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Studies of *RAS* Function in the Yeast *Saccharomyces cerevisiae*

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The three mammalian *RAS* genes, Ha-*ras*, Ki-*ras*, and N-*ras*, are capable of the malignant transformation of cultured animal cells (Barbacid 1987). Mutations in these genes have been linked to a large number of human cancers (Barbacid 1987). These genes encode closely related proteins that bind guanine nucleotides (Scolnick et al. 1979; Shih et al. 1980; Ellis et al. 1981) and are localized to the inner surface of the plasma membrane (Willingham et al. 1980; Papageorge et al. 1982). Normal *RAS* proteins also slowly hydrolyze GTP (Gibbs et al. 1984; McGrath et al. 1984; Sweet et al. 1984). These properties are similar to those of the G proteins, which has led to the widespread expectation that *RAS* proteins, like G proteins, are involved in the transduction of membrane signals that are linked to cellular proliferation or differentiation. Many of the mutations that activate the *RAS* genes result in the production of proteins with impaired GTP hydrolysis (Gibbs et al. 1984; McGrath et al. 1984; Sweet et al. 1984). This has suggested that, like G proteins, *RAS* proteins are active when bound to GTP but are inactive when bound to GDP. The biochemical function of the mammalian *ras* proteins is unknown.

We have been studying the function of the yeast *RAS* genes in the expectation that such study will lead to insights into the functioning of the mammalian *RAS* genes. *Saccharomyces cerevisiae* have two genes, *RAS1* and *RAS2*, that are structurally homologous to the mammalian *RAS* genes (DeFeo-Jones et al. 1983; Dhar et al. 1984; Powers et al. 1984). The yeast and mammalian *RAS* genes are functionally related as well, since mammalian *RAS* genes can complement yeast lacking their endogenous *RAS* genes (Kataoka et al. 1985b), and yeast *RAS* genes can malignantly transform cultured animal cells (DeFeo-Jones et al. 1985).

In the yeast *S. cerevisiae*, *RAS* proteins appear to control events related to growth arrest. The *RAS2* gene can be activated by a point mutation analogous to the point mutation of Ha-*ras*, which activates its oncogenic potential (Kataoka et al. 1984). Cells carrying the activated $RAS2^{val19}$ gene fail to arrest in the G_1 phase when starved, remain heat-shock sensitive when they reach a stationary phase, and fail to accumulate storage carbohydrates (Kataoka et al. 1984; Sass et al. 1986). These same sets of phenotypes are observed when the adenylyl cyclase pathway is activated, and they first led us to suspect that there is an interaction between *RAS* proteins and adenylyl cyclase (Uno et al. 1982).

The Interaction between *RAS* Proteins and Yeast Adenylyl Cyclase

In yeast, *RAS* proteins are required for the proper functioning of adenylyl cyclase (Toda et al. 1985). This is readily seen both from studies in vivo with mutant yeast strains (Table 1) and from studies in vitro (Fig. 1). Our in vitro systems use *RAS* proteins purified from an *Escherichia coli* expression system (Broek et al. 1985; Gross et al. 1985) and from an adenylyl cyclase complex purified from *S. cerevisiae* (Fig. 2) (Field et al. 1988). We conclude from our in vitro studies that *RAS* proteins interact directly with an adenylyl cyclase com-

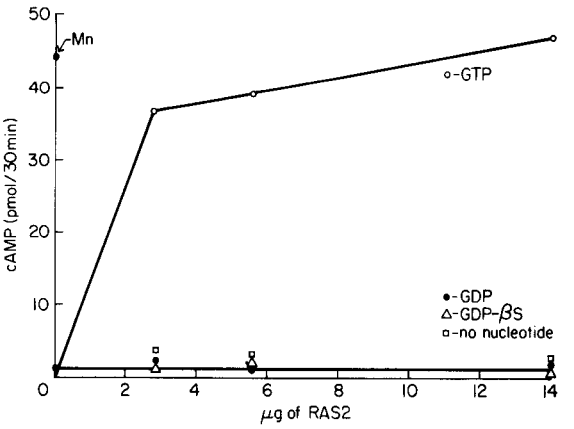

Figure 1. Stimulation of purified adenylyl cyclase complex by purified *RAS2* protein bound to various guanine nucleotides. The indicated amounts of wild-type *RAS2* protein were incubated with purified adenylyl cyclase (see text and Fig. 2). Mg^{++} was used in the assay except as indicated. Prior to incubation with adenylyl cyclase and to the reaction mix, *RAS2* proteins were preincubated with no nucleotide, 50 μM GTP, 50 μM GDP, and 50 μM GDPβS as indicated. (Reprinted, with permission, from Field et al. 1988.)

Table 1. Requirement of *RAS* and *CDC25* for the Production of cAMP in TPK^{w} Strains

Strain	Genotype[a]							cAMP level (pmole/mg protein)
	TPK1	*TPK2*	*TPK3*	*BCY1*	*RAS1*	*RAS2*	*CDC25*	
SP1	+	+	+	+	+	+	+	1.7
S7-1A	+	−	−	+	+	+	+	2.5
S13-58A	+	−	−	−	+	+	+	0.4
RS13-58A-1	w[b]	−	−	−	+	+	+	570
TF16.1	w[b]	−	−	−	+	+	−	0.2
TF18.2	w[b]	−	−	−	−	−	+	<0.3

cAMP levels were measured in cultures of cells growing logarithmically in a rich medium containing glucose.

[a]The genotypes of these strains are as indicated. The *TPK* genes encode the catalytic component of the cAMP-dependent protein kinase, whereas the *BCY1* gene encodes the regulatory component.

[b](w) An attenuated allele of *TPK1* (for details see, Nikawa et al. 1987b; Cameron et al. 1988).

plex. We cannot conclude that the *RAS* proteins act directly on adenylyl cyclase itself, since the complex copurifies with at least one other protein of 70,000 daltons (Fig. 2) (Field et al. 1988). We can conclude from in vitro work that *RAS* proteins that are bound to GTP stimulate adenylyl cyclase but that *RAS* proteins that are bound to GDP do not (Fig. 1). Thus, the activity of *RAS* proteins is controlled by the guanine nucleotide that they bind, consistent with the model of oncogenesis by mutant, activated *RAS*.

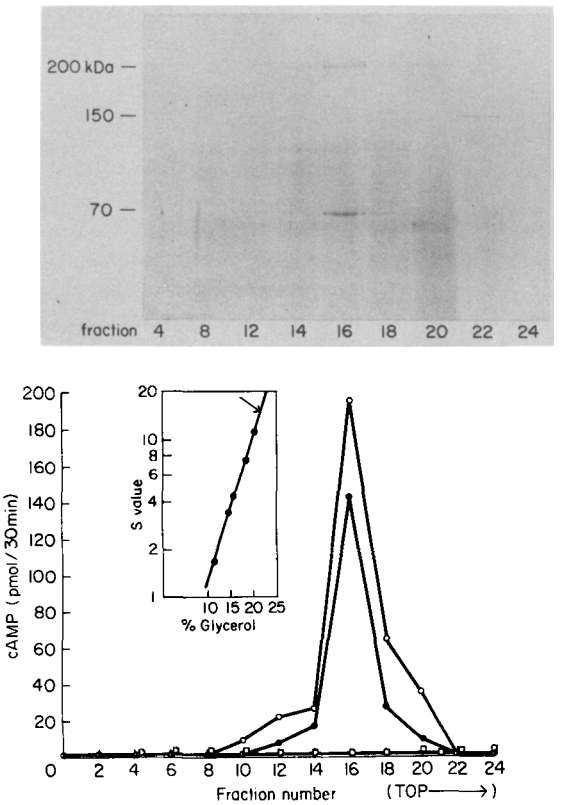

Figure 2. Purification of the adenylyl cyclase complex from yeast. A chimeric protein was expressed in yeast containing a defined peptide epitope at the amino terminus of the yeast adenylyl cyclase protein. Antibodies to the epitope were used to purify an adenylyl cyclase complex from yeast extracts by immunoaffinity chromatography. The complex was eluted with a synthetic peptide, and the complex was subjected to glycerol gradient sedimentation. The gradient fractions were analyzed on a silver-stained SDS polyacrylamide gel (*upper*) and also by adenylyl cyclase assays (*lower*). (*Inset*) S value determination by comparison with standards. Arrow indicates the peak of adenylyl cyclase activity. The assay conditions included Mn^{++} (○), Mg^{++} (□), Mg^{++} + 12 μg $RAS2^{val19}$ protein (●). (Reprinted, with permission, from Field et al. 1988.)

The Control of *RAS* Protein Activity

In *S. cerevisiae*, the *CDC25* protein appears to control *RAS* protein activity. *CDC25* alleles were first discovered as cell-cycle G_1-arrest mutants (Hartwell et al. 1973; Hartwell 1974). Cells lacking *CDC25* are deficient in cAMP (Table 1) and have aberrant adenylyl cyclase activity (Camonis et al. 1986; Martegani et al. 1986; Broek et al. 1987). Cells that contain the activated mutant $RAS2^{val19}$ gene do not require the *CDC25* product (Broek et al. 1987; Robinson et al. 1987). These data are consistent with the model shown in Figure 3 in which the *CDC25* product acts upstream of *RAS* and causes its activation, possibly by catalyzing nucleotide exchange.

Further evidence in favor of the model shown in Figure 3 has come from our discovery of mutant *RAS* genes that behave as though they interfere with *CDC25* activity. These mutant *RAS* genes were found in the course of a genetic screen for temperature-sensitive *RAS* mutants. In this screen, we found dominant temperature-sensitive lethal *RAS2* alleles. Significantly, lethality can be overcome by the presence of the *CDC25* gene on a high-copy plasmid but only if a wild-type *RAS2* or *RAS1* gene is also present. Lethality can also be overcome if the cells contain the mutationally activated $RAS2^{val19}$ gene. Thus, the mutant *RAS* proteins appear to interfere with the activation of wild-type *RAS* proteins, perhaps by forming a complex with *CDC25* proteins. The mutations in interfering *RAS* genes localize to the region that encodes part of a consensus nucleotide-binding site common to many GTP-binding proteins (Table 2) (Dever et al. 1987).

To explain our results with *CDC25* and *RAS*, we propose that *RAS* proteins and *CDC25* proteins normally undergo a transient and direct interaction, similar to models that have been proposed to explain the interaction of receptors with G proteins (Stryer 1986; Gilman 1987). As in those models, *CDC25* proteins interact with the GDP-bound form of *RAS* proteins and, by

Table 2. Amino Acid Sequence of Dominant Interfering *RAS* Mutants

RAS2	**GLY**	GLY	GLY	GLY	VAL	**GLY**	**LYS**	SER	ALA
						↓			
RAS2^{ala22}						ALA			
									↓
RAS2^{pro25}									PRO

The amino acid sequence of wild-type and dominant interfering mutant *RAS2* proteins are indicated from position 17 to position 25. The consensus sequences GXXXXGK for nucleotide-binding proteins is indicated in bold.

virtue of stabilizing the transitional state of nucleotide-free *RAS* protein, catalyze nucleotide exchange. We propose that the dominant temperature-sensitive *RAS* proteins remain bound to *CDC25* protein because alterations in the consensus nucleotide-binding site alter nucleotide affinity and stabilize a nucleotide-free *RAS-CDC25* protein complex.

Interfering Mutants in Signal-transduction Pathways

The discovery that there exist mutant forms of *RAS* that interfere with activation of normal *RAS* led us to think about interfering mutants in a more general sense. In the broadest possible terms, if there is a signal-transduction pathway wherein protein X interacts with protein Y, which then interacts with protein Z in a cascade of information flow, one can expect at least four types of dominant interfering mutant proteins (Fig. 4): X′ complexes ineffectively with Y, Y* complexes ineffectively with X, Y′ complexes ineffectively with Z, and Z* complexes ineffectively with Y. Genetic screens can be designed to search for mutations that produce these kinds of proteins, and such mutants may be valuable tools in the analysis of complex signaling pathways.

We have applied this approach to the *RAS*/adenylyl cyclase pathway of *S. cerevisiae*. We randomly mutagenized Ha-*ras* genes by the passage of plasmids carrying Ha-*ras* through a mutator strain of *E. coli* (Silhavy et al. 1984), and we screened the mutagenized plasmids for their ability to suppress the heat-shock sensitivity of strains of yeast carrying the *RAS2*^{val19} gene. One such mutant was found, and sequence analysis revealed that it contained an arginine for cysteine substitution at codon 186. This disrupts the Cys-A-A-X (where A is any aliphatic amino acid and where X is the terminal amino acid) consensus sequence of *RAS* proteins (Taparowsky et al. 1983; Powers et al. 1984) that functions as a target for the fatty acid addition that causes the membrane localization of *RAS* (Willumsen et al. 1984; Powers et al. 1986). We found that other mutations in this region that destroy the consensus sequence also result in Ha-*ras* genes that interfere with the phenotype of *RAS2*^{val19}.

Our analysis of the mutant Ha-*ras* proteins with a disrupted Cys-A-A-X consensus sequence indicates an unexpected complexity of *RAS* interactions. The mutant proteins remain cytosolic, in keeping with the findings of other investigators that the Cys-A-A-X consensus sequence is required for membrane localization (Willumsen et al. 1984). The Ha-*ras* mutants do not block *RAS2*^{val19} protein from localizing to the membrane. Their effect is therefore not likely to be due to

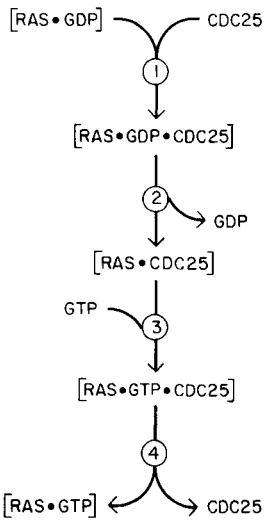

Figure 3. A model of *CDC25-RAS* interaction. (*Step 1*) *RAS* complexed to GDP binds to *CDC25* protein. (*Step 2*) *CDC25* stabilizes the nucleotide-free state of *RAS*, and GDP dissociates. (*Step 3*) GTP binds to *RAS*. This terminates the interaction with *CDC25*, and *CDC25* protein becomes free to interact with another *RAS* protein (*step 4*). According to this model, the dominant interfering *RAS* proteins cannot proceed through steps *3* and *4*, and instead remain bound to *CDC25*, preventing *CDC25* from interacting with wild-type *RAS* protein. (Reprinted, with permission, from Powers et al. 1988.)

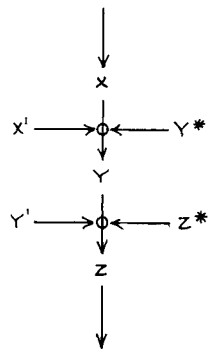

Figure 4. Classes of interfering mutants in signal-transduction pathways. A hypothetical cascade of interactions is shown, involving the sequential interactions of protein X with protein Y and of the latter with Z. X′, Y′, Y*, and Z* are hypothetical mutant proteins. See text for details.

dominant effects on *RAS* protein processing. Competition experiments indicate that the effects of the Ha-*ras* mutants are competed by overexpression of $RAS2^{val19}$ but not by overexpression of *CYR1*, the gene that encodes adenylyl cyclase (Kataoka et al. 1985a). From this, we tentatively conclude that these Ha-*ras* mutant proteins interfere with a cytosolic factor that may facilitate the interaction of *RAS2* with adenylyl cyclase. Alternatively, the mutant Ha-*ras* may interfere with a second function of *RAS*. Evidence for the multiple functions of *RAS* in yeast is given below.

We have also found mutant *CYR1* genes that interfere with the phenotypes of $RAS2^{val19}$. We used a strategy similar to the one described above. We passaged a plasmid carrying *CYR1* through a mutator strain of *E. coli* and readily found clones of *CYR1* that blocked the heat-shock sensitivity of the $RAS2^{val19}$ strains. The ease with which this screen yielded interfering mutations in *CYR1* led us to suspect that virtually any mutation that disrupted the enzymatic function of adenylyl cyclase could result in an interfering protein. Direct tests proved this hypothesis to be correct. The region encoding the catalytic portion of the adenylyl cyclase is located at the 3′ end of *CYR1* (Kataoka et al. 1985a). Frameshift or deletion mutations in this region result in the production of interfering forms of the *CYR1* product. Competition assays suggest that the effects of defective *CYR1* genes can be suppressed by overexpression of *RAS* proteins. It is likely, we think, that the mutant *CYR1* genes encode proteins that form ineffective complexes with *RAS* proteins. More generally, it may be true that proteins that are the targets of the *RAS* action can interfere with the *RAS* function when they are functionally incompetent.

Feedback Regulation of *RAS* Activity

In the course of screens for genes that when overexpressed can suppress the phenotypes induced by $RAS2^{val19}$, we cloned two genes of *S. cerevisiae* that encode cAMP phosphodiesterases *PDE1* and *PDE2* (Sass et al. 1986; Nikawa et al. 1987b). Together, these genes appear to encode the totality of cAMP phosphodiesterase activity measurable in yeast cell extracts (Nikawa et al. 1987a). Surprisingly, we found that cells that lacked these genes but that were otherwise normal did not accumulate enormous levels of cAMP (Table 3). One explanation for this result is that elevated levels of cAMP directly or indirectly feed back to turn off the further production of cAMP. Confirmation of this theory comes from examining cAMP levels in cells that lack the *PDE* genes but that contain the $RAS2^{val19}$ gene (Table 3) (Nikawa et al. 1987b). Such cells have enormously elevated levels of cAMP. In addition to confirming the existence of feedback, these studies strongly suggest that the $RAS2^{val19}$ protein is unresponsive to feedback controls. The simplest explanation of this is that feedback operates on *CDC25* activity, upon which the $RAS2^{val19}$ protein does not depend.

Feedback requires the activity of the cAMP-dependent protein kinase. The catalytic subunits of these genes are named *TPK1*, *TPK2*, and *TPK3* (Toda et al. 1987a). Cells with attenuated *TPK* genes have enormously elevated cAMP levels (Table 1) (Nikawa et al. 1987b). In cells lacking a fully cAMP-responsive protein kinase system, glucose feeding does not induce a biphasic response in cAMP levels (Fig. 5).

Evidence for Additional Functions of *RAS*

Most of the effects of *RAS* on yeast cells can be explained by their action on adenylyl cyclase. The phenotype of cells containing $RAS2^{val19}$ can readily be understood as a consequence of the perturbation of cAMP production: cAMP levels are elevated in cells containing $RAS2^{val19}$; activation of the cAMP-dependent protein kinases leads to phenotypes that closely resemble the $RAS2^{val19}$ phenotypes; and elevated expression of cAMP phosphodiesterases reverses the $RAS2^{val19}$ phenotypes (Sass et al. 1986; Nikawa et al. 1987a). Moreover, the lethality that otherwise results from disruption of both *RAS* genes can be overcome by disruption of the gene, *BCY1*, that encodes the cAMP-dependent protein kinase regulatory subunit (Toda et al. 1987b). The resulting unbridled protein kinase activity is sufficient to complement the loss of *RAS* function. However, there are subtle effects of disruption of both the *RAS1* and *RAS2* genes that do not appear to be identical to the effects of disrupting the adenylyl cyclase gene, and these effects suggest that *RAS* acts in

Table 3. Intracellular cAMP Levels of *RAS* and *PDE* Mutants

Strain	Genotype[a]				cAMP level (pmole/mg protein)		
	RAS1	*RAS2*	*PDE1*	*PDE2*	expt.1	expt.2	expt.3
SP1	+	G	+	+	1.7	1.6	1.8
TK161-R2V	+	V	+	+	2.7	n.d.[b]	3.5
DJ23-3C	+	G	−	−	3.6	n.d.[b]	n.d.[b]
DJ31-4D	+	V	−	−	2300	450	3000
DJ31-6A	+	V	−	−	n.d.[b]	3500	n.d.[b]

cAMP levels are shown for various strains growing logarithmically in a rich medium containing glucose.

[a]The genotypes are as indicated; (+) wild type; (−) a disruption; (G) wild-type *RAS2* ($RAS2^{gly19}$); (V) the activated $RAS2^{val19}$ allele (for details, see Nikawa et al. 1987b).

[b]n.d. indicates not determined.

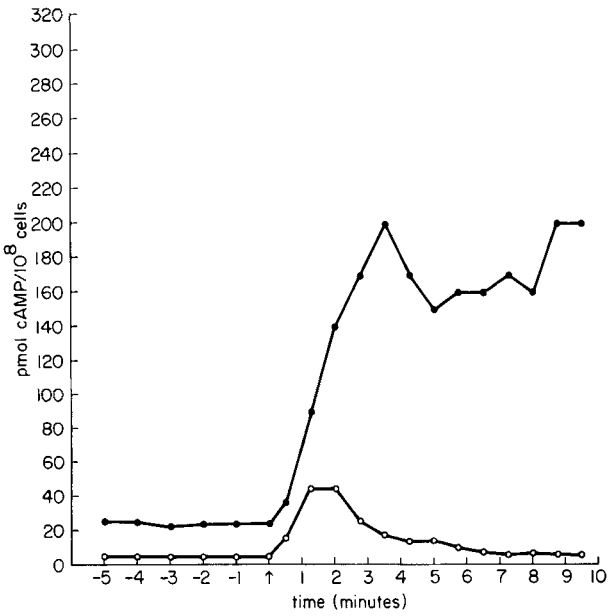

Figure 5. cAMP levels in response to glucose feeding in mutant *TPK* strains. Cells were grown in a rich medium containing acetate, and at zero time were fed 2% glucose. cAMP content was measured at the indicated times as described by Nikawa et al. (1987b). (○) Biphasic response of wild-type yeast cells. (●) Response of cells that lack *BCY1*, the gene encoding the regulatory component of the cAMP-dependent protein kinase, and cells that contain only an attenuated *TPK1*, one member of the gene family encoding the catalytic components of the cAMP-dependent protein kinase (for details, see Table 1; Toda et al. 1987a; Cameron et al. 1988).

ways that cannot be explained readily by its interaction with adenylyl cyclase.

There are two major differences between cells lacking *RAS* and cells lacking *CYR1*. First, haploid spores that lack the *CYR1* gene are often viable, although they give rise to very slow-growing colonies, whereas haploid spores that lack both *RAS1* and *RAS2* genes are almost never viable (Toda et al. 1987c). Second, overexpressing the *TPK* genes can readily suppress the growth defect resulting from lack of *CYR1* but cannot so readily suppress the growth defects resulting from lack of *RAS* genes. Indeed, such *RAS*-deficient strains suppressed by *TPK* genes are often temperature-sensitive. Thus, it appears that *RAS* may have additional functions other than the stimulation of adenylyl cyclase.

The results described above can each be explained in many ways. For example, one may propose that there is a second gene encoding adenylyl cyclase. We have rigorously eliminated this possibility. If such a second adenylyl cyclase existed, it would have to produce 1000-fold lower cAMP levels than the *CYR1* gene. One can also propose that it is better to have no cAMP than to have a little. However, there is one telling piece of evidence that rules out this possibility. We have shown that many strains which lack *CYR1* and *RAS*, but which are viable because *TPK* genes are highly expressed, are temperature-sensitive. This temperature sensitivity is cured by expressing *RAS* in such cells. A lack of *CDC25* function produces the same defects as a lack of *RAS* function. Hence, we conclude that *RAS* has additional functions other than stimulating adenylyl cyclase and that these functions are also controlled by *CDC25*.

Comparison of Mammalian and Yeast *RAS*

The similarities between the mammalian and yeast *RAS* genes are striking. The mammalian Ha-*ras* can complement yeast lacking their own *RAS* genes. Purified Ha-*ras* protein can stimulate purified yeast adenylyl cyclase (Table 4) (Broek et al. 1985). Genetic experiments, not presented here, demonstrate that Ha-*ras* can provide the additional functions of *RAS* in yeast, complementing the loss of *RAS* even in strains that lack adenylyl cyclase.

There are other similarities between mammalian and yeast *RAS*. Mammalian Ha-*ras* protein, like yeast *RAS*, is probably subject to feedback inhibition (Bar-Sagi and Feramisco 1986). We have evidence, too, that Ha-*ras* can interact with *CDC25*. The analogous mutations can be introduced into Ha-*ras*, causing the dominant interfering mutants of *RAS2*. When these mutant Ha-*ras* genes are expressed in yeast, they also appear to block *CDC25* activity. Similar mutants are also interfering in animal cells (Feig and Cooper 1988), suggesting that there is a mammalian protein that catalyzes nucleotide exchange in mammalian *RAS* proteins. Perhaps there is a *CDC25* homolog in mammals.

Two other questions of similarity are raised by our studies of yeast. First, in yeast, *RAS* absolutely controls its effector pathway. It is not clear if this is so in mammals, although we suspect it is. Second, in yeast it is likely that *RAS* has more than one function. It is quite possible that in mammalian cells, *RAS* proteins also have more than one function. This might explain some of the difficulty of assigning a function to mammalian *RAS*.

There are obvious differences between the mammalian and yeast *RAS* proteins. The most glaring difference appears to be in the immediate biochemical

Table 4. Activation of Purified Adenylyl Cyclase by Various *RAS* Proteins

RAS protein	Cation	Maximum activity[a] (pmole cAMP/30 min)
None	Mn^{++}	130
None	Mg^{++}	5.9
RAS2	Mg^{++}	260
$RAS2^{val19}$	Mg^{++}	220
Ha-*ras*	Mg^{++}	73
Ha-ras^{val12}	Mg^{++}	58

Adenylyl cyclase complex was purified from yeast as described in the legend to Fig. 2. cAMP production was measured in the presence of Mn^{++} on Mg^{++} ions and in the presence or absence of added *RAS* proteins that had been preincubated with GTP. $RAS2^{val19}$, the activated form of yeast *RAS2*, and Ha-*ras*, the wild-type protein encoded by the human Ha-*ras* gene, were made intact in *E. coli* expression systems as described by Broek et al. (1985).

[a]The adenylyl cyclase activity observed at saturating amounts of *RAS* proteins.

function of the *RAS* proteins in their respective hosts. It is unlikely that mammalian *RAS* functions to stimulate adenylyl cyclase in vertebrates (Birchmeier et al. 1985). Indeed, a fundamentally different model of *RAS* action has been proposed in mammalian cells. The model of *RAS* action in yeast is rather like the models of action for G proteins and transducin (Gilman 1987). This model is strongly supported by experiment. A radically different model, emerging from the discovery of a GTPase-activating protein and speculative analogies between *RAS* proteins and bacterial elongation factor EF-Tu, has been proposed previously (see Adari et al. 1988; Cales et al. 1988; McCormick et al., this volume). Our own prejudice is that this model is wrong. There are too many similarities between the yeast and mammalian *RAS* to abandon the yeast model altogether. Our own studies point to the complexity of *RAS* interactions with its effectors, and although the identities of the individual effectors may have evolved during speciation, we feel that the patterns of *RAS* interactions may have changed little in evolution.

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