GENETICS OF CELL PROLIFERATION

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Scientists in our section study oncogenes, genes that drastically alter the proliferative capacity of normal cells. In particular, we have concentrated our efforts on the *RAS* oncogenes, which were among the first genes implicated in human cancer. The study of *RAS* has been facilitated by the recent discovery that close homologs exist in the yeast *Saccharomyces cerevisiae,* an organism readily subjected to thorough genetic analysis. Studies of the *RAS* pathway in yeast have led us to examine the cAMP pathway in fine detail. In addition to the above, we have recently isolated and are now studying three new human oncogenes. Much of the work on the yeast *RAS* was done in collaboration with F. Tamanoi (this section), J. Broach (Princeton University), K. Matsumoto (Tottori University, Japan), and I. Uno and T. Ishikawa (University of Tokyo, Japan); some of the work with associated yeast genes was done in collaboration with M. Zoller (Molecular Genetics Section); and the work on other vertebrate oncogenes was performed in collaboration with J. Fogh (Sloan Kettering Institute, Rye, New York).

Yeast *RAS* Genes

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In the yeast *S. cerevisiae*, there are two genes, *RAS1* and *RAS2*, that are closely homologous to the mammalian *RAS* genes (Defeo-Jones et al., *Nature 306:* 707 [1983]; Dhar et al., *Nucleic Acids Res. 12:* 3611 [1984]; Powers et al., *Cell 36:* 607 [1984]). We have cloned both genes (*RAS1* and *RAS2*) from plasmid libraries and have determined the complete nucleotide sequence of their coding regions. They encode proteins that have nearly 90% homology with the first 80 positions of the mammalian *RAS* proteins, and nearly 50% homology with the next 80 amino acids. Yeast *RAS1* and *RAS2* proteins are more homologous to each other, with about 90% homology for the first 180 positions. After this, at nearly the same position that the mammalian *RAS* proteins begin to diverge from each other, the two yeast *RAS* proteins diverge radically. The yeast *RAS* proteins, like the proteins encoded by the mammalian genes, terminate with the sequence CysAAX, where *A* is an aliphatic amino acid. Thus, the yeast *RAS* proteins have the same overall structure and interrelationship as the family of mammalian *RAS* proteins. The domains of divergence may correspond to functional domains of the *RAS* proteins. Monoclonal antibody directed against mammalian *RAS* proteins immunoprecipitates protein in yeast cells containing high copy numbers of the yeast *RAS2* gene.

We and other investigators have previously shown that at least one functional *RAS* gene is essential for the germination of haploid yeast spores (Kataoka et al., *Cell 37:* 437 [1984]; Tatchell et al., *Nature 309:* 523 [1984]). This was demonstrated by constructing doubly heterozygous diploid yeast cells containing wild-type *RAS1* and *RAS2* alleles and *RAS1* and *RAS2* alleles each disrupted by a different auxotrophic marker. These diploid cells were then sporulated, and the resulting tetrads were analyzed. Only spores containing at least one functional *RAS* gene could germinate. We then introduced into these doubly heterozygous diploid cells a *RAS2* gene under the transcriptional control of the galactose inducible *GAL10* promoter (see Kataoka et al., *Cell 40:* 19 [1985]) linked to a third auxotrophic marker. These diploid cells were then sporulated, and tetrads were germinated on either glucose-containing medium (YPD) or galactose-containing medium (YPGal). The results confirm that at least one functional *RAS* gene is required for germination. *ras1*$^-$ *ras2*$^-$ *GAL10-RAS2* spores germinate only in the presence of galactose, the in-

ducer for the *GAL10* promoter. Cells with the genotype *ras1*$^-$ *ras2*$^-$ *GAL10-RAS2* were grown in YPGal and then shifted to YPD. The growth of these cells was then monitored, and cultures were plated on to YPGal agar to measure cell viability. Within several cell generations, cell growth ceased and cells lost viability. These experiments indicated that the *RAS* genes are needed not only for spore germination, but also for the continued growth and viability of yeast cells.

Certain missense mutations drastically alter the biological activity of mammalian *RAS* genes. In particular, the Ha-*ras*^{Val12} gene, which encodes valine instead of glycine at the twelfth codon of the human Ha-*ras* gene, can induce the tumorigenic transformation of NIH-3T3 cells. To test the consequences of a similar mutation of *RAS2* on the properties of yeast cells, we constructed the *RAS2*^{Val19} gene using site-directed mutagenesis (Kataoka et al., *Cell 37:* 437 [1984]). *RAS2*^{Val19} thus encodes valine instead of glycine at position 19, which corresponds to position 12 of the mammalian Ha-*ras* protein. We then examined the effects of introducing this gene into yeast cells. Our first observation was that diploid cells containing *RAS2*^{Val19} could not be induced to sporulate by incubation under conditions of nutritional deprivation (Kataoka et al., *Cell 37:* 437 [1984]). Next, we observed that haploid cells carrying *RAS2*^{Val19} lost viability if starved for nitrogen, sulfur, or phosphorus and failed to arrest in G_1 under those conditions. Wild-type cells normally arrest in G_1 and retain viability for long periods when nutritionally deprived. In addition to these phenotypes, we also noted that cells carrying *RAS2*^{Val19} failed to accumulate carbohydrate stores as cells entered the stationary growth phase (Toda et al., *Cell 40:* 27 [1985]). Thus, in general, *RAS2*^{Val19} cells appeared to show a defective response to nutritional stress.

The cluster of cellular properties just described closely resemble the phenotype of cells carrying the *bcy1* mutation (Matsumoto et al., *Proc. Natl. Acad. Sci. 79:* 2355 [1982], *Cell 32:* 417 [1983], *Exp. Cell Res. 146:* 151 [1983]; Uno et al., *J. Biol. Chem. 258:* 10867 [1983]). The *bcy1* mutation was first isolated by Matsumoto and co-workers as a mutation that suppresses the lethality that otherwise results from the disruption of adenylate cyclase (Matsumoto et al., *Proc. Natl. Acad. Sci. 79:* 2355 [1982]). Cells carrying *bcy1* appear to lack the regulatory subunit of the cAMP-dependent protein kinase and hence have lost the requirement for cAMP (Uno et al., *J. Biol. Chem. 257:* 14110 [1982]). These observations suggest that the *RAS* genes might be participating in the cAMP pathway. In support of this idea, we found that *bcy1* suppressed the lethality that otherwise results from disruption of both *RAS* genes.

The adenylate cyclase activity of the yeast *S. cerevisiae* is stimulated by guanine nucleotides in the presence of magnesium (Casperson et al., *J. Biol. Chem. 258:* 7911 [1983]). In this respect, yeast adenylate cyclase resembles the adenylate cyclase of mammalian cells, which can be stimulated by a guanine-nucleotide-binding complex called G_s (Gilman, *Cell 36:* 577 [1984]). Since the yeast *RAS* proteins also bind guanine nucleotides (Tamanoi et al., *Proc. Natl. Acad. Sci. 81:* 6924 [1984]), we reasoned that they might also modulate adenylate cyclase. This was tested directly by the assay of membranes from wild-type yeast cells and *ras1*$^-$ *ras2*$^-$ cells. Membranes from either contained appreciable adenylate cyclase activity when assayed in the presence of manganese ion, but *ras1*$^-$ *ras2*$^-$ membranes displayed negligible levels of activity when assayed in the presence of magnesium and a nonhydrolyzable guanine nucleotide analog. These results were confirmed in a striking manner by membrane-mixing experiments. We prepared membranes from *RAS1 RAS2* yeast carrying the *cyr1-1* mutation and membranes from *ras1*$^-$ *ras2*$^-$ yeast. The *cyr1-1* mutation disrupts the catalytic subunit of adenylate cyclase. Membranes from these two sources were assayed separately and together after membrane mixing and fusion. The data indicate that membrane mixing and fusion regenerate a guanine-nucleotide-stimulated adenylate cyclase activity (Toda et al., *Cell 40:* 27 [1985]).

The addition of purified yeast *RAS2* protein to membranes from *bcy1 ras1*$^-$ *ras2*$^-$ cells restores adenylate cyclase activity to about 50-fold above background level (Broek et al., *Cell* [1985] in press). Adenylate cyclase activity in the *bcy1 ras1*$^-$ *ras2*$^-$ membranes can also be increased dramatically by addition of yeast *RAS1* protein. Restoration of adenylate cyclase activity by *RAS* proteins is dependent on the presence of guanine

nucleotide, with the nonhydrolyzable GTP analog, GppNp, yielding twice the activity of that observed in the presence of GDP. Incubation of the *RAS2* protein with GTP prior to mixing with the *bcy1 ras1*$^-$ *ras2*$^-$ membranes results in adenylate cyclase activity comparable to that restored with *RAS2* bound to GDP. In contrast, preincubation of *RAS2*^{Val19} with GTP results in activation of adenylate cyclase to levels identical to that induced by *RAS2* proteins bound to GppNp. These results confirm our genetic studies and indicate that there are proteins in membranes that can distinguish between *RAS* proteins complexed with GTP and *RAS* proteins complexed with GDP; furthermore, they provide for the first time an in vitro bioassay for the effector function of *RAS*.

Yeast Genes in the *RAS*/cAMP Pathway

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In our effort to understand thoroughly the function of *RAS* in yeast, we have begun cloning genes which encode proteins that operate along the *RAS*/cAMP pathway. To do this, we have used standard genetic strategies.

BCY1 was cloned from a centromere-linked library (constructed by M. Rose, Massachusetts Institute of Technology, and generously provided by him) by transforming *bcy1* cells with the library and selecting cells capable of resistance to heat shock. *bcy1* cells, like *RAS2*^{Val19} cells, are heat-shock-sensitive (55°C for 30 min), presumably because they cannot enter G_0. We obtained one clone that displays all of the genetic features expected of a *BCY1* clone. Gene disruptions of *BCY1* were constructed and used to transform haploid yeast cells by gene replacement. The transformants display the same phenotype of cells containing the spontaneously occurring *bcy1* mutation.

CYR1, the gene encoding adenylate cyclase, was cloned in a similar manner by transforming *cyr1-2* mutants (temperature-sensitive for adenylate cyclase) with centromere-linked libraries and selecting cells that grew at the nonpermissive temperature. The nucleotide sequence of this gene has been determined (>6000 bp), and an amino acid sequence of the product has been deduced. We are not yet certain which methionine represents the start codon. Aminoterminal deletion mutants of the adenylate cyclase gene give rise to proteins that are catalytically active but do not appear to be regulated by *RAS*. High-level expression of such genes suppresses the lethality of the double *ras1*$^-$ *ras2*$^-$ mutation. We conclude from this that, in yeast, all of the lethal effects due to loss of *RAS* function are mediated through adenylate cyclase. We have succeeded in expressing enzymatically active yeast adenylate cyclase in *Escherichia coli*.

CAK1, a cAMP-dependent protein kinase catalytic subunit, was cloned by complementation of *cdc25*, a cell-division control mutant that arrests in G_1 at the nonpermissive temperature. *cdc25* is suppressed by *RAS2*^{Val19} and by high-level expression of adenylate cyclase. We therefore reasoned that *CAK1* would suppress *cdc25*, which it does. The nucleotide sequencing of this gene is nearly complete, and it shows extensive homology with the sequence of the bovine cAMP-dependent protein kinase catalytic subunit. Gene-disruption experiments indicate that cells lacking *CAK1* are viable. We therefore conclude that cells must contain a second cAMP-dependent protein kinase. Indeed, we observe by hybridization analysis another yeast gene weakly homologous to *CAK1*, which we are now cloning. High-copy plasmids of *CAK1* suppress lethality due to loss of endogenous *RAS* function or loss of adenylate cyclase. These results are formal proof that the effects of adenylate cyclase and hence cAMP are mediated through the cAMP-dependent protein kinase system.

CDC25 was cloned together with *CAK1* by transforming a *cdc25* strain with a centromere-linked library. *CDC25* is not one of the known genes in the adenylate cyclase pathway. We are nearly finished with its nucleotide sequence, and this work and the work on *CAK1* are proceeding in collaboration with M. Zoller (Molecular Genetics Section).

In addition to the genes described above, four recessive mutations, supA, B, C, and D, have been isolated that suppress the phenotype of *RAS2*^{Val19}. One gene, *PSI*, has been isolated that, in high copy, suppresses *RAS2*^{Val19}. The relationship of these genes to the classic cAMP pathway has yet to be determined.

Studies of the Vertebrate *RAS* Proteins

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Some tumor cells contain mutant *RAS* genes that are capable of transforming NIH-3T3 cells. Those genes that have been analyzed arise from the wild-type nontransforming *RAS* genes by mutations producing single amino acid substitutions at position 12 or 61 of the encoded protein. We have performed random bisulfite-induced mutagenesis on the cloned wild-type human Ha-*ras* gene to determine whether mutations at other positions can activate the transforming potential of that gene (Fasano et al., *Proc. Natl. Acad. Sci. 81:* 4008 [1984]). Most mutations are not activating, but mutations that specify single amino acid substitutions at position 12, 13, 59, or 63 of the encoded protein do activate the transforming potential of the Ha-*ras* gene. Some, but not all, mutant *RAS* proteins show an altered electrophoretic mobility in SDS-polyacrylamide gels.

Using the approach described in the previous section, we have been able to test if expression of the normal mammalian Ha-*ras* protein is sufficient for viability in yeast cells lacking their own endogenous *RAS* genes. To this end, we constructed a *GAL10*-Ha-*ras* transcription unit that utilized a full-length cDNA clone of the human Ha-*ras* mRNA under the control of the galactose-inducible *GAL10* promoter. This unit, closely linked to a *LEU2* marker, was inserted into diploid yeast cells that were doubly heterozyous for their endogenous *RAS* genes. Cells were induced to sporulate, and tetrads were examined after germination on YPD or YPGal plates. Approximately 40% of spores with the genotype *ras1*$^-$ *ras2*$^-$ *GAL10*-Ha-*ras* were capable of germination when plated on YPGal, from which we conclude that the human Ha-*ras* protein can supply essential *RAS* function to yeast. Direct biochemical experiments yielded the same result. Adenylate cyclase activity in *bcy1 ras1*$^-$ *ras2*$^-$ membranes was dramatically increased by the addition of purified human Ha-*ras* protein. These results indicate that the effector function of yeast and mammalian *RAS* proteins has been conserved in evolution. In particular, these results suggest that *RAS* proteins may be involved in regulating adenylate cyclase in vertebrates.

To test our ideas about *RAS* function derived from studies in yeast, we have developed a frog oocyte microinjection system. We have found that purified mammalian Ha-*ras* proteins (Gross et al., *Mol. Cell. Biol. 5:* 1015 [1985]) can induce immature oocytes to progress from prophase to metaphase. The Ha-*ras*^{Val12} is 200-fold more potent than Ha-*ras*^{Gly12} in inducing oocyte maturation. This effect is blocked by cholera toxin, which increases cAMP production in oocytes. We have observed no significant changes in cAMP concentrations due to injection of Ha-*ras* protein and conclude that the yeast *RAS* system is not entirely functionally analogous to the mammalian system. Nevertheless, it is clear that the oocyte system provides an excellent model system for testing ideas concerning *RAS* protein function.

Isolation and Characterization of Other Oncogenes

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The transfer of genomic DNA into NIH-3T3 cells has led to the discovery that genes present in some tumor cells are capable of inducing foci of morphologically transformed NIH-3T3 cells. Most of the transforming genes detected in this way have now been identified as members of the *RAS* gene family, either Ha-*ras*, Ki-*ras*, or N-*ras*. The transforming *RAS* genes detected by the NIH-3T3 focus assay have structural gene mutations that account for their transforming activity. Since the great majority of tumor DNAs tested fail to induce transformed foci, we have speculated that the focus assay has a bias for *RAS* genes containing structural mutations. Therefore, we have begun a series of experiments to explore alternative assays for transforming genes present in NIH-3T3 cells after DNA transfer. The system is a modification of the one described by D. Blair and coworkers (National Institutes of Health). Like theirs, our assay also relies on the ability of transformed NIH-3T3 cells to form tumors in nude mice, but it incorporates methods of cotransfection to heighten sensitivity. Using this assay, we have detected three human transforming genes from the DNA of MCF-7, a human breast carci-

noma cell line. One of these is N-*ras*, which is amplified in MCF-7 cells but does not appear to contain structural mutations. We have not yet established whether the other two genes, which we have called *mcf2* and *mcf3*, are associated with any genetic abnormality in MCF-7.

We are still in the process of characterizing *mcf2* and *mcf3*. *mcf3* appears to be the human homolog of the v-*ros* gene and has the structure predicted for a transmembrane receptor. It has been activated by a gene rearrangement resulting in truncation of the region encoding the putative extracellular domain. In addition, we have isolated and are characterizing a gene called *mas1*, which was isolated from a human mastoid epidermal carcinoma.

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