Marseille, France: Molecular mechanisms of T-cell-mediated cell death.

SESSION 8: Effectors and Inhibitors in Cytolytic Pathways
Chairperson: D. Hockenbery, Fred Hutchinson Cancer Research Center, Seattle, Washington


P. Anderson, Dana-Farber Cancer Institute, Boston, Massachusetts: Cytotoxic-granule-associated RNA-binding proteins as mediators of apoptotic cell death.

M. Rothe, Genentech, Inc., South San Francisco, California: Individual roles and signaling mechanisms of the two TNF receptors.

V.M. Dixit, University of Michigan Medical School, Ann Arbor: A20, a novel cytokine inducible zinc finger protein that inhibits apoptosis.

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SESSION 9: Developmental Control of Cell Death

Chairperson: D. Hockenbery, Fred Hutchinson Cancer Research Center, Seattle, Washington

H. Steller, Howard Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge: Programmed cell death in Drosophila.

J. Morgan, Roche Institute of Molecular Biology, Nutley, New Jersey: Role of immediate-early genes in programmed cell death.

N.M. Bonini and S. Benzer, California Institute of Technology, Pasadena: Cell death in the developing Drosophila eye.

In Vitro Selection from Combinatorial Libraries

October 17–October 20

FUNDED IN PART BY

SmithKline Beecham Pharmaceuticals

ARRANGED BY

G.F. Joyce, The Scripps Research Institute, La Jolla, California

SESSION 1: Theoretical Aspects

Chairperson: L.E. Orgel, The Salk Institute, San Diego, California


S. Kauffman, The Santa Fe Institute, New Mexico: Search on high-dimensional molecular fitness landscapes.

J. McCaskill, Institute for Molecular Biotechnology, Jena, Germany: Spatially resolved amplification and selection.

P. Schuster, Institute for Molecular Biotechnology, Jena, Germany: The search for RNA structures.

G.D. Stormo, University of Colorado, Boulder: Nucleic acid libraries.

D.C. Youvan, Massachusetts Institute of Technology, Cambridge: Computational optimization of combinatorial mutagenesis.
SESSION 2: Nucleic Acid Libraries
Chairperson: J. Szostak, Massachusetts General Hospital, Boston

A. Ellington, Indiana University, Bloomington: In vitro selection of aptamers that bind to viral proteins.
J.D. Keene, Duke University Medical Center, Durham, North Carolina: Selection of nucleic acid epitopes to the antigen-combining sites of antibodies.

L. Leung, Gilead Sciences, Foster City, California: Discovery and development of a thrombin inhibitor selected from a combinatorial ssDNA library.
L.E. Orgel, The Salk Institute, San Diego, California: In vitro selection of DNAs with unusual reactivity.

SESSION 3: Selection of Ribozymes
Chairperson: L.A. Loeb, University of Washington, Seattle

J.M. Burke, University of Vermont, Burlington: In vitro selection of hairpin ribozymes.
C. Drlica, Public Health Research Institute, New York, New York: Biological considerations concerning hammerhead ribozyme selection.
R. Green, University of California, Santa Cruz: Ribozyme selections as structural probes.
G.F. Joyce, The Scripps Research Institute, La Jolla, California: Better ribozymes through evolutionary chemistry.

SESSION 4: Phage Presentation Libraries
Chairperson: L. Gold, University of Colorado, Boulder

C.F. Barbas, The Scripps Research Institute, La Jolla, California: Synthetic antibodies and their use as scaffolds for the design of conformationally constrained peptides.
H.R. Hoogenboom, Cambridge Antibody Technology Limited, United Kingdom: Antibodies without immunization from phage display libraries.
R.C. Ladner, Protein Engineering Corporation, Cambridge, Massachusetts: Selection of binding proteins.

SESSION 5: Selection of Peptides and Small Organics
Chairperson: R.A. Lerner, The Scripps Research Institute, La Jolla, California

A. Schwienhorst, Max-Planck-Institute for Biophysical Chemistry, Gottingen, Germany: Experimental approaches to function space.
P. Schatz, Affymax Research Institute, Palo Alto, California: Peptide libraries linked to DNA-binding proteins.
K.S. Lam, Arizona Cancer Center, Tucson: Application of selective technology in various model systems.
M. Gallop, Affymax Research Institute, Palo Alto, California: Encoded synthetic libraries.
KB-binding Proteins: Their Role in Development and Growth Control

October 25–October 28

Funded by
Cold Spring Harbor Laboratory Corporate Sponsor Program

Arranged by
P.A. Baeuerle, Gene Center, Martinsried, Germany
R. Franz, Cold Spring Harbor Laboratory, New York
G.J. Nabel, Howard Hughes Medical Institute, University of Michigan, Ann Arbor

Session 1: NF-κB/REL Subunits: Biochemistry and Transcriptional Regulation I
Chairperson: P.A. Baeuerle, Gene Center, Martinsried, Germany

T. Maniatis, Harvard University, Cambridge, Massachusetts: The role of NF-κB in virus induction of the human B-interferon gene and the involvement of the high-mobility-group-protein HMG I(Y).
A. Israel, Institut Pasteur, Paris, France: The RBP-Jκ family represents κB-site-specific transcriptional activators.
R. Franz, Cold Spring Harbor Laboratory, New York: Alteration of Rel in a disease model.
G.J. Nabel, Howard Hughes Medical Institute, University of Michigan, Ann Arbor: NF-κB and HIV replication.
D.W. Ballard, Vanderbilt University School of Medicine, Nashville, Tennessee: A novel NF-κB complex containing p65 homodimers: Implications for transcriptional control at the level of subunit dimerization.

Session 2: NF-κB/REL Subunits: Biochemistry and Transcriptional Regulation II
Chairperson: T. Maniatis, Harvard University, Cambridge, Massachusetts

M.J. Lenardo, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland: NF-κB regulation of differentiation genes that control early T-cell development in the mouse thymus.
S. Ghosh, Yale University, New Haven, Connecticut: Regulation of NF-κB during B-lymphocyte differentiation.
I.M. Verma, The Salk Institute, San Diego, California: Regulation of κB/κB protein and direct association of Rel with TBP required for κB-driven transcription.
E. Serfling, University of Wurzburg, Germany: The κB-like site of the interleukin-2 promoter is a target of suppression of IL-2 transcription in T cells by agonists of protein kinase A.
C. Scheidereit, Max-Planck-Institute for Molecular Genetics, Berlin, Germany: Nuclear translocation control of NF-κB: Functional domains and role of phosphorylation.

Coffee break
SESSION 3: Dorsal Development and Signal Transduction
Chairperson: G.J. Nabel, Howard Hughes Medical Institute, University of Michigan, Ann Arbor

S.A. Wasserman, University of Texas Southwestern Medical Center, Dallas: Regulation of dorsal nuclear import.
R. Steward, Princeton University, New Jersey: Function of the cactus and dorsal protein.
B. Sha, The Rockefeller University, New York, New York: Analysis of mice deficient in NF-κB.
R. Bravo, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey: RelB: Role in mouse development?
M. Levine, University of California, San Diego, La Jolla: Characterization of DAF: A Rel protein in Drosophila that mediates an acute phase response.
Y. Engstrom, Stockholm University, Sweden: κB-binding proteins regulate the immune response in Drosophila and other insects.

SESSION 4: REL, p65, and Oncogenesis I
Chairperson: R.G. Roeder, The Rockefeller University, New York, New York

A. Manning, The Upjohn Company, Kalamazoo, Michigan: Inhibition of NF-κB activation in endothelial cells: Inhibition of leukocyte adhesion.

SESSION 5: REL, p65, and Oncogenesis II
Chairperson: R. Franz, Cold Spring Harbor Laboratory, New York

T.D. Gilmore, Boston University, Massachusetts: v-rel transformation: Functions required for transformation of chicken spleen cells by the v-rel oncogene.
P.J. Enrietto, State University of New York, Stony Brook: The role of c-rel in normal avian development and cell growth.

SESSION 6: IκB, Structural Motif, Protein/Protein Interactions
Chairperson: P.J. Enrietto, State University of New York, Stony Brook

P.A. Baehner, Gene Center, Martinsried, Germany: How is IκB released from NF-κB?
A.S. Baldwin, University of North Carolina, Chapel Hill: Regulation of NF-κB activity by cytoplasmic and nuclear mechanisms.
N.R. Rice, National Cancer Institute-Frederick Cancer Research and Development Center, Maryland: IκB.
SESSION 1: Overview of Human Molecular Genetics

F.C. Collins, National Center for Human Genome Research, National Institutes of Health, Bethesda, Maryland: Human molecular genetics and its impact on clinical genetics.

SESSION 2: Mapping, Sequencing, and Cloning Genes

E. Lander, Whitehead Institute for Medical Research, Cambridge, Massachusetts: How to find genes.
J. Sulston, Sanger Centre, Cambridge, United Kingdom: Sequencing DNA and why do it for a worm?
M.-C. King, University of California, Berkeley: The molecular genetics of human cancers.

SESSION 3: Genomes and Computers

T. Marr, Cold Spring Harbor Laboratory, New York: Genome informatics: What it does and a hands-on experiment.

SESSION 4: Applications and Consequences of Molecular Human Genetics

C.T. Caskey, Howard Hughes Medical Institute, Baylor College of Medicine, Houston, Texas: DNA-based diagnostics: Technical advances and commercial developments.

Roundtable Discussion:
The human genome and biotechnology.
Mechanisms of Developmental and Tumor Angiogenesis

November 7–November 10

Funded by
Cold Spring Harbor Laboratory Corporate Sponsor Program

Arranged by
D. Hanahan, University of California, San Francisco
J. Folkman, Children’s Hospital, Boston, Massachusetts: Introduction.

Session 1: Developmental Angiogenesis
Chairperson: R. Auerbach, University of Wisconsin, Madison

F. Dieterlen, Institut d’embryologie cellulaire et moleculaire du CNRS, Nogent Sur Marne, France: Ontogenic emergence of the endothelial network analyzed in the avian model.
W. Risau, Max-Planck-Institute, Bad Nauheim, Germany: Molecular mechanisms of vasculogenesis and embryonic brain angiogenesis.
M.L. Breitman, Mount Sinai Hospital, Toronto, Canada: The Tek endothelial receptor tyrosine kinase is required for mouse development.
D. Coffin, McLaughlin Research Institute, Great Falls, Montana: Growth factors in vascular morphogenesis.

Session 2: Positive Controls of Angiogenesis I
Chairperson: H.F. Dvorak, Harvard Medical School, Boston, Massachusetts

E. Keshet, The Hebrew University, Jerusalem, Israel: Role of VEGF in ischemia-induced angiogenesis.
K.H. Plate, Philipps-Universitat Marburg, Germany: Molecular mechanisms involved in glioma angiogenesis.
P.A. D’Amore, Harvard Medical School, Boston, Massachusetts: Endothelial cell-pericyte interactions in the vasculature.
SESSION 3: Positive Controls of Angiogenesis II
Chairperson: M. Klagsbrun, Harvard Medical School, Boston, Massachusetts

T. Maciag, American Red Cross, Rockville, Maryland: Mechanisms of fibroblast growth factor action.
G. Christofori, University of California, San Francisco: The angiogenic switch: Angiogenic factors and molecular events in multistage tumor development.
K. Alitalo, University of Helsinki, Finland: Roles of Tie and FLT4 receptor tyrosine kinases in megakaryoblastic differentiation and angiogenesis.
M. Bernfield, Harvard Medical School, Boston, Massachusetts: The Syndecans: A family of cell surface co-receptors for matrix and growth factors.

SESSION 4: Complex Controls of Angiogenesis
Chairperson: S.M. Schwartz, University of Washington, Seattle

E.H. Sage, University of Washington, Seattle: Regulation of angiogenesis by SPARC and type I collagen.
E.F. Wagner, IMP Research Institute, Vienna, Austria: Polyoma middle T expression and the control of endothelial cell growth.
V. Bautch, University of North Carolina, Chapel Hill: Transgenic mouse models of angiogenesis.
M.S. Pepper, University of Geneva, Switzerland: Angiogenesis in vitro: Cytokine interactions and extracellular proteolysis.

SESSION 5: Negative Control of Angiogenesis
Chairperson: D. Hanahan, University of California, San Francisco

N.P. Bouck, Northwestern University, Chicago, Illinois: Tumor suppressor gene control of thrombospondin and angiogenesis.
R. Weiner, University of California, San Francisco: The 16-kDa amino-terminal fragment of prolactin: An inhibitor of angiogenesis.
J. Folkman, Children's Hospital, Boston, Massachusetts: Circulating endothelial inhibitors.

Nitric Oxide: Molecular Mechanisms of Synthesis and Action

November 14–November 17

FUNDED BY
Cold Spring Harbor Laboratory Corporate Sponsor Program

ARRANGED BY
M.A. Marletta, University of Michigan College of Pharmacy, Ann Arbor

SESSION 1: NOS: Regulation and Expression
Chairperson: S.R. Tannenbaum, Massachusetts Institute of Technology, Cambridge

C.F. Nathan, Cornell University Medical College, New York, New York: Inducible NOS: Molecular aspects of regulation.
T. Michel, Brigham & Women's Hospital, Boston, Massachusetts: Biosynthesis and molecular regulation of endothelial nitric oxide synthase.
T.R. Billiar, Presbyterian University Hospital, Pittsburgh, Pennsylvania: From rodents to humans: Characterization of hepatic inducible NOS.
J. Cohen, Royal Postgraduate Medical School, London, United Kingdom: Cytosine interactions with the inducible NOS.
J.M. Cunningham, Brigham & Women's Hospital, Boston, Massachusetts: Regulation of arginine transport.
SESSION 2: NOS: Mechanism

**Chairperson:** T. Michel, Brigham & Women’s Hospital, Boston, Massachusetts

M.A. Marletta, University of Michigan College of Pharmacy, Ann Arbor: Current studies on NOS.

S. Kaufman, National Institute of Mental Health, National Institutes of Health, Bethesda, Maryland: Studies on mechanisms of rat brain NOS.

B.M. Mayer, Universitat Graz, Austria: Characterization of NOS-binding domains for L-arginine and tetrahydrobiopterin.

B.S.S. Masters, The University of Texas Health Science Center at San Antonio: Molecular aspects of the constitutive cerebellar NOS.

D.J. Stuehr, Cleveland Clinic, Ohio: Characteristics of macrophage NOS subunits and their assembly into functional dimeric enzyme.

SESSION 3: In Vivo Reactions of NO

**Chairperson:** C.F. Nathan, Cornell University Medical College, New York, New York

B. Demple, Harvard School of Public Health, Boston, Massachusetts: An NO-sensing gene regulator controlling resistance to activated macrophages.


L.K. Keefer, National Cancer Institute, National Institutes of Health, Frederick, Maryland: Genotoxicity of NO and its progenitors: An update.

E.M. Schuman, California Institute of Technology, Pasadena: NO, ADP ribosyltransferase activity, and intercellular signaling in long-term potentiation.

J. Moss, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland: Effects of NO on modification of proteins by NAD and ADP-ribose.

G. Enikopolov, Cold Spring Harbor Laboratory, New York: NO and gene expression in neuronal cells.

SESSION 4: NO/Guanylate Cyclase System

**Chairperson:** M.A. Marletta, University of Michigan College of Pharmacy, Ann Arbor

F. Murad, Molecular Geriatrics, Lake Bluff, Illinois: The NO-cGMP signal transduction system.

J. Burstyn, University of Wisconsin, Madison: Mechanisms of activation of soluble guanylyl cyclase.

J.R. Stone, University of Michigan, Ann Arbor: Purification of soluble guanylate cyclase from bovine lung and initial spectral characterization.

J. Corbin, Vanderbilt University, Nashville, Tennessee: Specificity and mechanisms of cGMP receptors.

J.M.C. Ribeiro, University of Arizona, Tucson: NO synthesis, storage, and delivery in the salivary glands of a blood sucking bug, *Rhodnius prolixus.*
SESSION 5: NO: Metal Complexes

Chairperson: L.K. Keefer, National Cancer Institute, National Institutes of Health, Frederick, Maryland

F.T. Bonner, State University of New York, Stony Brook: Aspects of aqueous NO chemistry.
W.B. Tolman, University of Minnesota, Minneapolis: Chemical modeling of the interactions of NO with copper sites in biology: Binding and activation of NO by biomimetic copper complexes.
V.S. Sharma, University of California, San Diego, La Jolla: Reactions of NO with heme-proteins.
D.J. Singel, Harvard University, Cambridge, Massachusetts: Magnetic resonance studies of NO biochemistry.

The Genetics of Manic Depressive Illness

December 8–December 11

FUNDED BY

The Charles A. Dana Foundation

ARRANGED BY

J.R. DePaulo, The Johns Hopkins Hospital, Baltimore, Maryland
K.R. Jamison, The Johns Hopkins Hospital, Baltimore, Maryland
J. Mallet, Centre National de la Recherche Scientifique, Cedex, France
P. McGuffin, University of Wales College of Medicine, Cardiff, United Kingdom
R.M. Post, National Institute of Mental Health, National Institutes of Health, Bethesda, Maryland
J.A. Witkowski, Banbury Center, Cold Spring Harbor Laboratory, New York.

SESSION 1: Brief Reports of Current Genetic Studies

Chairperson: D. Botstein, Stanford University School of Medicine, California

W.F. Byerley, University of Utah School of Medicine, Salt Lake City
N.B. Freimer, University of California, San Francisco
D.S. Gerhard, Washington University School of Medicine, St. Louis, Missouri
E.S. Gershon, National Institute of Mental Health, National Institutes of Health, Bethesda, Maryland
C. Gilliam, New York State Psychiatric Institute, Columbia University, New York
J.R. Kelsoe, University of California, San Diego, La Jolla
SESSION 2: Issues in Diagnosis

Chairperson: R.M. Post, National Institute of Mental Health, National Institutes of Health, Bethesda, Maryland

S. Simpson, The Johns Hopkins Hospital, Baltimore, Maryland: Defining the bipolar phenotype.
J.A. Egeland, University of Miami North Research Office, Hershey, Pennsylvania: Variable age of onset and diagnostic stability for BP1 disorder.
K.R. Jamison, The Johns Hopkins Hospital, Baltimore, Maryland: Clinical description of manic-depressive illness.
V. Reus, University of California, San Francisco: Children of bipolar probands: A longitudinal high-risk study.
W.C. Drevets, Washington University School of Medicine, St. Louis, Missouri: PET imaging of mood disorders: Implications for family studies.
R.M. Post, National Institute of Mental Health, National Institutes of Health, Bethesda, Maryland: Roundtable discussion.

SESSION 3: Candidate Gene Approach

Chairperson: C. Gilliam, New York State Psychiatric Institute, Columbia University, New York

A.J. Lewy, Oregon Health Sciences University, Portland: Biological markers in circadian phase disorders.
R.R. Crowe, University of Iowa Hospitals and Clinics, Iowa City: Discuss candidate gene approaches.
D. Schaid, Mayo Clinic, Rochester, Minneapolis: Relative risk methods for candidate genes using cases and their parents.
J.C. Hall, Brandeis University, Waltham, Massachusetts: Rhythm disorders in Drosophila.
T.A. Wehr, National Institute of Mental Health, National Institutes of Health, Bethesda, Maryland: Biological rhythms.

SESSION 4: Genetic Strategies I

Chairperson: N.J. Risch, Yale University School of Medicine, New Haven, Connecticut

D.R. Cox, Stanford University School of Medicine, California: Development of efficient strategies for linkage analysis using meiotic maps spanning the human genome.
T. Reich, Jewish Hospital, St. Louis, Missouri: The power of the genebank database to detect genes of moderate effect (oligogenes).
SESSION 5: Genetic Strategies II
Chairperson: J. Mallet, Centre National de la Recherche Scientifique, Gif-sur-Yvette Cedex, France

R. Plomin, Pennsylvania State University, University Park: Quantitative trait loci: Is manic-depression a dimension or disorder?
R.A. Price, University of Pennsylvania, Philadelphia: Polygenic (QTL) approaches to identifying genes for depression.
D.W. Fulker, University of Colorado, Boulder: A sib-pair approach to interval mapping of quantitative trait loci.

SESSION 6: Future Developments
Chairpersons: D.R. Cox, Stanford University School of Medicine, California
P. McGuffin, University of Wales College of Medicine, Cardiff, United Kingdom
Science museums and centers occupy an important position in American science education, providing "informal" learning opportunities that usually cannot be found within the formal school context. Science museums tend to accentuate technology and physical phenomena, which are amenable to hands-on exhibits that can stand up to use by large numbers of young people. With the exception of natural history, biology content is underrepresented, primarily because its subject matter does not translate well into interactive exhibits—living things by and large are not very amenable to manipulation by large numbers of students. Molecular genetics has proved a particularly difficult topic, because it deals almost entirely with phenomena that cannot be seen or manipulated in the usual sense. Our institution is unique in its focus on this difficult subject area. Our solution to the problem of dealing with things invisible has been to concentrate on providing high-level laboratories that help students learn about genes using the same modes of inference employed by research biologists. This emphasis on lab work places us in the company of a handful of science centers, notably the Lawrence Hall of Science, in Berkeley, California, and Fernbank Science Center, in Atlanta, Georgia.

Another unique attribute is our building, which was constructed in 1925 as Union Free School, the first "modern" elementary school in the village of Cold Spring Harbor. Designed by the noted New York architectural firm, Peabody, Wilson & Brown, the building’s symmetrical brick facade and details, such as dentil moldings and quoins, echo the Georgian revival architecture popular among builders of Long Island’s "Gold Coast" estates of the period. It is likely not coincidence that school board member and patron of construction of the building Jean Brown Jennings lived at the large Georgian-style estate "Burrwood" in nearby Lloyd Harbor with her husband Walter Jennings, a founder of Standard Oil Company. We think that Mrs. Jennings and others who were responsible for the construction of this beautiful building, as well as those who spent a good portion of their childhoods here, would be happy to see it preserved and tastefully updated as a showcase of modern science. In an architectural sense, the DNALC is truly a house of science, unlike most science centers, which typically strive to look futuristic. Many of these facilities are out of context with the architecture of their urban surroundings and appear unstuck in time, perhaps in a subtle way perpetuating the stereotype of science as an ivory tower out of touch with mainstream culture. We believe that our antique-style building sends the healthier message that science is forward looking, yet rooted in time and culture.
We Own a Completely Refurbished Facility

The DNALC has gone through several rounds of renovations, totaling $1.5 million, since we took lease of the facility from the Cold Spring Harbor Central School District in September 1987. Initial renovations in 1987–1989 included installing new heating/air-conditioning and electrical systems and converting a former classroom into the Bio2000 Biochemistry Laboratory, the teacher’s lounge into a bookstore, and the unfinished basement into staff offices and a research/prep laboratory. Other former classrooms, a library, and auditorium were converted into galleries in which were installed The Search for Life, a multimedia exhibit on the history of genetic technology loaned by the National Museum of American History.

Bridge financing by the Banbury Fund and tax-exempt financing from the Nassau/Suffolk County Industrial Development Agency made possible the purchase of the DNALC property in January 1993. A concept design study funded by longtime friends Mary Jeanne and Henry Harris provided a guide for the redevelopment of the main floor and exterior of the building in 1993. Prior to beginning renovations in April was the arduous task of clearing the building of some 9000 cubic feet of exhibit materials and hardware from The Search for Life exhibit, including two tractor trailers of parts! Major entrances were rebuilt and automatic fire sprinklers were installed to bring the building into full compliance with all handicap access, fire, and safety codes. Three galleries were renovated for future exhibits on human genetics and research at Cold Spring Harbor Laboratory. A BioMedia Computing Laboratory, equipped with 13 Macintosh computers and video projection, was installed in renovated space adjacent to the existing Bio2000 Biochemistry Laboratory. This project, as well as renovation of a

(Left to right) General contractor Bill Baldwin, DNALC Director David Micklos, CSHL Buildings and Group Director Jack Richards, and Centerbrook architect Jim Childress discuss renovation plans.
Demolition and reconstruction of front porch of DNALC.
computation/design office in the last remaining undeveloped basement space (in 1994), will be the final phase in the development of the DNALC as a model Human Genome Education Center. Planned for 1995 is construction of a 3500-square-foot BioMedia addition to the south side of the building, which will include a genetics laboratory and research library. The addition will also include modern lavatory facilities and an atrium/lunchroom—practical features needed to effectively handle our growing numbers of student visitors.
The 18 by 50-foot *Cellarium*, a room-size mural of the interior of a human cell, creates a splendid backdrop in the main gallery. This cell mural was completed during the summer by five art students from The Cooper Union for the Advancement of Science and Art (Hope Gangloff, Marius Jaskowski, and Jackie O'Neill) and the New York Institute of Technology (Donna Conversano and Elvia Reynolds), working under the direction of staff designer Susan Lauter. A 1/12th scale master drawing was divided into a 2 by 2-inch grid, and a corresponding 2 by 2-foot grid was chalked onto the ceiling and walls of the gallery. Each grid square on the master was scaled up on a 2-foot square of drafting tissue. Then, the back of each tissue drawing was rubbed with graphite and traced into its appropriate grid position in the gallery. The variety of colors seen in the mural were obtained by mixing only 12 colors of latex interior house paint. Twenty-five gallons of paint were applied using brushes and natural sea sponges.

The centerpiece of the renovation is a 104-seat multimedia auditorium, or *Multitorium*. The vaulted ceilings and plaster moldings of the original 1925 auditorium were preserved and complemented with a bold color scheme of blues, rust, and terra cotta. A frieze carries the names of 35 Nobel laureates who have contributed to our understanding of genetics and the molecular basis of heredity. Deeply banked seating provides a superb view for all, and the wide aisles provide an unusual feeling of spaciousness. A remote control allows one to shift easily between all major types of visual presentation—video, laser disk, computer, compact disk, slides, and overhead/opaque projection. The audio system makes use of JBL power amplifiers and three-way JBL studio monitors, computerized feedback control, and wireless radio-frequency microphones.
Long Island Discovery

The Multitorium is the venue for Long Island Discovery, which opened in September and received 6400 viewers during its first quarter of operation. This 28-minute "electronic field trip" was especially designed to help students explore the rich history of Long Island. Beginning with the mammoth glaciers that created the unique 118-mile-long landscape, the production presents a collage of the Long Islanders who shaped its history, including Indians, settlers, and whalers; Captain Kidd, the pirate; Anna Strong, the Revolutionary War spy; Jupiter Hammon, the first published African-American poet; Charles Lindbergh, the first trans-Atlantic aviator; and William Levitt, the architect of suburban living. The fast-paced presentation makes full use of the Multitorium's audiovisual capabilities. Thousands of images from 18 slide projectors and 3 video projectors are trained on three 5 by 7-foot glass screens and three 32-inch television monitors. Digital stereo surround sound is provided through ten JBL speakers, including an 18-inch sub-woofer.

Long Island Discovery was produced by Cablevision Systems Corporation, one of the nation's largest cable operators. The project was conceived by Charles Dolan, Cablevision Chairman/CEO and Cold Spring Harbor Laboratory Trustee, after seeing a similar presentation in Ireland. Charles recognized a fit between the DNALC's desire for a state-of-the-art auditorium and Cablevision's need for a dedicated facility to make its production widely available to local school children. Cablevision provided major funding for the renovation of the Multitorium and galleries, and the urgency to bring Long Island Discovery quickly to the public provided the impetus to complete construction in only 5 months+.

Renewal of National Science Foundation Support

The new year brought news of the renewal of key National Science Foundation (NSF) support of our training programs for high school and college faculty. A 3-year grant of $854,150 continues support for the DNA Science Workshop for high school teachers, which has received NSF funding since 1987. Through 1993, DNALC staff have instructed 1639 teachers at 74 workshops held in 31 states, Canada, and Puerto Rico. Building upon our past success, the new NSF program provides a two-tier model for organizing a critical mass of leadership teachers in molecular genetics at the national level and for disseminating genetics instruction at the local level. At the first level, Targeted DNA Science Workshops provide first-order training activity for underserved teachers and act as a screen for teachers with leadership potential. At the second level, the Leadership Institute in Human and Molecular Genetics provides recognition and super-order training for innovative leaders among the estimated 3500 high school teachers who have taken training workshops on molecular genetics/biotechnology. The aim is to utilize super-lead teachers as regional educational resources to ramify new teaching technologies in their regions and provide liaison between school systems and genetic information sources (such as genetic disease foundations/support groups and the National Institutes of Health). Following their training, lead teachers are pledged to provide a minimum of 30 hours in-service instruction for elementary and secondary teachers in their regions.

Our analysis of databases assembled by the National Academy of Science indicates that there are approximately 30 major, ongoing training programs for precollege teachers in genetics/biotechnology that are administered through
Leadership Institute participants (left) recreate a vintage photo of an early class at the Biological Laboratory (right, circa 1895).

academic institutions in the United States. However, most high-caliber institutes are offered in major urban centers of the east, midwest, and far west, thus effectively excluding rural and nonurban teachers in the south, midwest, southwest, Rocky Mountains, northwest, and Puerto Rico. Thus, the targeted workshops are designed to provide educational equity to underserved teachers in rural/nonurban areas by offering them the same high-quality instruction available to their urban peers. The first round of targeted workshops was held at John McDonogh High School in New Orleans and the University of Utah in Salt Lake City.

The Leadership Institute, held July 6–30, drew together 23 innovative teachers from 16 states. The participants’ previous experiences included teaching advanced laboratory units in molecular genetics, leading teacher-training workshops, developing model curriculum materials, administering equipment-sharing consortia, and conducting a state-wide survey of biotechnology education. To build upon this expertise, the institute provided 160 hours of advanced laboratory experimentation, scientist seminars, computer explorations, leadership training, classroom observations, and independent projects. One highlight was an informal question-and-answer period with the Laboratory’s Director James D. Watson, who shared the Nobel prize for the discovery of the structure of DNA. Others were "high-technology rotations" through the laboratories of Cold Spring Harbor scientists working in the technology-intensive areas of X-ray crystallography, protein biochemistry, and two-dimensional protein electrophoresis. Leadership teachers were free to attend seminars of three of the Laboratory’s postgraduate training courses, which were scheduled concurrently. Leadership teachers were housed along with visiting scientists on the Laboratory’s 100-acre campus. A summer stay at Cold Spring Harbor, and the chance to interact informally with staff and visiting scientists in residence, was a fitting honor for the leadership teachers, who represent the top 1% of high school biology instructors nationwide.

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The continuation of our successful Advanced DNA Science Workshop for college faculty was assured upon renewal of our NSF grant, which provides $291,275 of support during the next 2 years. The workshop curriculum articulates with and extends that of the DNA Science Workshop to include more sophisticated techniques such as restriction mapping; Southern hybridization (nonradioactive); genomic library construction and analysis, cloning by polymerase chain reaction (PCR); and human DNA fingerprinting by PCR. The curriculum will be formally published in 1994 as Laboratory DNA Science: An Introduction to Recombinant DNA Technology and Methods of Genome Analysis.

During the summer, a pair of 2-week Advanced DNA Science Workshops were conducted at the University of Puerto Rico, Rio Piedras (July 26–August 6), and the University of Washington, Seattle (August 16–27). The program attracted 43 faculty from predominately teaching institutions in seven states and Puerto Rico. An additional 34 faculty from six different states were trained at a pair of workshops (Columbia University, May 24–June 1, and California State University, Northridge, June 14–25) sponsored by the Department of Education’s Fund for the Improvement of Postsecondary Education.

Human Genome Diversity and Genetic Sensitivity

The rapid proliferation of screening tests to detect the genetic basis of human diseases ultimately may lead to social and legal judgments about what is genetically normal versus abnormal. Reducing predictive medicine to pinpointing often tiny genetic variances from the norm may also produce a mindset that overly focuses on the genetic differences between people. This runs counter to scientific evidence from the study of population genetics, which concludes that human beings are more alike genetically than they are different. Embodied in each person’s genes is an unbroken flow of human evolution dating back millions of years. Noted human geneticist Luca Cavalli-Sforza has said that understanding the common ancestry of all human beings may be the best inoculation against racism for young people growing up in a world that increasingly emphasizes cultural, racial, and ethnic differences.

In addition to fostering formal genetic literacy, biology educators also will be faced with the task of turning out compassionate citizens who understand that, as Laboratory Director James Watson has said, “Some people get a bad start in life because they are born into poverty, and some people get a bad start in life because they are born with a bad set of genes.” Most teachers attempt to stimulate this type of higher-order social and ethical analysis through case studies, role-playing, and panel discussion. Each of these methods essentially asks students to identify with an abstract situation largely removed from their own experiences. These methods often require a “briefing” of the “facts” of a case or technology, which can be substantially colored by the teacher’s presentation or selection of materials.

As a remedy to this situation, we began in earnest in 1993 to develop plans for a Human Genome Diversity—Student Allele Database (HGD-SAD) project that will provide an experiment-based means for high school and college students to investigate human genome diversity. The project centers around a hands-on laboratory that enables students to produce a personal “DNA fingerprint” of the alleles (DNA variations) they have inherited on chromosome 1 (D1S80). Students then have the option of submitting their results to a Student Allele Database.
maintained at the DNALC. Via computer, students can perform various statistical
tests to compare their own alleles with those of students from around the United
States and Europe. The exercise of making a personal DNA fingerprint and then
comparing it to the fingerprints of other students challenges students to consider
their place in the family of humankind, thus opening the door to genetic
sensitivity. Students’ questions about their own participation in the project
engender much of the ethical and personal decision making of DNA typing and
genetic data banking:

- Should I submit my alleles to the Student Allele Database?
- Does it matter if my anonymity is safeguarded?
- Are my alleles rare or frequent (in my class, in the world)?
- Do I share alleles with people of other races?
- Are the allele frequencies in my class different from those in other parts of
  the world?
- How genetically alike or different are people of (different countries, dif-
  ferent races)?
- What if my DNA fingerprint was a diagnosis of disease susceptibility?

HGD-SAD also offers an example of the interdisciplinary nature of human
genome research, which blends genetics, biochemistry, statistics, computational
science, and social science. The commitment to map and sequence the human
genome has called attention to the need for efficient computer strategies to col-
lect, organize, store, and access the billions of nucleotides of DNA sequence to
be generated over the next decade. HGD-SAD provides a simple analog of the
growing use of computers in human genome research and illustrates a means to
link together experimental and computer resources to encourage understanding
of biological concepts. The project also provides an unprecedented opportunity
for biology students and teachers to work in concert with genome researchers as
they develop computational systems for genetic research.

The Student Allele Experiment

The student allele experiment is based on a forensic DNA typing kit optimized for
educational use by DNALC staff member Mark Bloom. The object of the student
allele experiment is to amplify and visualize a locus on the short arm of
chromosome 1, termed D1S80, at which varying numbers of copies of a repeated
DNA sequence create polymorphisms. These variable numbers of tandem
repeats (VNTRs) are inherited in a Mendelian fashion as distinguishable alleles.
The VNTR polymorphism at D1S80 has a repeat unit of 16 base pairs and dis-
plays 29 alleles that determine 435 possible genotypes. The size of a D1S80 al-
lele depends on the number of VNTR copies present on the chromosome 1 in-
herited from the mother or father. For example, the maternally inherited allele may
have 21 copies of the repeat unit, whereas the paternally inherited allele may
have 14 copies. These different alleles can be separated by size using elec-
trophoresis.

The experiment consists of three parts: sample isolation, amplification by
PCR, and analysis by polyacrylamide gel electrophoresis (PAGE). Participating
schools receive a kit that contains two simple reagents (sterile saline and 10%
Chelex) and supplies for a class of students to prepare DNA extracts from their
own cells. Students obtain cheek epithelial cells by a 10-second rinse with saline
mouthwash (*bloodless and noninvasive*). Incubation at boiling temperature lyses the cheek cells and releases the DNA into solution. Cellular debris is separated from the crude DNA extract by centrifugation with Chelex, a chelating agent that binds inhibitors of the PCR.

The student DNA samples, identified only by number, are then passed on to a partner institution for PCR amplification and allele separation. A sample of this crude DNA extract is added to a "cocktail" of amplification reagents, including Taq polymerase, the four nucleotide phosphates, magnesium, and primers that identify the D1S80 locus on chromosome 1. Polymerase chain reaction is used to amplify the D1S80 locus of each student sample, and the amplification products are electrophoresed on a polyacrylamide gel, together with an allelic ladder of 27 amplified D1S80 alleles. A photostat of the stained gel is then provided to the participating teacher. Comparison of bands present in each lane with the allelic ladder allows students to determine their own D1S80 genotypes. Students have the choice of submitting their genotypes and demographic information, without personal identifiers, to the *Student Allele Database*.

HGD-SAD offers an appropriate point of collaboration for high schools and genome research centers. Electrophoretic analysis of human alleles is the next logical step for the growing numbers of high school biology teachers who are conversant in modern molecular genetics and currently perform electrophoretic analysis of viral DNA with their students. Preparation of student DNA samples requires only a clinical centrifuge and hotplate, which are available at most high schools or can be borrowed from a partner genome research center. Although the equipment needed for human allele detection—a DNA thermal cycler and polyacrylamide electrophoresis apparatus—is currently beyond the means of even the most advanced high school teachers, it is commonplace in any genome research center. A several hour commitment by a technical staff member at a genome research center is all that is required to generate the student genotypes.

Several options are open to high school teachers who would like to do more in-class experimentation. Amplified alleles can be separated in agarose using mini-gel apparatuses common in high schools. Although agarose systems can differentiate a number of alleles, they do not offer resolution great enough to score alleles for submission to the archival portion of the *Student Allele Database*. Efficient amplification of D1S80 alleles from crude extracts requires an automated DNA thermal cycler, the least expensive of which now costs approximately $2800. Therefore, we are working with the biocomputing center at the University of Chicago to develop a "washing machine" thermal cycler that will provide a low-cost alternative for amplifying student samples. Working from a design used in early efforts to automate thermal cycling at Cetus Corporation, teachers
at the 1993 Leadership Institute built a prototype machine, essentially composed of a washer valve and plumbing supplies, that successfully amplified the D1S80 alleles. We are currently working with the University of Chicago biocomputing center to construct a computer controller that can be distributed with a ready-to-assemble kit.

With funds from the Stone Foundation, we have equipped a Student Allele Reference Laboratory to qualify all allele submissions to the archival Student Allele Database. The reference laboratory will also process student samples referred from teachers in rural and nonurban areas nationwide who lack a nearby partner institution. The laboratory is equipped for large-format PAGE and a computerized documentation system that allows submitted students' gels to be automatically read and digitized for archival storage. Tom White, Vice President of Research & Development of Roche Molecular Systems, has agreed to donate 750 PCRs and provide 750 reactions at a reduced price each year over the next 5 years to support the reference laboratory and activities to "seed" the project in different regions of the country.

The Student Allele Database

The Student Allele Database program software is being jointly developed here and in collaboration with Helen Donis-Keller at Washington University and John Kruper at the University of Chicago. Program software will be developed to run on three platforms: Macintosh, UNIX, and MS-DOS. Initial applications are being developed to run on Macintosh computers, which are common in high schools, followed by DOS/MS Windows versions. Several applications that will be part of
the package have already been developed in the Donis-Keller laboratory for use in a UNIX environment, which is common in genome research centers. These utilities will be the starting points for designing a robust and scalable client-server application to administrate SAD materials. The database will be composed of four major utilities, all of which make use of a user-friendly graphical interface:

- On the client side, a Public Data Utility (PDU) is accessible to all users and provides data entry forms for recording allele data and demographic identifiers. The PDU also provides a workspace where data can be temporarily stored and evaluated, or compared to data stored in the archive. The PDU operates both in "stand-alone" mode (allowing entry, display, and analysis of local data) and in a "networked client" mode (allowing data submission to the archived dataset and comparison of local data to archival data).

- On the server side, an Archival Data Utility (ADU) is accessed through the PDU on a "read only" basis for users. The archival database contains submitted student cases that have been refereed to meet standards for scientific use and incorporates relational database technology to allow fast and efficient processing of simultaneous queries. The ADU can be accessed by the system administrator to enter and/or edit refereed cases. The ADU incorporates key features of research databases, including scalability, data integrity, and disaster recovery.

- A Statistics Utility is accessible to all users to enable data analysis using files from the PDU and ADU. A number of statistical and graphical tools will be added over time, including allele frequency estimation, heterozygosity and polymorphic information content (PIC), Hardy-Weinberg equilibrium, single allele isoclines, analysis of variance, and genetic distance.

- A Bulletin Board is accessible to all users, allowing students and teachers to share findings, interpretations, lesson plans, and curricular materials. In addition, the Bulletin Board operates an Internet Relay Chat (IRC) system to provide interactive dialogue between participants. With this, teachers and students can discuss in real time their local efforts and ask questions of designated professor "mentors" (drawn from the partner regional genome research centers) during scheduled on-line "seminars."

- The Bulletin Board and Archival Data Utility reside on a Sun Sparcstation 10-30 server with one gigabyte SSCI disk storage currently available at the DNALC. A fiber optic line will link the DNALC to the main Laboratory campus, providing high-speed access to national and international networks. Partner research institutions here and abroad will likely prefer to access the database through Internet. However, secondary biology teachers will likely tie into the system via Access Excellence, a biology education network that is reached through America Online.

International Collaborations

Although human genome diversity is not a large element of the Human Genome Initiatives of the National Institutes of Health and the Department of Energy, it is an important component of research funded by the international Human Genome Organization (HUGO). Our Italian collaborator, Marcello Siniscalco is active in the HUGO project, and his institution, Porto Conte Research and Training Laboratories (PCRTL) in Sardinia, has been the site of planning workshops for the HUGO-sponsored project, The People of Europe. As a reference laboratory in the
HUGO genome diversity project, PCRTL maintains a high-speed Internet connection. Thus, it was appropriate that Dr. Siniscalco agreed that PCRTL will serve as the European node for the Human Genome Diversity–Student Allele Database, (HGD-SAD), mirroring the SAD on its system and qualifying European submissions to the archival database.

To cement the new collaboration, a pilot Human Genome Diversity Training Workshop was conducted at the PCRTL as part of the European Week of Science in November. Funded by the Italian Ministry of University and Scientific and Technological Research, the workshop built upon previous DNALC collaborations with PCRTL to develop activities for the Italian Week of Science in 1991 and 1992. The pilot workshop was attended by 19 secondary science teachers (representing 11 European countries) who produced their own D1S80 fingerprints and tested a new method of preparing DNA from saliva. Highlights of the workshop were talks by Luca Cavalli-Sforza and Alberto Piazza, both authorities on human genetic diversity. They showed the participants methods for analysis of genetic distance using D1S80 data from Asian Indians, American Blacks, American Caucasians, and Sardinian school students (obtained by Dr. Siniscalco’s group). Several alleles were found among the Sardinian students that had never been seen among sample populations in North America.

Local Activities and Training Workshops

The Bio2000 Laboratory was kept very busy during the academic year, with 3200 precollege students participating in lab field trips on Variability and Inheritance; Corn Genetics and Mendelian Inheritance; Cells, Chromosomes, and Mutations; DNA Structure and Recombination; Bacterial Transformation; DNA Restriction Analysis; and Human DNA Fingerprinting. The Great Moments in DNA Science Lecture Series, held in the spring, continued as a popular element of our annual calendar of events, drawing the attendance of 730 local students and teachers:

Michael Gilman, Cold Spring Harbor Laboratory: To Grow or Not to Grow—How a Cell Decides.
Seth Grant, Columbia University: How Do Genes Control the Way the Brain Stores Memories?
William Lennarz, SUNY at Stony Brook: Sperm Meets Egg.
David Micklos, Cold Spring Harbor Laboratory: Eugenics—From Science to Social Quackery.

In February, we conducted the final in a series of four Human Genetics and Genome Analysis Workshops funded by the Department of Energy. This program draws together opinion leaders and policy makers who must help society make sense and proper use of genetic technology. The program has provided an opportunity to formalize the existing strong ties between the DNALC and our sister organization, Banbury Center. The combination of high-level seminars and collegial exchange in beautiful surroundings at Banbury and hands-on labs at the DNALC has proven a winning formula for our bright, but nonscientist, clientele of government administrators, lawyers, ethicists, reporters, educators, and members of patient support groups. Jan Witkowski’s keen scientific insight and broad contacts in basic/clinical molecular genetics ensure an extremely high caliber of featured speakers from around the country.
Kenneth Culver, National Institutes of Health: The First Human Gene Therapy Trials.

Neil Holtzman, John Hopkins Medical Institute: Map or Maze—The Future of Human Genetics.

Marsha Saxton, Massachusetts Office on Disability: Genetics and Cultural Attitudes to Disability.

Patricia Ward, Baylor College of Medicine: DNA-based Diagnosis for Human Genetic Diseases.


Our summer workshop schedule took on added intensity with the addition of the month-long Leadership Institute for 23 high school faculty, as well as increased offerings for precollege students. Summer has traditionally been our
season for teacher-training workshops, and Mark Bloom continued his hectic schedule of four 2-week Advanced DNA Science Workshops, reaching 79 college faculty at workshops held in New York, Seattle, Los Angeles, and San Juan. Dave Micklos instructed a lightened load of DNA Science Workshops, reaching 67 high school faculty at workshops in New Orleans, Salt Lake City, and the DNALC. Demand for student spaces in the two local DNA Science Workshops has been increasing over the last several years, with 37 high-ability students participating in summer 1993. Seventy-eight minority/disadvantaged students (5–6th grade) were instructed, free of charge, at four Fun With DNA Workshops taught by Robert Willis at the DNALC and A. Phillip Randolph High School in Harlem. To satisfy the high demand for our "kiddie camps," we entered into a new collaboration to administer five “Fun With DNA” camps at Portledge School in Locust Valley. This arrangement allowed us to accommodate an additional 101 5–6th grade students. In addition, 23 7–8th graders attended the pilot of the new World of Enzymes Workshop, designed for past "graduates" of Fun With DNA.

Corporate Advisory Board

Under the strong leadership of Doug Fox, Vice President of Marketing for the Times Mirror newspaper group, the Corporate Advisory Board has risen to the challenge of increasing unrestricted annual support from the Long Island business community. In 1993, the Board raised a total of $66,650. Corporate Advisory Board members help explain the DNALC's role as a center of educational excellence and key element of the community infrastructure to support high-technology industry on Long Island. Board members are drawn from a gamut of Long Island businesses, including communications, banking, accounting, insurance, law, real estate, retail sales, and health care/biotechnology. Guiding the effort, and working closely with DNALC and Laboratory staff, are members of the executive committee:

Douglas B. Fox, Chairman
Rocco S. Barrese, Dilworth and Barrese
Thomas J. Calabrese, NYNEX
Gary E. Frashier, Oncogene Science, Inc.
Patricia Peterson, Dale Gale Agency
Paul A. Vermylen, Jr., Meenan Oil Company

Michael Aboff
Howard M. Blankman
Harry G. Charlston
Richard M. Clark
Kenneth Daley
Robert E. Diller
Mrs. Sinclair Hatch
Arther D. Herman
John J. Lack
Ralf Lange
Mrs. Lilo Leeds
Ms. Shelia Mahony
Glenn Prestwich, Ph.D.
Francis Roberts, Ph.D.
Peter G. Schiff
John Smith
Michael Vittorio
Lawrence Waldman

Staff and Interns

We were disappointed when Robert Willis left his position as Special Programs Manager at the end of the summer to return to a teaching position in Maryland. Robert helped to expand our programs for minority and disadvantaged students in the New York metropolitan area, a task to which we remain committed. The vacuum created by Robert's departure was filled ably and cost-effectively by
three part-time laboratory instructors: Diane Jedlicka of Roslyn Public Schools and Drs. Diane Esposito and Flavio Della Setta, who have research positions at Cold Spring Harbor Laboratory. With degrees in elementary and special education, Diane Jedlicka administers the Roslyn OMNI Gifted Program with DNALC Education Manager Jane Conigliaro. Diane Esposito is a staff associate in the laboratory of Michael Wigler and has had research experience at Columbia University, Memorial Sloan-Kettering Cancer Institute, and North Shore Community Hospital. Diane also brings strong ties to our Italian collaborator Marcello Siniscalco, with whom she worked for several years. A native of Italy, Flavio is a post-doctoral fellow in the laboratory of Kim Arndt. Flavio has research experience in DNA structure and transcription at the University of Rome, University of Paris, and University of Nancy, where he holds a faculty position. We also rely on the collaboration of Twana Adams, an energetic minority educator with strong ties to community action groups in Harlem.

We were further frustrated when Administrative Assistant Sandra Ordway left at the end of the year to accept a position at the law firm Scheine, Fusco, Brandenstein, and Rada. Sandy first came to the DNALC in 1988 to organize a corps of neighborhood volunteers, joining the staff in 1989 to orchestrate our increasingly complex schedule of visiting schools and off-site workshops. We moved quickly to hire new Administrative Assistant Judy Cumella, who was a purchasing agent at Medical Sterilization, Inc. Additional administrative help was provided by part-time employee Margot Kohler and volunteer Katya Davey.

We continued to get excellent laboratory, computational, and administrative support from our growing staff of student interns drawn from neighboring school districts. Mark Staudinger, of Cold Spring Harbor High School, assisted Mark Bloom with Advanced DNA Science Workshops in Los Angeles, Seattle, and San Juan prior to beginning his freshman year at Muhlenberg College. Cold Spring Harbor senior David Hollman focused primarily on the development of multimedia computer programs and assisted Leadership Institute participants with computer projects, leaving at the end of summer for his freshman year in the College of Engineering at Cornell University. Ken Bassett, of Massapequa High School, assisted Dave Micklos with the DNA Science Workshop in Salt Lake City and assumed the position of senior intern at the beginning of the 1993–1994 school year. Assisting at Fun With DNA summer camps were lab aides Tara Marathe of Colby College, Daryn Berger of Walt Whitman High School, Andrea Conigliaro of St. Anthony’s High School, and Michael Conigliaro of Cold Spring Harbor High School. New interns were Jessica Hinton of Huntington High School, Chris Como of Cold Spring Harbor High School, and Andy Diller of Sachem High School. Andy brought with him considerable lab experience and represents the fruition of our efforts to stimulate advanced biology instruction on Long Island. He had the opportunity to take two molecular genetics electives at Sachem, where instructor Fred Gillam runs the nation’s largest precollege program in laboratory molecular genetics. Andy’s father Bob is a member of the DNALC’s Corporate Advisory Board and as Vice President of Marketing for Brinkmann Instruments has been instrumental in obtaining many gifts of Eppendorf pipets and centrifuges.

**PUBLICATIONS**


1993 Workshops, Meetings, and Collaborations

January 9–10 National Science Foundation Follow-up Workshop, DNA Science, University of Nevada, Reno, Nevada
January 16–17 National Science Foundation Follow-up Workshop, Advanced DNA Science, University of Puerto Rico, Mayaguez, Puerto Rico
January 23–24 National Science Foundation Follow-up Workshop, DNA Science, University of Puerto Rico, Mayaguez, Puerto Rico
January 26 Site visit to Biotechnology Teaching Laboratory, State University of New York, Stony Brook
February 4–6 Department of Energy Workshop, Human Genetics and Genome Analysis, DNALC and Banbury Center
February 8–11 Department of Energy Contractors Workshop, Santa Fe, New Mexico
February 13–14 Department of Education Follow-up Workshop, Advanced DNA Science, University of Chicago, Chicago, Illinois
February 15–17 Coalition for Education in the Life Sciences Meeting, Marine Biological Laboratory, Woods Hole, Massachusetts
February 19 Biology Teachers Inservice Training Committee Meeting, National Research Council, Washington D.C.
February 23 Site visit by Dennert O. Ware, Boehringer Mannheim Corporation
February 27–28 National Science Foundation Follow-up Workshop, Advanced DNA Science, DNALC
March 4 Corporate Advisory Board Meeting and Reception, DNALC
March 8–10 Site visit by Ian Muchamore, Wellcome Centre for Medical Science, London, England
March 13–14 Howard Hughes Medical Institute Follow-up Workshop, DNA Science, Edison Career Center, Montgomery County, Maryland
March 30 Site visit by Dennis Bittisnich, Australian National University, Canberra, Australia
March 31–April 1 Scholar in Residence Program, SUNY, Purchase, New York
April 5 Site visit by Tom Zinnen and Michael Patrick, University of Wisconsin, Madison, Wisconsin
April 27 Great Moments in DNA Science Honors Student Seminar, DNALC
May 4 Great Moments in DNA Science Honors Student Seminar, DNALC
May 7 Corporate Advisory Board Meeting, DNALC
May 10–12 Collaboration with Ray Gladden, Carolina Biological Supply Company, Burlington, North Carolina
May 11 Great Moments in DNA Science Honors Student Seminar, DNALC
May 13–15 Collaboration with Helen Donis-Keller, Washington University, St. Louis, Missouri
May 18 Great Moments in DNA Science Honors Student Seminar, DNALC
May 20 Corporate Advisory Board Meeting and Reception, DNALC
May 24–June 4 Department of Education, Advanced DNA Science Workshop, Columbia University, New York, New York
June 1 Corporate Advisory Board Meeting and Reception, DNALC
June 7–11 National Science Foundation Workshop, DNA Science, John McDonogh High School, New Orleans, Louisiana
June 14–25 Department of Education Workshop, Advanced DNA Science, University of California, Northridge, California
June 28–July 2 National Science Foundation, DNA Science Workshop, University of Utah, Salt Lake City, Utah
June 28–July 2 Fun With DNA Workshop, Portledge School, Locust Valley, New York
June 28–July 2 DNA Science Workshop, DNALC
June 29–July 1 National Science Foundation Workshop, Exploring Human Genetics, Titusville Middle School, Poughkeepsie, New York

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<table>
<thead>
<tr>
<th>Date Range</th>
<th>Event Description</th>
</tr>
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<tr>
<td>July 6–30</td>
<td>National Science Foundation <em>Leadership Institute in Human and Molecular Genetics</em>, DNALC</td>
</tr>
<tr>
<td>July 26–30</td>
<td><em>Fun With DNA</em> Workshop, Portledge School, Locust Valley, New York</td>
</tr>
<tr>
<td>July 26–August 6</td>
<td>National Science Foundation Workshop, <em>Advanced DNA Science</em>, University of Puerto Rico, Rio Piedras, Puerto Rico</td>
</tr>
<tr>
<td>August 2–6</td>
<td><em>Fun With DNA</em> Workshop, Portledge School, Locust Valley, New York</td>
</tr>
<tr>
<td>August 16–20</td>
<td><em>Advanced Placement Workshop</em>, Stanford University, Palo Alto, California; Barker Welfare Foundation Minority Workshop, <em>Fun With DNA</em>, DNALC</td>
</tr>
<tr>
<td>August 16–27</td>
<td>National Science Foundation Workshop, <em>Advanced DNA Science</em>, University of Washington, Seattle, Washington</td>
</tr>
<tr>
<td>August 23–27</td>
<td>High School Student/Faculty Workshop, DNALC</td>
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<tr>
<td>August 30–September 2</td>
<td><em>Microbes, Meteorites, and the Mind</em> Workshop, DNALC</td>
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<tr>
<td>September 8</td>
<td>Premiere, <em>Long Island Discovery</em>, DNALC</td>
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<tr>
<td>September 15</td>
<td>Site visit by Kyle Vermillion, Westminster School, Atlanta, Georgia</td>
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<tr>
<td>September 30</td>
<td>Corporate Advisory Board Meeting/Museum preview and Open House, DNALC</td>
</tr>
<tr>
<td>October 8–9</td>
<td>National Science Foundation Follow-up Workshop, <em>DNA Science</em>, John McDonough High School, New Orleans, Louisiana</td>
</tr>
<tr>
<td>October 15</td>
<td>Site visit by Greg Baird and Laura Leber, Genentech, South San Francisco, California</td>
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<tr>
<td>October 24–27</td>
<td>International Conference on Public Understanding of Science and Technology, Chicago Academy of Sciences, Chicago, Illinois</td>
</tr>
<tr>
<td>October 29</td>
<td>Baring Brothers Workshop, Cold Spring Harbor, New York</td>
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<tr>
<td>November 11</td>
<td>Eugenics lecture, Banbury Center, Cold Spring Harbor, New York</td>
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<tr>
<td>November 18–21</td>
<td>National Association of Biology Teachers National Meeting, Boston, Massachusetts</td>
</tr>
<tr>
<td>November 29–December 3</td>
<td>Human Genome Diversity Training Workshop, Porto Conte Research and Training Laboratories, Sardinia, Italy</td>
</tr>
<tr>
<td>December 15–17</td>
<td>National Science Teachers Association Annual Meeting, Orlando, Florida</td>
</tr>
</tbody>
</table>
1993 PUBLICATIONS

General Books

Experiments with Fission Yeast: A Laboratory Course Manual
C. Alfa, P. Fantes, J. Hyams, M. McLeod, and E. Warbrick

Genetic Maps, 6th Edition
S.J. O’Brien (ed.)

Molecular Probes of the Nervous System: Selected Methods for Antibody and Nucleic Acid Probes, Vol. 1
S. Hockfield, S. Carlson, C. Evans, P. Levitt, J. Pintar, and L. Silberstein

The Cell Surface
Symposia on Quantitative Biology 57

The Development of Drosophila melanogaster
M. Bate and A. Martinez Arias (eds.)

Double Talking Helix Blues
J. Herskowitz and I. Herskowitz (illustrated by J. Cuddihy)

The Early Days of Yeast Genetics
M.N. Hall and P. Linder (eds.)

Vaccines 93: Modern Approaches to New Vaccines Including Prevention of AIDS
H.S. Ginsberg, F. Brown, R.M. Chanock, and R.A. Lerner (eds.)

CSHL Monograph Series

Molecular and Cellular Biology of the Yeast Saccharomyces, Vol. 2: Gene Expression
E.W. Jones, J.R. Pringle, and J.R. Broach (eds.)

Transcriptional Regulation, Book 2
S.L. McKnight and K.R. Yamamoto (eds.)

Reverse Transcriptase
A.M. Skalka and S.P. Goff (eds.)

The RNA World
R.F. Gesteland and J.F. Atkins (eds.)

Nucleases, 2nd Edition
S.M. Linn, R.S. Lloyd, and R.J. Roberts (eds.)

Current Communications in Cell & Molecular Biology Series

Superantigens: A Pathogen’s View of the Immune System
B. Huber and E. Palmer (eds.)

Video Tapes

Targeted Mutagenesis in Mice: A Video Guide
R.A. Pedersen, V. Papaioannou, A. Joyner, and J. Rossant

Winding Your Way Through DNA
University of California, San Francisco

Cancer Surveys Series

Vol. 16: The Molecular Pathology of Cancer
N. Lemoine and N.A. Wright (eds.)

Vol. 17: Pharmacokinetics and Cancer Chemotherapy
P. Workman and M.A. Graham (eds.)

Vol. 18: Breast Cancer
I.S. Fentiman and J. Taylor-Papadimitriou (eds.)

Genome Analysis Series

Vol. 5: Regional Physical Mapping
K.E. Davies and S.M. Tilghman (eds.)

Vol. 6: Genome Maps and Neurological Disorders
K.E. Davies and S.M. Tilghman (eds.)

Vol. 7: Genome Rearrangement and Stability
K.E. Davies and S.T. Warren (eds.)

Journals

Genes & Development (Volume 7, 14 issues)
T. Grodzicker and N. Hastie (eds.)

PCR Methods and Applications (Volume 2, 2 issues; Volume 3, 3 issues)
D. Bentley, R. Gibbs, E. Green, and R. Myers (eds.)

Other

A Limner’s View
F. McCurdy and E. Watson

CSHL Annual Report 1992
Banbury Center Annual Report 1992
Administration and Financial Annual Report 1992
Abstract/program books for 14 CSHL meetings
The Press continued its ambitious publishing program in 1993 by adding 20 new books and 2 new videotapes, bringing its total titles in print to more than 120, by deciding to increase the frequency of both its current journals, and by announcing plans for the launch of a third journal in 1994. Sales increased by 16%, to over $4.3 million, and the balance after expenses, including significant one-time charges, rose by 25%.

**Book Publishing**

New titles were added in nearly all categories of the book catalog. Sales of laboratory manuals continued to flourish, particularly the perennial bestsellers *Molecular Cloning* by Joe Sambrook, Ed Fritsch, and Tom Maniatis; Ed Harlow and David Lane’s *Antibodies*; and the recent success by Jeffrey Miller, *A Short Course in Bacterial Genetics*. There were new manuals on molecular probes of the nervous system and fission yeast biology. Like many of our most successful books of this kind, both were based on popular courses recently taught at the Laboratory. The techniques they describe thus have the robust reliability that only comes from repeated use by people with varying degrees of inexperience.

The Symposium volume, *The Cell Surface*, published in the 60th anniversary year of the Symposium’s founding, was a colorful addition to the famous book series and marked the start of Bruce Stillman’s stewardship of this prestigious annual meeting. The second volume of the authoritative monograph *Transcriptional Regulation*, edited by Steve McKnight and Keith Yamamoto, was published, and the entire book was almost immediately reprinted in paperback in response to the strong demand for an edition affordable by graduate students. A paperback edition of the widely appreciated memoir of Barbara McClintock, *The Dynamic Genome*, edited by Nina Fedoroff and David Botstein, was published with the elegant cover that won a national design award.

Not every new title can be mentioned here (see opposite page for a complete list), but two new books made a particularly dramatic impact both visually and scientifically. *The RNA World*, edited by Ray Gesteland and John Atkins, revisited a prebiotic era in which evolution may have depended on replicating RNA and reviewed current ideas about the structure and function of this ancient molecule. The introductory articles by James Watson and Francis Crick reminded us how quickly their attention focused on the other nucleic acid after they deduced the structure of DNA exactly 40 years ago. A notable feature of this book was the frontispiece—a hologram derived from the computerized construction of an RNA-protein complex. The book was widely and enthusiastically reviewed, and its originality was rewarded with strong sales.

Equally arresting was the lavish artwork in *The Development of Drosophila melanogaster*, a monograph on the molecular analysis of fruit fly embryology edited by Michael Bate and Alfonso Martinez Arias. This two-volume set includes 114 color plates plus a separate 50-page color atlas of the developmental stages of the insect’s growth created entirely with stunning computer graphics by Volker Hartenstein. In printing terms, this was the most complex scientific monograph yet published at Cold Spring Harbor, and necessarily the most highly priced. Yet it has been warmly welcomed as an essential resource by the world’s expanding population of fly labs.
Publishing is a vital element in the Laboratory’s program of professional educational activities, and our books have helped make Cold Spring Harbor’s name an honored one among scientists worldwide. In 1993, however, we embarked on two projects with appeal beyond our customary audience. A series of six videotapes, entitled *Winding Your Way Through DNA*, was produced in collaboration with the University of California at San Francisco, from a meeting at which a group of distinguished scientists, led by Harold Varmus, spoke to the public about the perils and possibilities of genetic technology. The tape set has been recognized as a unique teaching tool and a stimulus to classroom discussion of these important issues among college students.

Yet an understanding of how genes and cells work should begin earlier in a child’s life, at middle or elementary school level. The children’s books from the United Kingdom on these topics, by Fran Balkwill and Mic Rolph, which we have distributed in the United States for several years, have proved lastingly popular. Encouraged by their success, we published this year our own contribution to this genre—a remarkable audio tape and book combination entitled *Double Talking Helix Blues*. As written and performed by Joel and Ira Herskowitz and beautifully illustrated by Judy Cuddihy, the basis of heredity is spelled out in song and words, in a style that appeals to 8–12 year olds.

Both of these projects are experiments, undertaken in a controlled way, partly in fulfillment of our educational mission, but with the quite serious intention of examining the market potential for future publications of this kind. They also illustrate what no observer of our book publishing operation can fail to miss—the remarkable growth in the number of new titles produced annually and the increased complexity of those titles in production terms. Nancy Ford and her editorial and production staff in the book division have, as always, met the many challenges these changes have presented while maintaining the reputation for high quality our books earned long ago.

**Journal Publishing**

It was a year of expansion for our journals. *Genes & Development*, now in its seventh year, consistently published papers of high quality. The number of papers submitted to the journal increased and the scope of those published continued to broaden. *G&D* was again the most cited journal in genetics and in developmental biology and, as in previous years, showed a steady increase in circulation. Because of the rising demand to publish in the journal and to avoid creating issues that were discouragingly thick, the decision was taken to produce issues semimonthly in 1994.

The success of *PCR Methods and Applications*, launched in 1991, prompted an acceleration from quarterly to bimonthly publication with the start of Volume 3 in August. A series of articles was commissioned as a primer on getting started in this powerful but still tricky technology, and these articles will later be reassembled in the form of a laboratory manual. The journal’s circulation continued to rise and its vigorous financial health was again greatly aided by the impressive advertising revenues mustered by Nancy Kuhle.

As always, the success of our journals owes a great deal to the editors, advisory board members, and expert reviewers whose willingness to make critical, often difficult judgments on the papers offered is the basis for the quality of the journals’ content.
New journals continue to proliferate in the biomedical sciences despite shrinking library budgets, the escalating costs of serial publishing, and a weary sense among many that there are more than enough journals already. Yet science continues to evolve and bud off new fields of enquiry, some of which become vigorous and visible enough to support a specialist journal.

The growing tide of interest in the biological analysis of learning prompted us to consult a number of experts on whether their field had yet reached this stage of development. We were delighted to find that the consensus view was encouraging. The decision was therefore taken to launch a new journal, Learning & Memory, in 1994. A distinguished group of editors and advisors was readily assembled, attracted by the idea of a journal devoted exclusively to papers on this broad and fascinating topic.

Throughout the year, Judy Cuddihy and her colleagues in the journal division have succeeded in meeting the demands of producing flawless issues on an accelerated schedule.

Marketing, Fulfillment, and Finance

The expansion of our publishing program into new fields was complemented by vigorous marketing activities efficiently managed by Ingrid Benirschke. These activities centered on a series of attractive, subject-specific catalogs designed by Jim Suddaby that were mailed to targeted recipients worldwide. New titles were announced in three editions of the Notebook, and a New Year’s sale of older titles at reduced prices was highly successful. As always, the Press participated in the trade shows associated with the national meetings of societies for genetics, cell biology, neuroscience, and microbiology. In addition, we exhibited at international conferences in virology and genetics that took place back-to-back in the United Kingdom in August.

Closer to home, our bookstore in Grace Auditorium managed by Connie Hallaran and Nancy Hodson extended our marketing activities to the several thousand visitors who attended the Laboratory’s meetings and courses. Despite its narrow confines, the bookstore attracted more business than ever in 1993, with its eclectic mix of science books from Cold Spring Harbor and elsewhere, gifts, and travel necessities.

Vital support for book and journal sales in the form of customer service, order processing, and distribution was ably provided by Guy Keyes and his staff in our Plainview facility, from which more than 50,000 books were dispatched during the course of the year. Their computer system was ever more finely tuned to our needs, and by applying her ingenuity on a personal computer to the sales data, Nancy Hodson initiated many kinds of financial analysis that helped us make better and more timely business decisions.

New Projects

The development of projects for publication in 1995 and beyond continued unabated. More than 20 new publications of various kinds were initiated and will be described in future reports. We also began a new serial publication for purely internal use. Pressing News is a bimonthly roundup of news from each Press department and has significantly improved communication among the departments in our four separate locations.
It was a year in which communications technology moved from obscurity to the front pages, and terms such as the information superhighway, CD-ROMs, and multimedia became commonplace. Wary of the hype but intrigued by the potential of these technologies, we have begun to explore their utility in our marketplace. Our aim will be to adopt whatever of the new technologies are best-suited to the goal of keeping our Press at the forefront of scientific communication worldwide.

John R. Inglis
FINANCIAL STATEMENTS
## FINANCIAL STATEMENTS

### BALANCE SHEET
Year ended December 31, 1993
with comparative amounts for 1992

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<thead>
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<th>ASSETS</th>
<th>Unrestricted</th>
<th>Operating Funds</th>
<th>Endowment &amp; Similar Funds</th>
<th>Land, Building, &amp; Equipment Funds</th>
<th>Total All Funds</th>
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<td>Other</td>
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<td>Accrued interest receivable</td>
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<td>Publications inventory</td>
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<td>Other assets, principally deferred expenses</td>
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<td>Investment in employee residences</td>
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<td>Land, buildings, and equipment:</td>
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<td>Land and improvements</td>
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<td>Buildings</td>
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<td>Furniture, fixtures, and equipment</td>
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<td>Laboratory equipment</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>365,630</td>
</tr>
<tr>
<td>Library books and periodicals</td>
<td>-</td>
<td>-</td>
<td>365,630</td>
<td>-</td>
<td>74,613,928</td>
</tr>
<tr>
<td>Less accumulated depreciation and amortization</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>19,182,465</td>
</tr>
<tr>
<td>Land, buildings, and equipment, net</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>55,431,463</td>
</tr>
<tr>
<td>Construction in progress</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1,326,536</td>
</tr>
<tr>
<td>Total assets</td>
<td>$6,163,044</td>
<td>1,350,000</td>
<td>3,899,551</td>
<td>33,770,391</td>
<td>65,585,474</td>
</tr>
</tbody>
</table>
## Liabilities and Fund Balances

### Operating Funds

<table>
<thead>
<tr>
<th>Liabilities:</th>
<th>Unrestricted</th>
<th>Endowment &amp; Similar Funds</th>
<th>Land, Building, &amp; Equipment Funds</th>
<th>Total All Funds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accounts payable and accrued expenses</td>
<td>$915,125</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Notes payable</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bonds payable</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Deferred revenue</td>
<td>1,773,543</td>
<td>-</td>
<td>3,899,551</td>
<td>-</td>
</tr>
<tr>
<td><strong>Total liabilities</strong></td>
<td>2,688,668</td>
<td>-</td>
<td>3,899,551</td>
<td>31,362,113</td>
</tr>
</tbody>
</table>

### Fund balances:

| Unrestricted-undesignated              | 3,474,376    | -                          | -                                | -               |
| Unrestricted-designated                | -            | 1,350,000                  | -                                | -               |
| Endowment and similar funds           | -            | -                          | 33,770,391                       | -               |
| Land, buildings, and equipment:       | -            | -                          | -                                | -               |
| Expended                               | -            | -                          | -                                | 31,970,878      |
| Unexpended-Donor restricted            | -            | -                          | -                                | 242,786         |
| Unexpended-Board authorized            | -            | -                          | -                                | 2,009,697       |
| **Total fund balances**                | 3,474,376    | 1,350,000                  | -                                | 34,223,361      |

**Total liabilities and fund balances:**

|                             | $6,163,044   | 1,350,000                  | 3,899,551                        | 65,585,474      |
STATEMENT OF SUPPORT, REVENUE AND EXPENSES, AND CHANGES IN FUND BALANCES
Year ended December 31, 1993
with comparative amounts for 1992

<table>
<thead>
<tr>
<th>Operating Funds</th>
<th>Unrestricted</th>
<th>Endowment &amp; Similar Funds</th>
<th>Land, Building, &amp; Equipment Funds</th>
<th>Total All Funds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Support and revenue:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Public support</td>
<td>$1,804,344</td>
<td>6,327,427</td>
<td>1,898,789</td>
<td>347,400</td>
</tr>
<tr>
<td>Government grant awards</td>
<td>-</td>
<td>11,550,559</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Indirect cost allowances</td>
<td>8,510,065</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Other revenue:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Program fees</td>
<td>1,535,762</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rental income</td>
<td>289,891</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Publications</td>
<td>4,319,267</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dining services</td>
<td>1,661,674</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rooms and apartments</td>
<td>1,451,719</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Distribution from Robertson Funds</td>
<td>490,000</td>
<td>1,085,000</td>
<td>-</td>
<td>2,543,577</td>
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<tr>
<td>Investment income</td>
<td>204,790</td>
<td>-</td>
<td>-</td>
<td>2,543,577</td>
</tr>
<tr>
<td>Royalty &amp; licensing</td>
<td>283,870</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>146,282</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Total other revenue</td>
<td>10,314,409</td>
<td>17,877,986</td>
<td>1,898,789</td>
<td>347,400</td>
</tr>
<tr>
<td>Total support and revenue</td>
<td>20,697,664</td>
<td>18,962,986</td>
<td>4,442,366</td>
<td>648,571</td>
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<tr>
<td>Expenses:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Program services:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Research</td>
<td>-</td>
<td>14,630,971</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Summer programs</td>
<td>1,155,025</td>
<td>3,505,060</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Publications</td>
<td>4,314,244</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Banbury Center conferences</td>
<td>239,149</td>
<td>340,309</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DNA Education Center programs</td>
<td>28,491</td>
<td>420,937</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total program services</td>
<td>5,556,909</td>
<td>18,962,977</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Supporting services:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Direct research support</td>
<td>1,196,259</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Library</td>
<td>505,105</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Operation and maintenance of plant</td>
<td>5,008,217</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>General and administrative</td>
<td>3,387,597</td>
<td>-</td>
<td>134,539</td>
<td>-</td>
</tr>
<tr>
<td>Dining services</td>
<td>1,659,112</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Interest</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>926,162</td>
</tr>
<tr>
<td>Total supporting services</td>
<td>11,756,290</td>
<td>-</td>
<td>134,539</td>
<td>926,162</td>
</tr>
</tbody>
</table>
Depreciation and amortization

<table>
<thead>
<tr>
<th></th>
<th>1,350,000</th>
<th>33,580,731</th>
<th>67,859,360</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total expenses</td>
<td>17,313,199</td>
<td>18,897,277</td>
<td>3,447,804</td>
</tr>
<tr>
<td>Excess (deficiency) of support and revenue over (under) expenses</td>
<td>-</td>
<td>-</td>
<td>(2,799,233)</td>
</tr>
</tbody>
</table>

$3,384,465

134,539

3,447,804

39,792,819

10,400,050

Other changes in fund balances:

<table>
<thead>
<tr>
<th></th>
<th>1,350,000</th>
<th>33,580,731</th>
<th>67,859,360</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capital expenditures</td>
<td>(3,165,731)</td>
<td>(712,075)</td>
<td>3,877,806</td>
</tr>
<tr>
<td>Transfer to restricted funds</td>
<td>(425,000)</td>
<td>837,873</td>
<td>(412,873)</td>
</tr>
<tr>
<td>Transfer to endowment funds</td>
<td>(586,233)</td>
<td>(191,507)</td>
<td>1,213,683</td>
</tr>
<tr>
<td>Transfer to unrestricted funds</td>
<td>614,603</td>
<td>-</td>
<td>(614,603)</td>
</tr>
<tr>
<td>Net increase (decrease) in fund balances</td>
<td>(177,896)</td>
<td>-</td>
<td>4,494,034</td>
</tr>
</tbody>
</table>

| Fund balances at beginning of year | 3,652,272 | 1,350,000 | 29,276,357 |
| Fund balances at end of year      | $3,474,376 | 1,350,000 | 33,770,391 |

Net increase (decrease) in fund balances: 4,494,034

Fund balances at beginning of year: 3,652,272

Fund balances at end of year: $3,474,376

Endowments and Similar Funds 9.2%

Foundation and Private Contributions and Grants 18.9%

Interest and Miscellaneous 2.1%

Corporate Contributions and Grants 5.0%

Auxiliary Activities 20.7%

Federal Grants 44.1%
## COMPARATIVE OPERATING HISTORY
### 1989-1993
(Dollars in Thousands)

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Income:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Main Lab:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grants &amp; contracts</td>
<td>13,062</td>
<td>13,535</td>
<td>15,172</td>
<td>16,800</td>
<td>18,136</td>
</tr>
<tr>
<td>Indirect cost reimbursement</td>
<td>6,412</td>
<td>6,558</td>
<td>7,170</td>
<td>8,388</td>
<td>8,383</td>
</tr>
<tr>
<td>Other</td>
<td>4,034</td>
<td>3,976</td>
<td>5,056</td>
<td>5,520</td>
<td>6,049</td>
</tr>
<tr>
<td>CSH Press</td>
<td>4,450</td>
<td>4,223</td>
<td>3,079</td>
<td>3,709</td>
<td>4,319</td>
</tr>
<tr>
<td>Banbury Center</td>
<td>1,012</td>
<td>1,120</td>
<td>1,090</td>
<td>1,104</td>
<td>1,281</td>
</tr>
<tr>
<td>DNA Learning Center</td>
<td>662</td>
<td>585</td>
<td>744</td>
<td>822</td>
<td>796</td>
</tr>
<tr>
<td><strong>Total income</strong></td>
<td>29,592</td>
<td>29,997</td>
<td>32,311</td>
<td>36,343</td>
<td>38,964</td>
</tr>
<tr>
<td><strong>Expenses:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Main Lab:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grants &amp; contracts</td>
<td>13,062</td>
<td>13,535</td>
<td>15,172</td>
<td>16,800</td>
<td>18,136</td>
</tr>
<tr>
<td>Operation &amp; maintenance of plant</td>
<td>3,412</td>
<td>3,759</td>
<td>3,904</td>
<td>4,241</td>
<td>4,777</td>
</tr>
<tr>
<td>General &amp; administrative</td>
<td>2,377</td>
<td>2,414</td>
<td>2,468</td>
<td>2,634</td>
<td>2,785</td>
</tr>
<tr>
<td>Other</td>
<td>3,165</td>
<td>2,973</td>
<td>3,375</td>
<td>4,141</td>
<td>4,385</td>
</tr>
<tr>
<td>CSH Press</td>
<td>3,934</td>
<td>3,708</td>
<td>3,488</td>
<td>3,548</td>
<td>4,134</td>
</tr>
<tr>
<td>Banbury Center</td>
<td>1,038</td>
<td>1,125</td>
<td>1,063</td>
<td>1,070</td>
<td>1,226</td>
</tr>
<tr>
<td>DNA Learning Center</td>
<td>635</td>
<td>615</td>
<td>752</td>
<td>843</td>
<td>768</td>
</tr>
<tr>
<td><strong>Total expenses</strong></td>
<td>27,623</td>
<td>28,129</td>
<td>30,222</td>
<td>33,277</td>
<td>36,211</td>
</tr>
<tr>
<td>Excess before depreciation and designation of funds</td>
<td>1,969</td>
<td>1,868</td>
<td>2,089</td>
<td>3,066</td>
<td>2,753</td>
</tr>
<tr>
<td><strong>Depreciation</strong></td>
<td>(1,399)</td>
<td>(1,485)</td>
<td>(1,898)</td>
<td>(2,358)</td>
<td>(2,522)</td>
</tr>
<tr>
<td><strong>Designation of funds (1)</strong></td>
<td>(400)</td>
<td>(250)</td>
<td>(100)</td>
<td>(600)</td>
<td>0</td>
</tr>
<tr>
<td><strong>Net operating excess</strong></td>
<td>$170</td>
<td>133</td>
<td>91</td>
<td>108</td>
<td>231</td>
</tr>
</tbody>
</table>

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
Cold Spring Harbor Laboratory is a nonprofit research and educational institution chartered by the University of the State of New York. Less than half (44.1%) of our annual support is derived from Federal grants and contracts, and thus we rely heavily on support from the private sector: foundations, corporations, and individuals. 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."

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 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, One Bungtown Road, Post Office 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, One Bungtown Road, Post Office Box 100, Cold Spring Harbor, NY 11724, or call 516-367-8840.
**FINANCIAL SUPPORT OF THE LABORATORY**

Cold Spring Harbor Laboratory, Banbury Center, and the DNA Learning Center receive a substantial portion of their funding through grants from the Federal Government and through grants, capital gifts, and annual contributions from private foundations, corporations, and individuals. The following section summarizes funding that occurred during 1993.

## GRANTS January 1, 1993–December 31, 1993

### COLD SPRING HARBOR LABORATORY

<table>
<thead>
<tr>
<th>Grantor</th>
<th>Program/Principal Investigator</th>
<th>Duration of Grant</th>
<th>1993 Funding*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FEDERAL GRANTS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>NATIONAL INSTITUTES OF HEALTH</strong></td>
<td>Cancer Research Center, Dr. Mathews</td>
<td>1/92–12/96</td>
<td>$3,415,962</td>
</tr>
<tr>
<td></td>
<td>Cancer Center Support, Dr. Stillman</td>
<td>8/90–7/95</td>
<td>4,478,704</td>
</tr>
<tr>
<td></td>
<td>Oncogene Program Project, Dr. Wigler</td>
<td>3/88–2/93</td>
<td>187,088</td>
</tr>
<tr>
<td><strong>Research Support</strong></td>
<td>Dr. Anderson</td>
<td>4/90–3/93</td>
<td>53,188</td>
</tr>
<tr>
<td></td>
<td>Dr. Arndt</td>
<td>4/88–3/93</td>
<td>36,090</td>
</tr>
<tr>
<td></td>
<td>Dr. Arndt</td>
<td>1/91–12/94</td>
<td>371,931</td>
</tr>
<tr>
<td></td>
<td>Dr. Bar-Sagi</td>
<td>7/91–6/96</td>
<td>445,726</td>
</tr>
<tr>
<td></td>
<td>Dr. Beach</td>
<td>12/84–1/93</td>
<td>90,411</td>
</tr>
<tr>
<td></td>
<td>Dr. Beach</td>
<td>9/66–8/94</td>
<td>298,491</td>
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<tr>
<td></td>
<td>Dr. Beach</td>
<td>7/66–6/93</td>
<td>57,153</td>
</tr>
<tr>
<td></td>
<td>Dr. Beach</td>
<td>8/90–7/93</td>
<td>210,663</td>
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<tr>
<td></td>
<td>Dr. Beach</td>
<td>4/93–3/97</td>
<td>195,847*</td>
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<tr>
<td></td>
<td>Dr. Cheng</td>
<td>9/91–8/93</td>
<td>134,572</td>
</tr>
<tr>
<td></td>
<td>Dr. Davis</td>
<td>7/92–11/93</td>
<td>267,774</td>
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<tr>
<td></td>
<td>Dr. Davis</td>
<td>9/85–11/94</td>
<td>289,165</td>
</tr>
<tr>
<td></td>
<td>Dr. Franza</td>
<td>10/91–8/94</td>
<td>553,112</td>
</tr>
<tr>
<td></td>
<td>Dr. Franza</td>
<td>1/91–12/94</td>
<td>141,245</td>
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<tr>
<td></td>
<td>Dr. Futcher</td>
<td>4/93–3/97</td>
<td>226,514*</td>
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<tr>
<td></td>
<td>Dr. Futcher</td>
<td>1/85–12/94</td>
<td>904,703</td>
</tr>
<tr>
<td></td>
<td>Dr. Garrels</td>
<td>9/92–8/97</td>
<td>501,836</td>
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<tr>
<td></td>
<td>Dr. Gilman</td>
<td>12/89–11/94</td>
<td>412,183</td>
</tr>
<tr>
<td></td>
<td>Dr. Greider</td>
<td>8/91–7/96</td>
<td>410,446</td>
</tr>
<tr>
<td></td>
<td>Dr. Greider</td>
<td>9/85–3/94</td>
<td>534,055</td>
</tr>
<tr>
<td></td>
<td>Dr. Helfman</td>
<td>8/93–5/98</td>
<td>181,580*</td>
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<tr>
<td></td>
<td>Dr. Helfman</td>
<td>7/92–6/96</td>
<td>487,637</td>
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<td>Dr. Hernandez</td>
<td>9/91–8/96</td>
<td>426,221</td>
</tr>
<tr>
<td></td>
<td>Dr. Hernandez</td>
<td>3/92–2/96</td>
<td>254,299</td>
</tr>
<tr>
<td></td>
<td>Dr. Herr</td>
<td>7/89–6/94</td>
<td>400,528</td>
</tr>
<tr>
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**DEPARTMENT OF ENERGY**

| Research Support              | Dr. Marr                                                                  | 7/91–2/94         | 571,805       |
|                               | Dr. Martienssen                                                           | 8/91–8/93         | 63,698        |
|                               | Dr. Peterson                                                             | 5/92–5/93         | 25,941        |
| Meeting Support               | 58th Symposium: DNA and Chromosomes                                       | 1993              | 20,000*       |

**U.S. DEPARTMENT OF AGRICULTURE**

| Research Support              | Dr. Ma                                                                    | 9/92–9/94         | 124,650       |
|                               | Dr. Sundaresan                                                           | 8/91–8/94         | 101,773       |
| Workshop Support              | CSHL Workshop on Molecular Markers for Plant Breeding, Dr. Sundaresan     | 10/93–9/94        | 6,000*        |

**NONFEDERAL GRANTS**

| Research Support              | Muscular Dystrophy Research Dr. Greider                                   | 11/93–10/94       | 3,000*        |
|                               | Dr. Nawa                                                                 | 11/92–10/93       | 200,000       |
|                               | Dr. Nawa                                                                 | 12/93–11/94       | 30,000*       |
|                               | Dr. Gilman                                                               | 7/91–5/94         | 149,314       |
|                               | Dr. Marshak                                                              | 7/93–6/95         | 103,000*      |
|                               | Dr. Mathews (Scholar Award)                                              | 1/93–12/93        | 38,575*       |
|                               | Dr. Mathews (Scholar Award)                                              | 1/92–12/94        | 110,013       |
|                               | Dr. Sutton                                                               | 1986–2012         | 51,755        |
|                               | Dr. Wigler, Professorship                                                | 4/92–12/93        | 9,379         |
|                               | Dr. Wigler                                                               | 7/91–6/96         | 56,438        |
|                               | Dr. Helfman                                                              |                  |              |

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+ Includes direct and indirect cost
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* New Grants Awarded in 1993
+ Includes direct and indirect cost
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**Course Support**


**Meeting Support**

- Abbott Laboratories: Molecular and Cell Biology, Plasminogen Activation (1993) 2,500*
- American Diagnostica: Molecular and Cell Biology, Plasminogen Activation (1993) 5,000*
- American Cyanamid Co.: Vaccines (1993) 10,000*
- Amgen, Inc.: Liver Gene (1993) 2,000*
- Anheuser-Busch Co.: Yeast Cell Biology (1993) 500*
- Becton Dickinson Labware: Liver Gene (1993) 100*
- Boehringer Mannheim, Company, Germany: Molecular and Cell Biology, Plasminogen Activation (1993) 1,000*
- Behringwerke/Hoechst: Molecular and Cell Biology, Plasminogen Activation (1993) 1,000*
- Boehringer Mannheim, Company, Italy: Molecular and Cell Biology, Plasminogen Activation (1993) 1,000*
- Chiron Corporation: Liver Gene (1993) 500*
- Collen Foundation: Molecular and Cell Biology, Plasminogen Activation (1993) 5,000*
- Genetics Institute: Molecular and Cell Biology, Plasminogen Activation (1993) 1,000*
- Labatt Brewing Co., Miles, Inc.: Yeast Cell Biology (1993) 100*
- Pharmacia Biotech, Inc.: Liver Gene (1993) 100*
- Pharmacia Laboratory: Vaccines (1993) 11,988*
- Pierce Chemical Co.: Cytoskeleton and Cell Function (1993) 1,000*
- The Upjohn Company: Liver Gene (1993) 500*
- U.S. Army Research: Cytoskeleton and Cell Function (1993) 3,000*
- Wellcome Trust: Gene Therapy (1993) 7,200*

* New Grants Awarded in 1993
+ Includes direct and indirect cost
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* New Grants Awarded in 1993
+ Includes direct and indirect cost
**NONFEDERAL SUPPORT**

*New Grants Awarded in 1993*

*Includes direct and indirect cost*

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* New Grants Awarded in 1993
+ Includes direct and indirect cost
During 1993, the Laboratory received capital and program contributions from foundations, individuals, and corporations totaling $4,092,050.

Capital contributions were composed of gifts to the Cancer Research Fund, the William J. Matheson Chair, the Charles S. Robertson Chair, and the Second Century Unrestricted Fund, each of which are individual components of the Laboratory's permanent endowment. Capital contributions also include planned gifts, the principal of which will accrue to the Laboratory upon the death of the donor.

Program contributions were composed of gifts to the Genetic Diseases Fund and to the William Maxwell Cowan and Henry Wendt Neuroscience Fellowships. Program contributions are gifts intended to be used in their entirety to fund ongoing Laboratory programs.

Sherburn Becker  
The Banbury Fund  
Booth Ferris Foundation  
John Cleary  
Maxwell Cowan  
Daphne Seybolt Culpeper Foundation  
Fort Hill Foundation  
Robert B. Gardner, Jr. *  
Townsend and Elise Knight *  
David H. Koch Foundation  
Charles Henry Leach II *  

George and Mary Lindsay  
William R. Miller  
Oncor  
Dr. David B. Pall  
Edward L. Palmer  
Perkin Fund  
Rippel Foundation  
Alan and Edith Seligson  
Owen Smith  
James Watson  
Henry Wendt  
Taggart Whipple *

Total $4,092,050

* Planned Gift
Corporate Sponsor Program

The Corporate Sponsor Program provides support for the meetings held in both Grace Auditorium on the main Laboratory campus and Banbury Center. 1993 marked the 10th year of the Program, and it is the long-term financial stability provided through the generosity of the Program's members that enables us to keep our meetings exciting and at the cutting edge of modern biological research. In 1993, there were 37 members (see below) of the Corporate Sponsor Program, and the meetings that they supported were attended by 4600 scientists.

The members of the Program receive special privileges in acknowledgment of their contributions. We waive all on-site fees for eight representatives of each company at our meetings. Three of these scientists may attend meetings at Banbury Center, where attendance is otherwise only by invitation of the organizers. Corporate Sponsors receive gratis copies of Cold Spring Harbor Laboratory Press publications including the journals *Genes & Development* and *PCR*. Beginning in 1994, we will make Grace Auditorium available to Corporate Sponsor Program members for sponsorship of scientific meetings on topics of their own choice. These meetings will be of the same excellent standard as the regular meetings and will garner kudos from being held here at the Laboratory. They will be strictly limited in number.

We also acknowledge our Sponsors in all relevant publications, including the books of abstracts given to all meeting participants. The names of the sponsoring companies are listed on the poster describing the meetings. This is mailed to approximately 7000 scientists throughout the world.

Akzo Pharma International B.V.  
Alafi Capital Company  
American Cyanamid Company  
Amgen Inc.  
Applied Biosystems, Inc.  
Armstrong Pharmaceuticals-Medeva PLC  
BASF Bioresearch Corporation  
Becton Dickinson and Company  
Boehringer Mannheim Corporation  
Bristol-Myers Squibb Company  
Chugai Pharmaceutical Co., Ltd.  
Diagnostic Products Corporation  
The Du Pont Merck Pharmaceutical Company  
Forest Laboratories, Inc.  
Genentech, Inc.  
Glaxo  
Hoffmann-La Roche Inc.  
Johnson & Johnson  
Kyowa Hakko Kogyo Co., Ltd.  
Life Technologies, Inc.  
MetPath  
Millipore Corporation  
Monsanto Company  
New England BioLabs, Inc.  
Oncogene Science, Inc.  
Pall Corporation  
The Perkin-Elmer Corporation  
Pfizer Inc.  
Sandoz Research Institute  
Schering-Plough Corporation  
SmithKline Beecham Pharmaceuticals  
Sterling Winthrop Inc.  
Sumitomo Pharmaceuticals Co., Ltd.  
Takeda Chemical Industries, Ltd.  
Toyobo Co., Ltd.  
The Upjohn Company  
Wyeth-Ayerst Research

Total $737,750
Cold Spring Harbor Laboratory Association (CSHLA)

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James L. Spingarn, Vice President
Mrs. Edward Greenberg, Treasurer
Mrs. E. Maxwell Geddes, Jr., Secretary
G. Morgan Browne, Asst. Secretary/Treasurer

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Mrs. Philip R. von Stade
James D. Watson, Ph.D.

President’s Report

It was not easy to step into the shoes of my respected predecessors, Ed Pulling and George Cutting. In another way, it was very easy because Ed and George had attracted a large membership of Laboratory supporters. I also inherited a super board of directors, Joan Pesek who knows all the ropes, a good staff, and a calendar of events half full.

With the mandate of the Association in mind, we planned the year to provide information to our directors, as well as our membership, about the Laboratory so that they could expand their scientific knowledge and be good ambassadors in the community. Each of our four Director’s Meetings included an update of science plus a special report from a young investigator. We wished to reach out to as many new people as possible to attract them to and acquaint them with the science being done here. To accomplish this, we needed to arrange varied events, some informational, some entertaining, and some to raise funds, all followed by tours of the Laboratory. Finally, we hoped that our present members would continue their support and that our outreach would bring in new members who would support the Annual Fund.

Looking beyond basic research to scientific environmental studies, at the annual meeting in January, Dr. George M. Woodwell of the Woods Hole Research Center spoke on global warming, alarming us with his view of the future of our ecosystems. In conjunction with the American Museum of Natural History, Dr. Melanie L.J. Stiassny gave a lecture on tropical cichlid fish, an interesting opportunity for our community to appreciate a little known threat to aquatic species.

Subsequent panel discussions focused on diseases related to the latest basic genetic research of Cold Spring Harbor scientists: Breast Cancer, Alzheimer’s Disease, and Manic Depression. We hope to offer more of these discussions in the future, making the public lecture concurrent with a Banbury meet-
ing on the same subject where the same panelists are participating. Because of its prevalence in this community, two evenings were devoted to Lyme disease.

Music has always been part of the CSHLA’s calendar. This year, we were most fortunate to have a concert of Iolanthe performed by the Blue Hill Troupe. George Cutting had made the arrangements with our neighbor Jay Whitman, who not only brought the Troupe, but was a star performer as well. Mary Ann and Harry Charlston, with Wendy Hatch, chaired an enthusiastic committee that had the pre-performance dinner and the auditorium filled before the invitations were mailed. It was a delightful evening for which we were extremely grateful to the members of the Troupe. The benefit netted $19,995 for the Annual Fund.

The opportunity for another special art occasion came in the fall. To benefit the Laboratory and to celebrate Faith McCurdy’s birthday, her family and friends attended “A Limner’s View,” the architectural drawings of Faith McCurdy. To complement the show, Liz Watson prepared a catalog that described in a charming way each drawing. Again, on that weekend, the show was enjoyed by many newcomers to the grounds.

Annually, at the renowned Cold Spring Harbor Symposium, the Association invites one of the most distinguished attendees to be the speaker for the Dorcas Cummings Memorial Lecture. Dr. Eric S. Lander (Director, MIT Center for Genome Research) gave an illuminating talk on DNA, one that was highly understandable to our audience which came away with a fresh awareness of the part proteins play in activating genes. Perhaps some referred to the Franny Elder Glossary after the lecture? The following members deserve special thanks for hosting the traditional dinners in their homes in honor of the selected worldwide scientists attending the Symposium:

Mr. and Mrs. Henry Babcock
Mr. and Mrs. Loren Berry
Mr. and Mrs. David Clark
Mr. and Mrs. John Cleary
Dr. and Mrs. Paul Cushman
Mr. and Mrs. W. Tucker Dean
Mr. and Mrs. Donald Deming
Mr. and Mrs. Duncan Elder
Mr. and Mrs. Edward Gerry
Mr. and Mrs. John Grace
Mr. and Mrs. Henry U. Harris
Mrs. Sinclair Hatch
Mr. and Mrs. James Large
Mr. and Mrs. George Lindsay
Mr. and Mrs. David L. Luke III
Dr. and Mrs. Larry Nathanson
Mrs. Francis T.P. Plimpton
Mr. and Mrs. John Reese
Mr. and Mrs. Thomas Saunders
Mr. and Mrs. Owen Smith
Mr. and Mrs. James Spingarn
Mr. and Mrs. John Stevenson

On a stifling hot evening, June 19th, some 400 guests assembled on the lawn of Watson’s house, Airs lie, for the “Secret of Life” Garden Party. Although I was unable to attend, I have been informed of the wonderful success of this event. It was perfectly planned by Chairpersons Lisa Schiff and Laurie Nesi, working with Jane Greenberg and Joan Pesek. DNA tee shirts, posters to clarify the scientific “secret,” decorations with a science theme—every part of the evening introduced the Laboratory to those who attended. Following the reception, all 400 guests were entertained by hosts and hostesses who had previously toured the Laboratory. They were:

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Heartfelt thanks go to all who worked on this evening. It brought 140 new members to the Association, netted $23,985, and gave everyone a marvelous time.

As discussed above, outreach events are part of our agenda. John and Joyce Phelan gave a lovely cocktail party at Airlie for their friends from Garden City. In November, Price Waterhouse provided their new auditorium and held a reception in New York City for "An Evening with Jim Watson, The Genetics of Cancer." The program included Dr. Bayard Clarkson (Honorary Trustee), Dr. Jim Watson, and Dr. Michael Gilman, for the purpose of introducing New Yorkers to the Cold Spring Harbor Laboratory. Those in attendance were excited by what they heard and the event generated several new members for the Association.

Add all the above events to the information and publicity disseminated by the news articles (and local) reporting new discoveries at Cold Spring Harbor Laboratory, the 40th Anniversary of the Discovery of the Structure of DNA by Drs. Watson and Crick, and finally the glorious Nobel prize won by Dr. Rich Roberts, whom we all know, and you have the elements that have made the year 1993 so successful. When our Association Directors wrote to the membership in the fall, 358 members renewed their support and 160 new members joined overall, bringing the total to a high of 889 members. With Jim Spingarn heading up the Major Gifts Committee and Carol Large in charge of membership, working with Wendy Hatch, Mary Ann Charlston, John Cleary, and other Directors, the Annual Fund grew to a record total of $526,241.

These unrestricted Annual Funds that support science are critical, enabling the Laboratory to attract and retain the most desirable young scientists and assist them with start-up money. At a time when federal grants are extremely competitive and slow to come through, our newcomers, although qualified, may have to wait months to receive their grants. It was extremely rewarding to find that the administration was able to allocate $100,000 over budget for scientific support this year because of the generosity of the Association Members! I thank all of our members for their wonderful gifts.
So it is with anticipation that I embark on 1994, ready to work with Bruce and Grace Stillman while Jim and Liz have a sabbatical in England. There are plans to offer more tours and many events, and in addition, the Scientific Support Committee expects to offer more social opportunities to scientific staff and their spouses. Should anyone have extra tickets for concerts or theatre, they would be much appreciated.

Warm thanks are extended to all the Directors who have made my first year easy. Of course I will miss Wendy Hatch, a gem whom we will keep working with us, and I look forward to having Coe Kerr, Vernon Merrill, and Doug Morris as new Directors. This report would be incomplete without a huge thank you to the remarkable Administrative and Development staff.

Mary D. Lindsay

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<td>Other</td>
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<td><strong>Total</strong></td>
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