GENETICS OF CELL PROLIFERATION

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Our laboratory has continued work on growth control in eukaryotes. Two experimental systems are employed: the yeast *Saccharomyces cerevisiae* and cultured mammalian cells.

The yeast studies have grown from a study of the function of mammalian *RAS* oncogenes. Close structural and functional homologs exist in yeast, and they control adenylate cyclase. Two other human oncogenes are under intensive scrutiny: the *ros* gene, which appears to be expressed in glioblastomas and encodes a transmembrane tyrosine kinase, and the *mas* gene, which encodes a protein that resembles the visual rhodopsins in structure.

Growth Control in *S. cerevisiae*

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The effort of this section is directed to define the components of the *RAS/CYR* (adenylate cyclase) pathway in yeast and to study their interactions. Work from previous years (see last year's Annual Report) resulted in cloning the two *RAS* genes *RAS1* and *RAS2*; *SUPH,* a gene required for maturation of *a* factor and *RAS* proteins (Powers et al., *Cell 47:* 413 [1986]); *CDC25,* a gene required for progression through G_1 (Broek et al., *Cell* [1987] in press); *CYR,* which encodes adenylate cyclase; *TPK1, TPK2,* and *TPK3,* which encode the cAMP-dependent protein kinase catalytic subunits (T. Toda et al., in prep.); *BCY1,* which encodes the cAMP-dependent protein kinase regulatory subunit (Toda et al., *Mol. Cell. Biol.* [1987a] in press); *PDE2,* which encodes the high-affinity cAMP phosphodiesterase (Sass et al., *Proc. Natl. Acad. Sci. 83:* 9303 [1986]); and a variety of auxiliary genes of unassigned function (*JUN1, JUN2, SUPC, SCH1, SCH2,* and *SCH9*). There have been several new developments in this area. *CDC25* appears to control *RAS* function, and we have found dominant lethal *RAS* mutations. Biochemical studies now clearly indicate the complete GTP dependence of *RAS.* Progress has been made in purifying yeast adenylate cyclase. Study of the *TPK* genes has led to a better understanding of their role in regulating physiologic responses. We have cloned and sequenced both yeast cAMP phosphodiesterase (*PDE1* and *PDE2*) genes. Disruption of the *PDE*s or mutations in the cAMP-dependent protein kinases (*TPK* genes) have revealed an extraordinarily powerful feedback mechanism for cAMP homeostasis. Progress has been made in characterizing additional genes related to the *RAS/CYR* pathway (*SCH1, SCH2, SCH9, JUN1, JUN2,* and *SUPC*). Our progress is described in greater detail below.

SUPH was originally isolated as an allele capable of suppressing the phenotypic effects of the mutant of $RAS2^{val19}$. The *supH* alleles were found to be *a*-specific sterile. In collaboration with members of I. Herskowitz's laboratory at the University of California (San Francisco), we showed that *supH* strains fail to secrete *a*-factor, fail to palmitylate yeast *RAS* proteins, and fail to palmitylate many other yeast proteins that are normally palmitylated. We derive two conclusions from these findings: (1) The *SUPH* gene product is required for the normal maturation of proteins containing the Cys-*A*-*A*-*X* (where *A* is any aliphatic amino acid and *X* is the terminal amino acid) consensus sequence for palmitylation, and (2) this processing event is required for the maturation and/or secretion of *a*-factor (Powers et al., *Cell 47:* 413 [1986]).

CDC25 was first described by Hartwell et al. (*Genetics 74:* 267 [1973]) as the wild-type allele of a temperature-sensitive mutation causing G_1 arrest. This gene was cloned, sequenced, and characterized (Broek et al., *Cell* [1987] in press). It encodes a 1589-amino-acid protein with no obvious structural similarities to known proteins. Disruption of *CDC25* causes a lethal phenotype that can be suppressed by $RAS2^{val19}$ but not by wild-type *RAS2.* Most recent biochemical experiments indicate that the adenylate cyclase of *S. cerevisiae* lacking *CDC25* has a very low level of activity in the absence of guanine nucleotides. From biochemical and genetic data, we have concluded that the *CDC25* product controls *RAS* protein function (Broek et al., *Cell* [1987] in press). Indeed, we have identified a mutant *RAS* protein that behaves as though it complexes irreversibly with *CDC25* (see below).

RAS1 and *RAS2* genes have been previously characterized (Kataoka et al., *Cell 37:* 437 [1984]; *Cell 40:* 19 [1985]; Powers et al., *Cell 36:* 607 [1984]; Toda et al., *Cell 40:* 27 [1985]). Recent biochemical experiments with *RAS* proteins purified from *Escherichia coli* expression systems indicate that they fail to stimulate adenylate cyclase when bound to a guanine dinucleotide (Field et al., *Mol. Cell. Biol.* [1987] in press). These results strongly confirm the model of *RAS* activation by point mutations that destroy intrinsic GTPase activity. In addition, we have determined that, in vitro, *RAS* bound to guanine diphosphates does *not* compete with *RAS* bound to guanine triphosphates for stimulation of adenylate cyclase (Field et al., *Mol. Cell. Biol.* [1987] in press).

In an unrelated series of experiments, a dominant temperature-sensitive lethal *RAS2* mutation was found during a search for genes capable of suppressing the phenotypic effects of $RAS2^{val19}$. This temperature-sensitive defect cannot be suppressed by high-copy *RAS2* or by high-copy *CDC25* alone, but it can be suppressed by high-copy *CDC25* when at least one copy of wild-type *RAS* is present. These

experiments indicate the existence of a novel class of *RAS* mutants capable of blocking the *RAS* pathway in a dominant fashion. The simplest explanation of the particular allele we have discovered is that it encodes a protein that forms an ineffective complex with *CDC25*. The existence of dominant interfering mutations in regulatory components has important implications for strategies to understand *RAS* function as well as the functions of other oncogenes.

We are still testing the hypothesis that *RAS* has an alternate function in addition to stimulating adenylate cyclase. This hypothesis appears to be needed to explain two curious observations. First, disruption of the *CYR1* gene is not always lethal, whereas disruption of both *RAS* genes invariably is (Toda et al., in *Oncogenes and Cancer,* Japan Scientific Societies Press, Tokyo, Japan [1987b] in press). Second, disruptions of *CYR1* can be readily suppressed by high-copy *TPK* genes, but disruptions of *RAS* suppressed by these genes are temperature-dependent. We are exploring the hypothesis of alternate *RAS* function by both genetic and biochemical analyses.

CYR1 encodes adenylate cyclase. Its sequence and preliminary structural characterization were previously published (Kataoka et al., *Cell 43:* 493 [1985]). Our current effort is directed at determining how the activity of adenylate cyclase is regulated. As a first step, we have begun to purify this enzyme from *S. cerevisiae.* For this purpose, we have constructed a modified *CYR1* gene encoding a product with a novel amino-terminal domain. This domain consists of a peptide derived from an influenza virus protein, for which we have obtained monoclonal antibodies from R. Lerner and I. Wilson at the Research Institute of Scripps Clinic. A single-step affinity purification with elution by peptide has led to over 100-fold purification of yeast adenylate cyclase activity. This purified activity is still *RAS*-responsive. These studies should reveal whether there are other proteins that purify as part of the adenylate cyclase complex. Recent studies of the phosphodiesterases and protein kinases indicate that there is a negative feedback control of cAMP production (see below). We are testing to determine if part of this feedback control results in a physical modification of adenylate cyclase.

BCY1 encodes the regulatory subunit of the cAMP-dependent protein kinase (Toda et al., *Mol. Cell. Biol.* [1987a] in press), and its properties were reported in last year's Annual Report. Disruption of *BCY1*, like the introduction of the *RAS2*^{val19} gene, induces severe aberrations in G_1 arrest, carbohydrate accumulation, carbon source utilization, germination, sporulation, and heat-shock resistance. Experiments described below indicate that the phenotypes of disruptions of *BCY1* appear to be mediated through its associated kinases. We have been unable to assign any role to *BCY1* other than its control of the *TPK* genes.

TPK1, TPK2, and *TPK3* encode the cAMP-dependent protein kinases (T. Toda et al., in prep.). Their properties were partly described in last year's report. More recently, we have obtained intragenic mutations in each of the *TPK* genes that suppress the heat-shock-sensitive phenotype of *BCY1* disruptions. Cells containing these *TPK*^{w} alleles and *bcy1* therefore have a kinase activity that is not responsive to fluctuations in cAMP. Yet such cells can arrest in G_1 upon starvation, can utilize nonfermentable carbon sources, can sporulate and germinate correctly, and can accumulate glycogen appropriately (S. Cameron et al., in prep.). Thus, the entire panoply of physiologic responses thought to be at least partly controlled by the cAMP effector system is performed normally despite the inability to modulate this effector system. Therefore, these processes must be under coordinate control, with the cAMP kinase system a contributing (but not exclusively determining) function. Most recently, study of the *TPK*^{w} genes has led to insights into the feedback control of cAMP production (see below).

PDE1 and *PDE2,* respectively, encode the low- and high-affinity cAMP phosphodiesterases of *S. cerevisiae* (Sass et al., *Proc. Natl. Acad. Sci. 83:* 9303 [1986]; J. Nikawa et al., in prep.). The *PDE2* gene was initially described last year. It has weak homology with several vertebrate phosphodiesterases. *PDE1,* recently isolated as a high-copy suppressor of *RAS2*^{val19}, has weak homology with phosphodiesterases of the slime mold, but no homology with *PDE2.* Thus, there are at least two distinct branches of phosphodiesterases in evolution.

Gene-disruption experiments with the phosphodiesterases have led to the surprising discovery of a rigorous feedback control of cAMP production. Strains lacking both *PDE* genes show only a modest twofold elevation of cAMP levels. However, strains lacking the *PDE* genes but containing the mutant *RAS2*^{val19} gene show a 1000-fold in-

crease in cAMP. These results imply that there is a strong feedback control of cAMP generation in *S. cerevisiae,* which likely passes through the *RAS* proteins. Further experiments with the *TPK* genes indicate that feedback probably results from the activity of the cAMP-dependent protein kinase. In particular, activation of the kinase by disruption of *BCY1* depresses cAMP levels, whereas the presence of the *TPK*^{w} alleles, in the absence of wild-type *TPK* genes, causes a great elevation of cAMP levels. Work is in progress to characterize these interactions at the biochemical level.

SCH9 and other genes (e.g., *JUN1, SCH1,* and *SCH2*) that interact with the *RAS/CYR1* pathway have been identified. We have not yet fully characterized these genes at the nucleotide, genetic, or biochemical level. *SCH9* has the structure of a protein kinase and can perform many functions of the cAMP-dependent protein kinases.

Human Oncogenes

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We are studying two human oncogenes that we recently isolated: the *ros* and *mas* genes. These genes were isolated using a modification of the NIH-3T3 transfection assay, which identifies oncogenes by their ability to induce tumorigenicity (Fasano et al., *Mol. Cell. Biol. 4:* 1695 [1984]). It is now clear that this assay identifies normal human genes that have the potential to act as oncogenes when amplified, overexpressed, or rearranged as a secondary consequence of DNA transfer (Birchmeier et al., *Mol. Cell. Biol. 6:* 3109 [1986]; Young et al., *Cell 45:* 711 [1986]). These oncogenes map to the distal portion of chromosome 6q (Rabin et al., *Oncogene Res.* [1987] in press).

THE HUMAN *ROS* GENE

We previously described the isolation of two human oncogenes, called *mcf2* and *mcf3*, using the DNA cotransfer and tumorigenicity assay (Fasano et al., *Mol. Cell. Biol. 4:* 1695 [1984]). Nucleotide sequence analysis of *mcf3* cDNA clones indicated that the *mcf3* gene arose by rearrangement of a human gene homologous to the v-*ros* gene. It is likely that this rearrangement occurred during or after DNA transfer and was responsible for activating the oncogenic potential of *ros*. This gene, like its v-*ros* counterpart, contains a transmembrane domain and a carboxy-terminal domain that is homologous to the known tyrosine protein kinases. The rearrangement creating *mcf3* resulted in the loss of a putative extracellular domain. It is likely that the normal *ros* gene, like the normal counterpart of the v-*erb* and v-*fms* genes, is a growth factor or hormone receptor. We are currently attempting to obtain a full-length cDNA clone to help us identify the extracellular domain and the physiologic ligand of this receptor. Expression studies in progress clearly indicate that the *ros* gene is expressed in a very high proportion (80%) of human glioblastoma cell lines and hence might be a useful tumor cell marker for that cancer (Birchmeier et al., *Cold Spring Harbor Symp. Quant. Biol. 51:* 993 [1986]). We have recently identified a glioblastoma cell line with a deletion of the *ros* sequences encoding extracellular sequences (C. Birchmeier et al., in prep.).

THE HUMAN *MAS* GENE

We have continued the characterization of another human oncogene, which we call *mas.* This gene was isolated using the same methodology used for isolating the *mcf3* gene described above (Young et al., *Cell 45:* 711 [1986]). Comparison of the transforming locus with the placental locus, cDNA cloning and sequencing, and S1-nuclease-protection experiments have led to the following conclusions. Like *mcf3, mas1* was activated in NIH-3T3 cells during or after gene transfer by a DNA rearrangement, and, like *mcf3,* there is no evidence that *mas* was activated in the tumor cells from which it was ultimately derived. Unlike *mcf3,* the transforming *mas* gene is not rearranged within coding regions. Rather, its ability to transform cells appears to be entirely related to its high level of expression, a consequence of a rearrangement involving DNA 5′ to coding sequences. The normal *mas* gene, cloned from a human placental cosmid library, is only weakly transforming.

Two aspects of the *mas* gene make it of considerable interest as an oncogene. First, NIH-3T3 cells transformed with *mas* are highly tumorigenic and grow to high saturation densities but are not radically morphologically altered compared with normal NIH-3T3 cells. Second, the protein encoded by *mas* is very hydrophobic. Its hydropathy profile

closely resembles that of the visual rhodopsins, and the *mas* product is predicted to have seven transmembrane domains. In this respect, it is novel among cellular oncogenes. Our preferred hypothesis is that the *mas* protein, like rhodopsin, is a signal-transducing receptor that activates a GTP-binding protein. An understanding of the mechanism whereby *mas* transforms cells is likely to lead to new insights into growth-control mechanisms. We have been attempting to develop antibodies for the protein and to test putative ligands.

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