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In Yeast, *RAS* Proteins Are Controlling Elements of Adenylate Cyclase

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Summary

S. cerevisiae strains containing *RAS2*^{val19}, a *RAS2* gene with a missense mutation analogous to one that activates the transforming potential of mammalian *ras* genes, have growth and biochemical properties strikingly similar to yeast strains carrying *IAC* or *bcy1*. Yeast strains carrying the *IAC* mutation have elevated levels of adenylate cyclase activity. *bcy1* is a mutation that suppresses the lethality in adenylate cyclase deficient yeast. Yeast strains deficient in *RAS* function exhibit properties similar to adenylate cyclase deficient yeast. *bcy1* suppresses lethality in *ras1*$^-$ *ras2*$^-$ yeast. Compared to wild-type yeast strains, intracellular cyclic AMP levels are significantly elevated in *RAS2*^{val19} strains, significantly depressed in *ras2*$^-$ strains, and virtually undetectable in *ras1*$^-$ *ras2*$^-$ *bcy1* strains. Membranes from *ras1*$^-$ *ras2*$^-$ *bcy1* yeast lack the GTP-stimulated adenylate cyclase activity present in membranes from wild-type cells, and membranes from *RAS2*^{val19} yeast strains have elevated levels of an apparently GTP-independent adenylate cyclase activity. Mixing membranes from *ras1*$^-$ *ras2*$^-$ yeast with membranes from adenylate cyclase deficient yeast reconstitutes a GTP-dependent adenylate cyclase.

Introduction

The *ras* genes are a highly conserved family of genes first discovered as the oncogenes of Harvey and Kirsten rat sarcoma viruses (Ellis et al., 1981). Mutant *ras* genes encoding altered proteins are found in many human and rodent tumor cells and are capable of the morphologic and tumorigenic transformation of NIH3T3 cells, an established murine cell line (Tabin et al., 1982; Reddy et al., 1982; Taparowsky et al., 1982, 1983; Shimizu et al., 1983; Yuasa et al., 1983; Capon et al., 1983). Little is known about the physiological function of the *ras* proteins in vertebrates. The yeast Saccharomyces cerevisiae contains two closely related but distinct genes, *RAS1* and *RAS2*, that encode proteins that are highly homologous to the mammalian *ras* proteins (Defeo-Jones et al., 1983; Powers et al., 1984; Dhar et al., 1984). We have been studying the *RAS* genes in yeast in the expectation that the powerful genetic approaches possible in that organism will enable us to understand *RAS* function in that organism. We and others have previously reported that while neither *RAS1* nor *RAS2* are by themselves essential genes, some *RAS* function is required for the continued growth and viability of haploid cells (Kataoka et al., 1984, 1985; Tatchell et al., 1984). Diploid yeast cells containing *RAS2*^{val19}, a *RAS2* allele with a missense mutation analogous to one that activates the transforming potential of mammalian *ras* genes, are defective in sporulation (Kataoka et al., 1984). In this manuscript we further characterize yeast strains carrying various mutant *RAS* alleles, demonstrating that they are phenotypically similar to strains with mutations in the cyclic AMP effector pathway and, indeed, that they have biochemical defects in that pathway.

Results

The Absence of *RAS2* Function Promotes Sporulation

In response to certain forms of nutrient starvation, diploid yeast cells undergo growth arrest, meiosis, and sporulation. We previously reported that diploid cells containing *RAS2*^{val19} sporulate poorly under these conditions. We have now examined the effect of deleting *RAS* function on the sporulation response. For this purpose, we utilized disrupted genes with auxotrophic markers inserted within *RAS* coding sequences. Diploid cells, including wild-type, homozygous *ras1*$^-$/*ras1*$^-$, homozygous *ras2*$^-$/*ras2*$^-$, and heterozygous strains, were constructed by transformations and matings (see Table 1). All diploid strains sporulated well in sporulation medium (SPO), which consists of 1% potassium acetate, and no diploid strains sporulated in a medium consisting of yeast extract, Bacto peptone, and 2% glucose (YPD). However, homozygous *ras2*$^-$/*ras2*$^-$ diploids, but not any other strains, sporulated efficiently in a medium consisting of yeast extract, Bacto peptone, and 1% potassium acetate (YPA). Thus, in contrast to diploid cells containing *RAS2*^{val19}, which generally sporulate poorly, diploid cells without a functional *RAS2* gene are able to sporulate even in relatively rich medium. In this respect, *ras2*$^-$/*ras2*$^-$ strains resemble diploid yeast strains which lack adenylate cyclase (Matsumoto et al., 1983a).

RAS2^{val19} Strains Have Reduced Viability following Nutrient Deprivation

Previous studies had indicated that yeast strains containing *RAS2*^{val19} lost viability when stored at 4°C or room temperature (Kataoka, 1984). This observation prompted us to examine cell viability under more defined conditions. To study this phenomenon, we constructed stable isogenic *RAS2* and *RAS2*^{val19} strains by the scheme first described by Scherer and Davis (1979). We constructed a plasmid, p*HIS3*-*RAS2*^{val19}, that contained both the intact *HIS3* and

Table 1. Sporulation Efficiency of Various *ras*$^-$ Diploid Cells

Diploid[a]	YPA[b]	SPO[b]	*RAS1*[c]	*RAS2*[c]
DC5/6	0.0 (3)	11.5 (3)	+/+	+/+
TK1-5	0.0 (3)	29.7 (3)	+/+	+/−
TK2-8	0.0 (2)	25.5 (2)	+/+	+/−
TK1-2	19.4 (3)	47.4 (3)	+/+	−/−
TK1-10	3.0 (2)	55.0 (2)	+/+	−/−
KPPK-1	0.0 (1)	11.8 (1)	+/−	+/−
S5S2/T3-22B	58.5 (3)	75.9 (3)	+/+	−/−
T3-23B/ST1	0.0 (2)	19.7 (2)	−/−	+/+
T3-23B/ST2	0.0 (1)	30.7 (1)	−/−	+/+
T3-23B/ST3	0.0 (1)	30.0 (1)	−/−	+/+
T3-23B/ST4	0.0 (1)	22.8 (1)	−/−	+/+

[a] See Table 3 for strain descriptions. No two strains are strictly congenic, although their genetic backgrounds are very similar.
[b] After overnight incubation in YPD medium cells were transferred into YPA medium and incubated for 4 days (YPA), or transferred to YPA medium for 2 days and then transferred to SPO medium for 2 days (SPO). All incubations were in liquid culture at 30°C. The percentage of sporulated cells in each experimental group was determined by microscopic examination of at least 500 cells. The numbers in parenthesis represent the number of independent times a given experimental group was tested.
[c] The genotypes were determined directly by Southern hybridization (strains TK1-5, TK2-8, TK1-2, TK1-10) or by inference from the presence of auxotrophic markers in haploid parents.

RAS2^{val19} genes (Kataoka, 1984). We used this plasmid to obtain His$^+$ transformants of the *his3* haploid yeast strain SP1 (see Table 3). We retained two such transformants in which the plasmid had integrated by a single homologous recombination into the *RAS2* locus, as determined by Southern hybridization analysis. From two independent transformants, TK161 and TK162, we obtained His$^-$ revertants and screened these by Southern hybridization using an oligonucleotide probe that distinguished the *RAS2*^{val19} from the *RAS2* allele. One revertant from each, TK161-R2V and TK162-R2V, contained a single copy of the *RAS2*^{val19} gene without duplication of the *RAS2* locus. We also identified His$^-$ revertant strains TK161-R2G and TK162-R2G, derived, respectively, from TK161 and TK162, which contained a single copy of the wild-type *RAS2* locus. The phenotype and genotype of these haploid yeast strains were stable. The strains were stored frozen to avoid loss of viability or the accumulation of secondary genetic changes. The phenotypic features of these strains were compared.

We grew cells from these strains in suspension to saturation in either rich (YPD) or synthetic (SD) medium and then measured colony-forming efficiency in rich medium (see Table 4). Unlike the *RAS2* strains TK161-R2G and TK162-R2G, the *RAS2*^{val19} strains TK161-R2V and TK162-R2V rapidly lost viability when grown to saturation in synthetic medium. Cells containing *RAS2*^{val19} did not lose viability as rapidly when grown to saturation in rich medium. Curiously, when these latter cells were diluted into fresh YPD we observed a transient rise in the proportion of multibudded cells within 3 hr of reinoculation

To test if *RAS2*^{val19} haploid cells lost viability under other conditions of growth arrest, we deprived cells either of all sources of nitrogen or of sulfur or of just their auxotrophic requirements and then measured viability after various incubation times. Under such conditions, the *RAS2*^{val19} cells rapidly lost viability (Table 4). Moreover, under these conditions cells containing *RAS2*^{val19} exhibited terminal arrest at all phases of the cell cycle. In contrast, haploid cells containing *RAS2* did not rapidly lose viability and were predominantly arrested in the unbudded state, the physiologically normal arrest point for S. cerevisiae when starved for nitrogen or sulfur (Pringle and Hartwell, 1981).

The inability of diploid yeast cells containing the *RAS2*^{val19} gene to sporulate efficiently previously hampered our studies of yeast containing *RAS2*^{val19} since we had been unable to utilize segregation analysis to make definitive phenotypic assignments. However, we fortuitously found that a haploid yeast strain, K382-19D, commonly used for chromosomal mapping (Klapholz and Esposito, 1982), mates with haploid yeast containing *RAS2*^{val19} to form diploids that can sporulate efficiently. We therefore performed tetrad analysis of diploids resulting from mating K382-19D with the *RAS2*^{val19} strain, TK161-R2V. The haploid progeny were tested for the formation of multibudded cells 3 hr after refeeding stationary cultures, for the ability to survive nitrogen starvation, and for the ability to sporulate when mated. The sensitivity to nitrogen starvation was determined by a convenient plate assay (see Table 2 and Figure 1B). Finally, DNA was prepared from each haploid progeny and the allelic assignment at *RAS2* was made on the basis of hybridization with an oligonucleotide probe. The results indicate perfect correlation between the multibudded phenotype, the sensitivity to nitrogen starvation, and the *RAS2*^{val19} genotype. In contrast, three of eight *RAS2*^{val19} progeny were clearly competent in sporulation, suggesting that the strain K382-19D contains dominant suppressor(s) of the *RAS2*^{val19} induced inhibition of sporulation.

RAS2^{val19} Strains Fail to Accumulate Carbohydrates

Yeast cells entering into stationary phase accumulate the carbohydrate stores glycogen and trehalose (Lillie and Pringle, 1980). Since *RAS2*^{val19} strains exhibit abnormal response to nutrient stress, we tested the progeny of crosses between K382-19D and TK161-R2V for their ability to accumulate carbohydrates when grown to saturation density in YPD medium. The results shown in Table 2 indicate a perfect correlation between the inability to accumulate glycogen or trehalose with the *RAS2*^{val19} genotype. This observation allowed us to distinguish between colonies of *RAS2* cells from colonies of *RAS2*^{val19} cells by a simple iodine staining procedure. Aqueous iodine/iodide solutions stain yeast colonies containing glycogen brown (Chester, 1968). Among progeny between the cross of K382-19D with TK161-R2V, all *RAS2* colonies stained brown, and all *RAS2*^{val19} colonies stained yellow (see Table 2 and Figure 1A).

Elevated Trehalase Activity in *RAS2*^{val19} Cells

The phenotype we have just described for yeast containing the *RAS2*^{val19} mutation closely resembles the phenotype of cells that contain the recessive mutation *bcy1*, isolated by Matsumoto et al. (1982), as a suppressor of adenylate cyclase deficient yeast. First, *bcy1* homozygous

Table 2. Properties of Segregants from K382-19D/TK161-R2V Tetrads

	RAS2 Genotype[a]	Iodine Staining[b]	Starvation Resistance[c]	Multi-budding[d]	Sporulation Efficiency[e]	Glycogen Accumulation[f]	Trehalose Accumulation[f]
K382-19D	G	+	+	NT	50.0	352.	302.
TK161-R2V	V	–	–	NT	<0.03	0.32	10.2
Segregants							
T41-2A	G	+	+	<1	25.6	122.	124.
-2B	G	+	+	1	37.1	40.2	81.6
-2C	V	–	–	10	0.7	0.75	4.0
-2D	V	–	–	10	0.3	0.75	17.6
-3A	V	–	–	31	12.9	1.4	16.0
-3B	G	+	+	<1	28.3	246.	256.
-3C	V	–	–	17	<0.3	0.88	14.8
-3D	G	+	+	1	35.8	108.	167.
-5A	G	+	+	1	11.1	14.4	292.
-5B	V	–	–	19	0.7	1.2	24.0
-5C	V	–	–	10	8.7	1.0	25.6
-5D	G	+	+	<1	46.0	460.	184.
-7A	V	–	–	14	8.7	5.2	14.0
-7B	G	+	+	<1	21.7	142.	134.
-7C	G	+	+	1	20.0	66.	105.
-7D	V	–	–	24	1.7	1.4	14.0

See Table 3 for a description of the genotype of K382-19D and TK161-R2V. Segregants were typed for all auxotrophic markers, which segregated in the expected proportions.

[a] The 19th amino acid encoded by *RAS2* was determined by oligonucleotide blotting. "G" represents glycine and "V" represents valine.

[b] Patches of the indicated strains were grown on YPD agar plates for three days and a solution of 0.2% iodine and 0.4% potassium iodide (Chester, 1968) was poured onto the plates. Patches were scored for staining after 1 min. "+" indicates a dark brown stain and "–" indicates no brown staining. See Figure 1A.

[c] Patches grown on YPD plates were replica plated onto solid medium lacking nitrogen (YNB-N): 1.7 g yeast nitrogen base (Difco) without amino acids and without ammonium sulfate, with 20 g glucose and 20 g agar per liter. After 9 days incubation at 30°C, YNB-N plates were replica plated onto YPD plates and incubated at 30°C. "+" indicates survival, and "–" indicates that little growth was observed after 1 day on YPD plates. See Figure 1B.

[d] Stationary phase cultures in YPD were diluted 1/10 into fresh YPD at 30°C. Three hours later, the cultures were sonicated and the percentage of multibudded cells determined microscopically. One hundred cells were scored from each group.

[e] Sporulation efficiency was examined in diploids resulting from mating the indicated strains with mating strains 14a (*MATa his1*) or 17α (*MATα his1*) from the CSHL collection. Patches of diploid cells were then transferred by replica plating onto YPA plates, incubated for one day, transferred to SPO plates and incubated for two days, all at 30°C. At least 300 cells from each group were examined microscopically.

[f] Glycogen and trehalose content of stationary phase cells was measured according to the procedure of Gunja-Smith et al. (1977) and Lillie and Pringle (1980), respectively. Heavily inoculated cultures were grown in YPD for 48 hr. Stationary phase was assessed by two criteria: the reduction of glucose in the medium and a lack of increase in cell number. Saturation cell densities of segregants were roughly comparable. Numbers represent the concentration of carbohydrate in μg per ml culture.

diploid strains, like diploid cells containing *RAS2*^{val19}, do not sporulate efficiently (Matsumoto et al., 1983a). Second, *bcy1* haploid cells lose viability rapidly when starved for nitrogen (Matsumoto et al., 1983b). Third, under certain growth conditions *bcy1* cells will become multibudded (Matsumoto et al., 1983b). Fourth, *bcy1* strains do not accumulate trehalose when grown to stationary phase (Uno et al., 1983) and do not accumulate glycogen (unpublished observations).

bcy1 strains are deficient in the regulatory subunit of the cyclic AMP-dependent protein kinase (Uno et al., 1982). As a result, *bcy1* strains appear to contain a constitutively active protein kinase catalytic subunit. One reflection of this defect is an elevated level of trehalase in cell extracts (Uno et al., 1983). Trehalase in wild-type cell extracts can be activated to the levels in *bcy1* cell extracts by treatment with cAMP, ATP, and the cyclic AMP-dependent protein kinase, while trehalase in *bcy1* extracts cannot be further activated by this treatment (Uno et al., 1983). A similar biochemical defect can be observed in yeast strains containing the *IAC* mutation that have high levels of adenylate cyclase activity and intracellular cyclic AMP. We therefore examined the trehalase activity of segregants from the cross between K382-19D and TK161-R2V described in Table 2. These results (Table 5) clearly indicate that, like *bcy1* and *IAC* strains, the *RAS2*^{val19} strains have elevated trehalase activity that cannot be significantly increased by treatment with the cyclic AMP and ATP.

Mutation at the *BCY1* Locus Suppresses Lethality in *ras1*$^-$ *ras2*$^-$ Strains

Since *bcy1* suppresses the lethality of mutants deficient in adenylate cyclase, and since, like adenylate cyclase deficient diploid yeast, *ras2*$^-$/*ras2*$^-$ diploid yeast sporulate in rich medium, we tested whether *bcy1* would suppress the lethality of *ras1*$^-$ *ras2*$^-$ haploid yeast. By making a series of crosses with the *bcy1* strain AM203-1B and strains of yeast with disrupted *RAS* genes we constructed *ras1*$^-$ *bcy1* (T16-2A and T16-3D) and *ras2*$^-$ *bcy1* (T17-7B) haploid strains (see Table 3). The presence of the disrupted *RAS* genes in these strains was determined by the auxotrophic markers used to disrupt gene function: *HIS3* to disrupt *RAS1*

Table 3. Strain Descriptions

Strain	Description
AM18-5C	*MATα cyr1-1.*
AM203-1B	*MATα his7 bcy1.*
DC5/6[a]	*MATa/MATα leu2/leu2 his3/+ his4/+ can1/can1.*
K382-19D	*MATα his7 tyr1 ura3 ade2 hom can1 cyh*[R] *spo11.* Chromosomal mapping strain from Klapholz and Esposito, 1982.
KP-2	*MATa leu2 his3 ura3 trp1 ade8 can1 ras2::URA3.* Transformant of SP1 with Nco I/Hind III fragment of *pras2::URA3.*
KPPK-1	*MATa/MATα leu2/leu2/ his3/his3 ura3/ura3 trp1/trp1 ade8/+ can1/+ RAS1/ras1::HIS3 RAS2/ras2::URA3.* Diploid resulting from crossing PK-1 with KP-2.
KPPK-1D	*MATa leu2 his3 ura3 trp1 ras1::HIS3.* Segregant from KPPK-1.
M76.3C	*MATa leu2 his3 cyr1-1.* Obtained from Dr. Szostak.
PK-1	*MATα leu2 his3 ura3 trp1 ras1::HIS3.* Transformant of SX50-1C with Eco RI/Bam HI fragment of *pras1::HIS3.*
S5S2	*MATa leu2 his3 can1 ras2::LEU2.* Segregant from TK1-5.
SP1[a]	*MATa leu2 ura3 trp1 his3 ade8 can1.*
ST-1[b]	*MATa leu2 his3 ura3 trp1 ade8 can1 ras1::URA3.*
ST-2[b]	*MATa leu2 his3 ura3 trp1 ade8 can1 ras1::URA3.*
ST-3[b]	*MATa leu2 his3 ura3 trp1 ade8 can1 ras1::URA3.*
ST-5[b]	*MATa leu2 his3 ura3 trp1 ade8 can1 ras1::URA3.*
SX50-1C[a]	*MATα leu2 ura3 trp1 his3.*
T-58B	*MATα leu2 his3 bcy1.* Segregant from AM203-1B/M76.3C.
T3-22B[c]	*MATα leu2 his3 ura3 trp1 ade8 ras2::URA3.*
T3-23B[c]	*MATα leu2 his3 ura3 trp1 can1 ade8 ras1::HIS3.*
T3-24C[c]	*MATα leu2 his3 ura3 trp1 ade8 can1 ras2::URA3.*
T3-28B[c]	*his3 leu2 ura3 trp1 ras2::URA3.*
T3-28C[c]	*his3 leu2 ura3 trp1 ade8.*
T3-28D[c]	*his3 leu2 trp1 ras1::HIS3.*
T3-35A[c]	*his3 leu2 ura3 trp1.*
T3-35C[c]	*his3 leu2 ura3 trp1 ras1::HIS3.*
T3-35D[c]	*his3 leu2 ura3 trp1 ade8 ras2::URA3.*
T16-2A[d]	*MATα leu2 his3 trp1 bcy1 ras1::HIS3.*
T16-3D[d]	*MATa leu2 his3 ura3 bcy1 ras1::HIS3.*
T16-11A[d]	*MATα his3 leu2 ura3 trp1 bcy1.*
T17-7B	*MATa leu2 his3 can1 bcy1 ras2::LEU2.* Segregant from T-58B/S5S2.
T26-11C	*MATα leu2 his3 can1 bcy1 ras1::HIS3 ras2::LEU2.* Segregant from T16-2A/S5S2.
T26-19C	*MATα leu2 his3 trp1 can1 bcy1 ras1::HIS3 ras2::LEU2.* Segregant from T16-2A/S5S2.
T27-10D	*MATα leu2 his3 ura3 can1 bcy1 ras1::HIS3 ras2::LEU2.* Segregant from T16-3D/S5S2.
T27-25B	*MATa leu2 his3 ura3 can1 bcy1 ras1::HIS3 ras2::LEU2.* Segregant from T16-3D/S5S2.
T28-3C	*MATα leu2 his3 ura3 trp1 ade8 can1 bcy1 ras1::HIS3 ras2::LEU2.* Segregant from T17-7B/T3-23B.
TK1-2	*MATa/MATα leu2/leu2 his3/+ his4/+ can1/can1 ras2::LEU2/ras2::LEU2.* Transformant of DC5/6 with Nco I/Hind III fragment of *pras2::LEU2.*
TK1-5	*MATa/MATα leu2/leu2 his3/+ his4/+ can1/can1 ras2::LEU2/+.* Transformant of DC5/6 with Nco I/Hind III fragment of *pras2::*LEU2.
TK1-10	Same as TK1-2. Independent transformant.
TK2-8	Same as TK1-5. Independent transformant.
TK161	*MATa leu2 his3 ura3 trp1 ade8 can1 RAS2::pHIS3-RAS2*^{val19}. Transformant of SP1 with Cla I linearized p*HIS3-RAS2*^{val19}.
TK161-R2G	*MATa leu2 his3 ura3 trp1 ade8 can1.* His$^-$ revertant of TK161.
TK161-R2V	*MATa leu2 his3 ura3 trp1 ade8 can1 RAS2*^{val19}. His$^-$ revertant of TK161.
TK162	Same as TK161. Independent transformant.
TK162-R2G	*MATa leu2 his3 ura3 trp1 ade8 can1.* His$^-$ revertant of TK162.
TK162-R2V	*MATa leu2 his3 ura3 trp1 ade8 can1 RAS2*^{val19}. His$^-$ revertant of TK162.

Mutant genotypes and mating types are listed if known. All strains are *RAS1 RAS2* unless stated otherwise.
[a] From CSHL collection.
[b] Transformant of SP1 with Eco RI/Bam HI fragment of *pras1::URA3.*
[c] Segregant from KPPK-1 tetrad.
[d] Segregant from T-58B/KPPK-1D.

and *LEU2* to disrupt *RAS2*. The presence of *bcy1* in these crosses was followed by iodine/iodide staining, by the multibudded phenotype, by defective sporulation in presumptive *bcy1/bcy1* diploids, and by back crosses to yeast strains deficient in adenylate cyclase. From these crosses we determined that *bcy1* is not allelic to either *RAS1* or *RAS2* and segregates 2:2 in complete tetrads.

We next crossed the *ras1*$^-$ *bcy1* strains T16-2A or T16-3D with the *ras2*$^-$ strain S5S2, and crossed the *ras2*$^-$ *bcy1* strain T17-7B with the *ras1*$^-$ strain T3-23B to obtain a set of diploid strains that were heterozygous at each of the *RAS1*, *RAS2*, and *BCY1* loci (see Table 3). These diploids were sporulated and after tetrad dissection the phenotype of viable progeny was examined after germination on YPD plates (see Table 6). All viable His$^+$ Leu$^+$ spores that germinated on YPD plates had the *bcy1* phenotype. The *bcy1* genotype was confirmed by back crosses to the *cyr1*-1 strain, M76.3C. Since the *HIS3* marker disrupted *RAS1* and the *LEU2* marker disrupted *RAS2*, we concluded that these cells lacked functional *RAS* genes. This conclusion was confirmed by Southern blotting which demonstrated the absence of intact *RAS* genes in these haploid cells (Figure 2). These results indicate that T16-2A, T16-3D, and T17-7B contain a suppressor of *ras1*$^-$ *ras2*$^-$ lethality and that this suppressor must be very closely linked to *bcy1*. We note that slightly less than half of the *ras1*$^-$ *ras2*$^-$ *bcy1* spores

Table 4. Viability of Strains under Various Conditions

Cell Strain[a]		TK161-R2G		TK161-R2V	
Growth Condition[b]		% Budded[c]	% Viable[c]	% Budded	% Viable
YPD		12	100	56	80
SD	complete	25	100	32	5.00
	–nitrogen	0	100	60	0.10
	–auxotrophic requirements	11	13	40	0.01
Cell Strain		**TK162-R2G**		**TK162-R2V**	
Growth Condition		**% Budded**	**% Viable**	**% Budded**	**% Viable**
YPD		18	100	35	70
SD	complete	6	90	35	5.0
	–nitrogen	2	40	30	1.0
	–sulfate requirements	1	90	14	2.0

[a] The four strains listed here are described in the text and in Table 3. The "G" strains have wild-type *RAS2*; the "V" strains have *RAS2*^{val19}.
[b] TK161-R2G and TK161-R2V were inoculated from log phase cultures into the indicated liquid medium at 3 × 10^5 cells/ml and incubated for four days. TK162-R2G and TK162-R2V were inoculated at 10^6 cells/ml and incubated for 36 hr. Culture conditions were either rich medium (YPD) or synthetic medium (SD) either supplemented with the required auxotrophic supplements (complete) or without (– auxotrophic requirements). For nitrogen starvations, ammonium sulfate and all auxotrophic requirements were omitted. For sulfate starvation, ammonium and magnesium sulfates were replaced with ammonium and magnesium chlorides. All cultures had reached stationary phase in YPD and in complete SD.
[c] The percentage of budded cells was determined by the microscopic examination of at least 200 cells. The percentage of viable cells was determined by the colony-forming efficiency of sonicated cultures on YPD agar.

were able to germinate. This poor penetrance may be explained in several ways. First, *ras1*$^-$ *ras2*$^-$ strains may lack some functions required for efficient germination which are not fully suppressed by *bcy1*; second, there may be a second suppressor unlinked to *bcy1* needed for survival of *ras1*$^-$ *ras2*$^-$ cells; third, and we think most likely, the cytoplasmic inheritance of wild-type cAMP-dependent protein kinase and BCY1 protein from the *bcy1*/$^+$ heterozygous parent renders germination of *ras1*$^-$ *ras2*$^-$ *bcy1* spores inefficient. These possibilities are under continued study.

Adenylate Cyclase Activity of *RAS* Mutants Has Altered GTP Responsiveness

The results we have presented strongly suggest that the effector pathway of yeast *RAS* interacts with the effector pathway of cyclic AMP. Two alternate classes of hypotheses might explain our observations. First, *RAS* might control an essential and parallel pathway with a phenotypic endpoint similar to the cyclic AMP effector pathway, with hyperactivity of either pathway being able to compensate for a deficiency in the other. Second, *RAS* proteins might be essential elements of the cyclic AMP effector pathway itself. There are many points at which *RAS* could in principle interact with the cyclic AMP pathway. One clear possibility is that the yeast *RAS* proteins modulate adenylate cyclase. In fact, the yeast *RAS2* protein, like the mammalian *ras* proteins, is a guanine nucleotide binding protein (Tamanoi et al., 1984), and certain guanine nucleotide binding proteins have been shown to modulate adenylate cyclase. Moreover, the yeast adenylate cyclase activity of crude membrane preparations is modulated by GTP in the presence of Mg^{++} ion (Casperson et al., 1983), although no mutants defective in this activity have previously been isolated.

To test the idea that *RAS* proteins modulate adenylate cyclase activity, we first examined the intracellular cyclic AMP levels of yeast strains carrying mutant *RAS* genes. Segregants from K382-19D/TK161-R2V, first described in Table 2, give a set of strains all containing *RAS1* and either *RAS2* or *RAS2*^{val19}. Intracellular cAMP was measured in mid log phase cultures (Table 7), and normalized for cellular protein. The average concentration of cAMP in eight *RAS2* strains was 1.8 pmol/mg protein; for the eight *RAS2*^{val19} strains it was 7.9 pmol/mg protein. The correlation between genotype and cyclic AMP level is perfect. Next we examined the cyclic AMP levels in yeast strains lacking one or both *RAS* genes. *ras1*$^-$ *RAS2* strains T3-28D and T3-35C had only slightly depressed cyclic AMP levels, while *RAS1 ras2*$^-$ strains T3-28B and T3-35D had at least 4-fold lower levels of cAMP than wild-type *RAS1 RAS2* strains. *ras1*$^-$ *ras2*$^-$ *bcy1* strains T26-19C and T27-10D had levels of intracellular cyclic AMP at least 20-fold lower than wild-type levels. The *bcy1* mutation itself does not affect intracellular cyclic AMP, as previously reported (Uno et al., 1983). Together these results indicate that the *RAS1* and *RAS2* proteins modulate cyclic AMP levels. These results also indicate that the *RAS2* gene is the major determinant of cyclic AMP levels, while *RAS1* may only be a minor determinant. This would explain why *ras2*$^-$/*ras2*$^-$ homozygous diploid strains can sporulate in rich medium but *ras1*$^-$/*ras1*$^-$ strains cannot. The results with *RAS2*^{val19} strains can explain its phenotypic consequences: elevated cyclic AMP levels lead to elevated activity of the cyclic AMP-dependent protein kinase and hence to a phenotype resembling that of *bcy1* and *IAC*.

We next tested directly the properties of the adenylate cyclase of crude membranes prepared from yeast strains carrying *RAS* mutations. Membranes from the wild-type strain SP1, from the *RAS2*^{val19} strain TK161-R2V, and from the *bcy1 ras1*$^-$ *ras2*$^-$ strain T27-10D were prepared by published methods, modified as described in Experimental Procedures. Adenylate cyclase activity was assayed in the

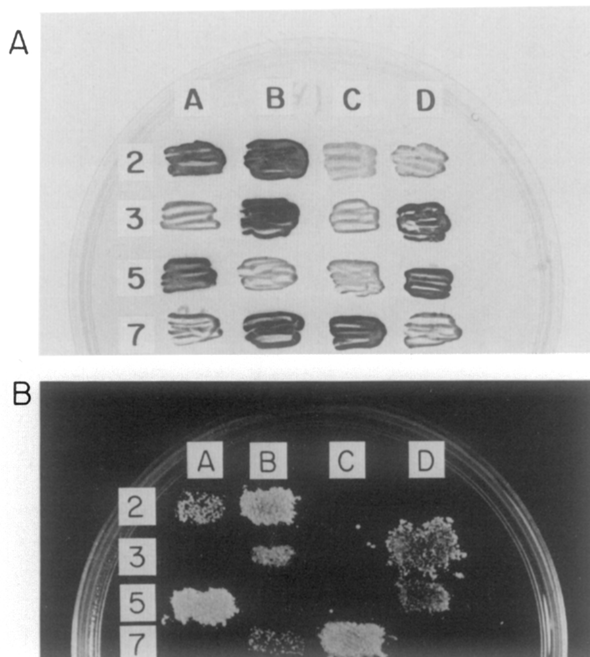

Figure 1. Segregation of Phenotypes from TK161-R2V/K382-19D

(A) Iodine/iodide staining for TK161-R2V/K382-19D segregants. The indicated progeny from TK161-R2V/K382-19D crosses (see Table 2) were streaked onto YPD plates, and allowed to grow for 2 days at 30°C. Then five milliliters of 0.2% iodine/0.4% potassium iodide was gently poured over the colonies and photographs taken 1 min later.

(B) Lethality of nitrogen starvation for segregants from TK161-R2V/K382-19D. The indicated progeny from TK-161-R2V/K382-19D diploids were streaked onto YPD plates. After 2 days incubation at 30°C, they were replica plated onto YNB-N plates that lack a source for nitrogen. After 9 days incubation at 30°C, the plates were again replica plated onto YPD plates and incubated at 30°C for one day.

presence of either manganese ions, or magnesium ions, or magnesium ions and the nonhydrolyzable GTP analog guanosine-5'(β,γ-imino)triphosphate, Gpp(NH)p (Table 8). In the presence of manganese ions, all three membrane preparations had comparable adenylate cyclase activities. Thus, all strains appeared to have equivalent amounts of the catalytic subunit of adenylate cyclase. As reported by Casperson et al. (1983), membranes from wild-type cells had a low adenylate cyclase activity in the presence of magnesium ion alone, which could be induced 4-fold by the addition of the nonhydrolyzable GTP analog. In contrast, the *ras1⁻ ras2⁻ bcy1* strain had low levels of activity in the presence of magnesium, and this activity was not significantly increased by the addition of the nonhydrolyzable GTP analog. Finally, membranes from the *RAS2*^{val19} strain had elevated levels of adenylate cyclase when assayed in the presence of magnesium ion alone. This level was not increased by the addition of Gpp(NH)p to the incubation mixture. These results suggest that cell strains carrying mutant *RAS* genes have altered regulation of adenylate cyclase. The *bcy1* mutation does not itself affect adenylate cyclase (Matsumoto et al., 1982).

These results were confirmed in a striking manner by membrane mixing experiments. Membranes were prepared from the *ras1⁻ ras2⁻ bcy1* strain T27-10D and from the adenylate cyclase deficient *cyr1*-1 strain AM18-5C and assayed separately and after mixing, with the addition of manganese ions, magnesium ions, or magnesium ions with Gpp(NH)p (Table 8). As expected, membranes from the *cyr1*-1 strain showed low levels of cyclase activity in the presence of manganese or magnesium ions or magnesium ions and Gpp(NH)p, while membranes from the *ras1⁻ ras2⁻ bcy1* strain had high levels in the presence of manganese ions, and low levels under other conditions. When membranes from the *ras1⁻ ras2⁻ bcy1* strain were mixed with membranes from the *cyr1*-1 strain, we observed an adenylate cyclase

Table 5. Trehalase Activity of Segregants from K382-19D/TK161-R2V

Segregant[a]	*RAS2* Genotype[a]	Trehalase Activity after Additions[b]				
		None	ATP	cAMP	ATP & cAMP	Ratio +/−[c]
T41-2A	G	1.2	1.1	1.3	4.9	4.5
-2B	G	1.3	1.2	1.2	7.0	6.1
-2C	V	7.0	7.1	7.0	6.8	1.0
-2D	V	6.0	6.4	6.2	6.3	1.0
-3A	V	6.5	6.3	6.2	5.9	0.9
-3B	G	1.4	1.3	1.4	3.9	3.0
-3C	V	3.3	3.2	3.4	5.8	1.8
-3D	G	1.2	1.1	1.2	6.6	5.9
-5A	G	1.1	7.0	1.1	4.7	4.3
-5B	V	4.9	4.9	4.9	4.9	1.0
-5C	V	5.7	5.7	6.0	6.0	1.0
-5D	G	1.5	1.2	1.4	4.9	4.1
-7A	V	5.7	5.8	5.9	7.2	1.3
-7B	G	1.3	1.2	1.3	4.0	3.5
-7C	G	1.2	1.2	1.2	6.1	5.2
-7D	V	6.8	6.3	6.9	6.8	1.0

[a] The *RAS2* genotype was determined as described in Table 2. "G" represents glycine; "V" represents valine.

[b] Trehalase activity (U/mg) was measured in crude cell extracts, as previously described, following incubation with either no additions, ATP, cAMP, or ATP and cAMP (Uno et al., 1983).

[c] The ratio of trehalase activity with and without addition of cAMP and ATP.

Table 6. Tetrad Dissection of *ras1/+ ras2/+ bcy1/+* Diploids

Cross: Genotype of Progeny			T16-2A/S5S2		T16-3D/S5S2		T17-7B/T3-23B	
RAS1	*RAS2*	*BCY1*	Viable	Non-viable	Viable	Non-viable	Viable	Non-viable
+	+	+	10	0	5	0	14	0
−	+	+	13	0	14	1	16	1
+	−	+	20	0	14	0	11	1
−	−	+	0	15	0	4	0	4
+	+	−	16	0	7	0	6	2
−	+	−	17	0	10	1	9	1
+	−	−	11	0	9	1	13	0
−	−	−	4	7	5	4	8	10

See Table 3 for strain description of haploid strains. Individual diploids from the indicated crosses were sporulated and tetrads dissected. The genotypes of all viable spores were determined as follows: The *RAS* phenotypes were deduced from the presence of auxotrophic markers used to disrupt the respective genes. The *bcy1* phenotypes were deduced from the cluster of phenotypes that identify this mutation and that segregate in 2:2 fashion. When possible, the genotypes of non-viable spores were assigned on the basis of the viable spores within a tetrad, assuming normal Mendelian segregation of genetic loci. The table summarizes data only from tetrads where complete genotypic determinations were possible. Overall spore viability was 65%.

activity that could be induced at least 10-fold in the presence of magnesium by the addition of Gpp(NH)p. These experiments demonstrate that there are GTP-responsive factors present in the *cyr1*-1 strain, absent from the *ras1⁻ ras2⁻ bcy1* strain, that fully reconstitute a GTP activatable adenylate cyclase activity in the presence of an intact catalytic subunit.

Discussion

The complex of phenotypes that we have ascribed to mutations in *RAS* are consistent with the notion that *RAS* function is involved in the cellular response to nutritional stress. Cells containing *RAS2*^{val19} are insensitive to changes in their nutritional environment or are unable to make appropriate physiological responses. Diploid cells fail to sporulate when starved. Haploid cells lose viability if growth is arrested by a variety of nutrient deprivations, and fail to accumulate carbohydrate stores when they enter stationary phase. Conversely, homozygous *ras2⁻* diploid cells sporulate prematurely in rich medium lacking only glucose as a carbon source, as though such diploid cells were overly sensitive to changes in their nutritional status. Significantly, an inability to respond appropriately to nutritional stress is a characteristic of many transformed mammalian cells (Pardee, 1974).

The phenotypic features just discussed demonstrate similarities between cells lacking *RAS* and cells containing *cyr1*-1 and between cells containing *RAS2*^{val19} and cells containing *bcy1*. *cyr1*-1 encodes a defective adenylate cyclase (Matsumoto et al., 1984). Cells containing *cyr1*-1 have low levels of adenylate cyclase and require exogenous cyclic AMP for growth (Matsumoto et al., 1982). *bcy1* was isolated as a suppressor mutation of *cyr1*-1 (Matsumoto et al., 1982). Although the gene product of *BCY1* is unknown, *bcy1* cells have drastically reduced levels of the cyclic AMP binding regulatory component of the cyclic AMP-dependent protein kinase (Uno et al., 1982). As a result, *bcy1* cells have constitutively high levels of such kinase activity. The same defect is seen in yeast cells containing the *IAC* mutation,

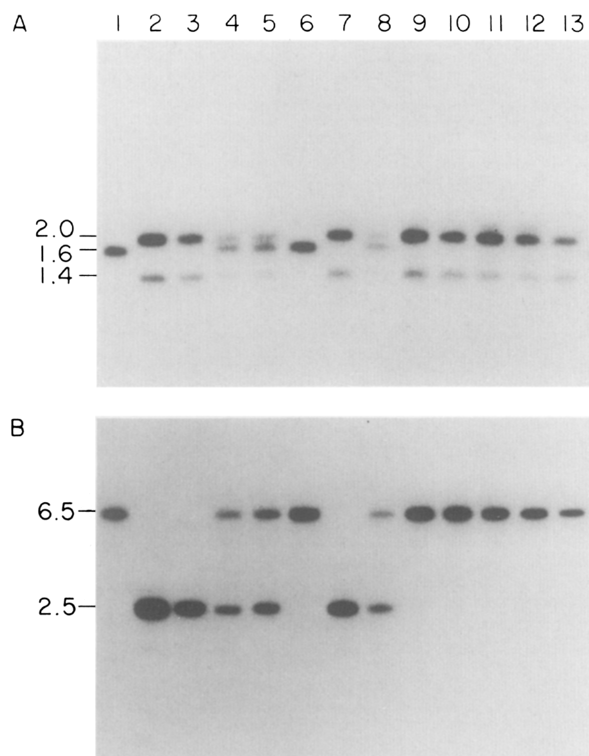

Figure 2. Blot Hybridization Analysis of *ras1⁻ ras2⁻ bcy1* segregants

Yeast DNAs from various strains were digested with Hind III (A) or Nco I/Hind III (B), electrophoresed in 0.7% agarose, transferred to nitrocellulose, and hybridized with ^{32}P-labeled probes.

(A) Nitrocellulose filter hybridized with the 1.6 kb Hind III fragment of the yeast *RAS1* gene nick translated to 1.0×10^8 cpm per μg DNA. (B) Nitrocellulose filter hybridized with the 1.2 kb *Hpa* I fragment of the yeast *RAS2* gene nick translated to 1.0×10^8 cpm per μg DNA. Strains from which DNAs were prepared were as follows. S5S2 (lane 1); T16-2A (lane 2); T16-3D (lane 3); S5S2/T16-2A (lane 4); S5S2/T16-3D (lane 5); T17-7B (lane 6); T3-23B (lane 7); T17-7B/T3-23B (lane 8); T26-11C (lane 9); T26-19C (lane 10); T27-10D (lane 11); T27-25B (lane 12); T28-3C (lane 13). Genotypes of these strains are indicated in Table 3. In (A), 1.6 kb marks the position of the intact *RAS1* gene, and 2.0 and 1.4 kb marks the positions of the *RAS1* gene disrupted with *HIS3*. In (B), 2.5 kb marks the position of the intact *RAS2* gene, and 6.5 kb the position of *RAS2* disrupted with *LEU2*.

Table 7. Intracellular Cyclic AMP Levels of *RAS* Mutants

Strain[a]	Genotype[a]	Cyclic AMP Level[b] (pmol/mg protein)
T41-2A	*RAS1 RAS2*	1.5
-2B	*RAS1 RAS2*	1.8
-2C	*RAS1* $RAS2^{val19}$	8.3
-2D	*RAS1* $RAS2^{val19}$	6.1
-3A	*RAS1* $RAS2^{val19}$	10.1
-3B	*RAS1 RAS2*	2.0
-3C	*RAS1* $RAS2^{val19}$	5.9
-3D	*RAS1 RAS2*	1.9
-5A	*RAS1 RAS2*	2.0
-5B	*RAS1* $RAS2^{val19}$	7.6
-5C	*RAS1* $RAS2^{val19}$	9.4
-5D	*RAS1 RAS2*	1.5
-7A	*RAS1* $RAS2^{val19}$	7.9
-7B	*RAS1 RAS2*	1.4
-7C	*RAS1 RAS2*	1.9
-7D	*RAS1* $RAS2^{val19}$	7.6
T3-28C	*RAS1 RAS2*	2.4
-28B	*RAS1* $ras2^-$	0.4
-28D	$ras1^-$ *RAS2*	1.5
-35A	*RAS1 RAS2*	2.4
-35D	*RAS1* $ras2^-$	0.6
-35C	$ras1^-$ *RAS2*	1.1
T-58B	*RAS1 RAS2 bcy1*	2.0
T16-11A	*RAS1 RAS2 bcy1*	1.9
T26-19C	$ras1^-$ $ras2^-$ *bcy1*	<0.1
T27-10D	$ras1^-$ $ras2^-$ *bcy1*	<0.1

[a] The T41 strains are segregants from the cross of K382-19D with TK161-R2V (see Table 2). The T3 strains are segregants from KPPK-1. See Table 3 for a description of the other strains.
[b] Intracellular cyclic AMP was measured as described previously (Uno et al., 1981).

which overproduce cyclic AMP (Uno et al., 1982). $RAS2^{val19}$ strains also appear to have a similar biochemical defect since extracts of cells carrying $RAS2^{val19}$, like *bcy1* and *IAC* strains, have elevated trehalase activities which cannot be further elevated by treatment with cyclic AMP and ATP. Moreover, the *bcy1* mutation is a suppressor of the lethal effects disrupting both *RAS1* and *RAS2* genes.

There are several hypotheses consistent with these observations. The simplest, and perhaps the most attractive, is that the *RAS* proteins, directly or indirectly, modulate adenylate cyclase activity. Yeast *RAS* proteins, like their mammalian counterparts, are GTP binding proteins (Tamanoi et al., 1984) and, in vertebrate systems, GTP binding proteins modulate adenylate cyclase (Gilman, 1984). Moreover, yeast adenylate cyclase activity is stimulated by GTP in the presence of magnesium (Casperson et al., 1983). We therefore directly tested the hypothesis that *RAS* proteins modulate cyclic AMP levels and adenylate cyclase activity. Measurements of cyclic AMP levels in mutant strains clearly indicate that at least one intact *RAS* gene is required to sustain measurable levels of cyclic AMP, and that $RAS2^{val19}$ strains have significantly elevated cyclic AMP levels. Analysis of the adenylate cyclase activity of membranes prepared from wild-type and mutant cells clearly indicates that $ras1^-$ $ras2^-$ *bcy1* yeast cells lack components needed for GTP stimulation of adenylate cyclase. The mixing of membranes from *RAS1 RAS2 cyr1*-1 strains with membranes from $ras1^-$ $ras2^-$ *bcy1* strains restores a GTP-responsive adenylate cyclase. Work in progress (Dan Broek) indicates that the addition of purified *RAS* proteins restores measurable adenylate cyclase activity to $ras1^-$ $ras2^-$ cell membranes. Curiously, membranes from $RAS2^{val19}$ strains have high levels of adenylate cyclase when assayed in the presence of magnesium, and this level is not further increased by incubation with GTP. This finding indicates a clear biochemical difference between wild-type and $RAS2^{val19}$ cells. The molecular basis for this difference is under continued study, and may be related to the observation that H-ras^{gly12} but not H-ras^{val12} has significant GTPase activity (Sweet et al., 1984; McGrath et al., 1984).

In the accompanying paper (Kataoka et al., 1985), we demonstrate that expression of the human H-*ras* gene can complement yeast lacking functional endogenous *RAS*

Table 8. Adenylate Cyclase Activity in Membranes

Strain		Assay Conditions		
Experiment 1[a]	Genotype[b]	Mn^{++}	Mg^{++}	Mg^{++}, Gpp(NH)p
SP1	*RAS1 RAS2*	49.2	3.7	14.3
TK161-R2V	*RAS1* $RAS2^{val19}$	51.9	14.2	16.1
T27-10D	$ras1^-$ $ras2^-$ *bcy1*	36.8	0.4	0.4
Experiment 2[c]				
T27-10D	$ras1^-$ $ras2^-$ *bcy1*	51.0	1.1	0.7
AM18-5C	*RAS1 RAS2 cyr1*-1	0.3	0.1	0.2
T27-10D + AM18-5C		29.5	2.3	23.5

[a] Membranes from the indicated strains were prepared and adenylate cyclase was assayed as described in Experimental Procedures. Membranes were assayed either in the presence of 2.5 mM Mn^{++} or 2.5 mM Mg^{++} or 2.5 mM Mg^{++} and 10 μM Gpp(NH)p. Adenylate cyclase activity is expressed in units of picomoles of cAMP generated per mg of membrane protein per minute. Essentially identical results were obtained in three independent experiments.
[b] The full genotypes of the indicated strains are given in Table 3.
[c] In this experiment membranes from the indicated strains were incubated either alone or together for 2 hr at 0°C in 25 mM MES (pH 6.2), 1 mM ATP, 0.06% Lubrol with or without 30 μM Gpp(NH)p. They were diluted as described in Experimental Procedures such that the final Lubrol concentration was 0.01%. Membranes were then incubated at 15°C for 60 min and assayed as before. Essentially identical results were obtained in three independent experiments.

genes. From this we have concluded that there has been conservation of the immediate biochemical function of *ras* during evolution. If the yeast *RAS* proteins interact directly with components of the cyclic AMP effector pathway, perhaps so do the mammalian *ras* proteins. If this is true, the task of understanding tumorigenic transformation by these proteins rests on an understanding of the cyclic AMP-dependent protein kinase which is believed to mediate most of the effects of cyclic AMP. While there is solid evidence that cyclic AMP does have a role in regulating cellular proliferation in mammalian cells (Green, 1978; Rozengurt, 1981), this role will inevitably depend on the proteins that are available for phosphorylation by the cyclic AMP-dependent protein kinase, and on how the cell is programmed to respond to these proteins. Thus, the effect of *ras* on cells may be under the control of the differentiated state of the cell, which, in turn, could be under the control of yet other oncogenes.

On the other hand, we have not demonstrated that *ras* proteins interact directly with adenylate cyclase, and we do not know the mechanism by which it modulates adenylate cyclase. There still remains considerable room for speculation on the normal function of *ras* protein in vertebrate cells.

Experimental Procedures

DNA

All plasmid DNAs used in these studies were described previously (Kataoka et al. 1984). Plasmid and yeast DNAs were prepared essentially as described by others (Tanaka and Weisblum, 1975; Struhl et al., 1979). DNA restriction endonucleases, polymerases, ligases, and kinases were used under conditions recommended by suppliers (New England Biolabs or Bethesda Research Labs). Nitrocellulose filter blot hybridizations were performed as described by Southern (1975). Filters were hybridized with appropriate DNA fragments ^{32}P-labeled by nick translation (Maniatis et al., 1975) or with oligonucleotides labeled by polynucleotide kinase and [$\gamma^{32}P$]ATP. The oligonucleotide 5′ AACACCAA-CACCACC was prepared and hybridized under conditions described previously (Fasano et al., 1984).

Cells

Liquid culture media used were: 1% Bacto-yeast extract, 2% Bacto-peptone, and 2% Dextrose (YPD); 0.67% yeast nitrogen base without amino acids (Difco) and 2% Dextrose (SD); 1% Bacto-yeast extract, 2% Bacto-peptone, and 1% potassium acetate (YPA); 1% potassium acetate (SPO); and 0.17% yeast nitrogen base without amino acids and without ammonium sulfate (Difco) and with 2% dextrose (YNB-N). SD medium was supplemented with auxotrophic requirements as needed to 80 μg/ml. Solid medium contained 2% agar (Difco) and composition described above except solid sporulation medium (SPO) contained in addition 0.25% Bacto-peptone, 2% potassium acetate, and 0.05% dextrose. General genetic manipulations of yeast were carried out essentially as described by Mortimer and Hawthorne (1969). Yeast transformations were performed according to Beggs (1978). Cell derivations are given in Table 3. Nomenclature for genotypes follows standard conventions: dominant alleles are in capital letters, recessive alleles are lower case, and genetic marker X integrated at loci Y is designated Y::X.

Cyclic AMP and Adenylate Cyclase Assays

Intracellular cyclic AMP levels were determined as previously described (Uno et al., 1981). Membrane extracts were prepared and stored as described (Casperson et al., 1983) with the following modification. Spheroplasts (from one liter cultures of mid log phase cells (1–2 × 10^7/ml) grown in YPD) were lysed after resuspending in 10–20 ml 50 mM MES (pH 6.2), 0.1 mM $MgCl_2$, 0.1 mM EGTA, and 2 mM PMSF by four strokes of a tightly fitted dounce homogenizer. Adenylate cyclase activity was assayed as described previously (Casperson et al., 1983) and ^{32}P-cAMP produced was determined as described previously (Solomon et al., 1973). For membrane mixing experiments, membranes from the desired strains were diluted to a volume of 30 μl and to a final concentration of 2 mg/ml in a solution consisting of 25 mM MES (pH 6.2), 1 mM ATP, 0.06% Lubrol, 2 mM β-mercaptoethanol, with or without 30 μM Gpp(NH)p. These membranes were then mixed and incubated at 0°C for 2 hr. After this, 60 μl of 25 mM MES (pH 6.2), 1 mM ATP, 4.2 mM $MgCl_2$, 16 mM theophiline, 33 U/ml creatine phosphokinase, 33 mM creatine phosphate was added and the membrane mixtures were incubated at 15°C for 1 hr. The reaction was initiated by addition of 10 μl of a solution containing [$\alpha^{32}P$] ATP (to a final 20–150 cpm/pmol), 10 mM cAMP [^3H], and 15,000 cpm [^3H] cAMP/per reaction. Production of ^{32}P-cAMP was assayed as above.

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