Copyright © 1989 by the Genetics Society of America

Isolation and Characterization of Temperature-Sensitive Mutations in the *RAS2* and *CYR1* Genes of *Saccharomyces cerevisiae*

Hiroshi Mitsuzawa,* Isao Uno,* Takehiro Oshima[†] and Tatsuo Ishikawa*

**Institute of Applied Microbiology, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan, and* [†]*Suntory Bio Pharma Tech Center, Chiyoda-machi, Oura-gun, Gunma 370-05, Japan*

Manuscript received August 31, 1988
Accepted for publication September 9, 1989

ABSTRACT

The yeast *Saccharomyces cerevisiae* contains two *ras* homologues, *RAS1* and *RAS2*, whose products have been shown to modulate the activity of adenylate cyclase encoded by the *CYR1* gene. To isolate temperature-sensitive mutations in the *RAS2* gene, we constructed a plasmid carrying a *RAS2* gene whose expression is under the control of the galactose-inducible *GAL1* promoter. A *ras1* strain transformed with this plasmid was subjected to ethyl methanesulfonate mutagenesis and nystatin enrichment. Screening of approximately 13,000 mutagenized colonies for galactose-dependent growth at a high temperature (37°) yielded six temperature-sensitive *ras2* (*ras2[ts]*) mutations and one temperature-sensitive *cyr1* (*cyr1[ts]*) mutation that can be suppressed by overexpression or increased dosage of *RAS2*. Some *ras2[ts]* mutations were shown to be suppressed by an extra copy of *CYR1*. Therefore increased dosage of either *RAS2* or *CYR1* can suppress the temperature sensitivity caused by a mutation in the other. *ras1 ras2[ts]* and *ras1 cyr1[ts]* mutants arrested in the G_1 phase of the cell cycle at the restrictive temperature, and showed pleiotropic phenotypes to varying degrees even at a temperature permissive for growth (25°), including slow growth, sporulation on rich media, increased accumulation of glycogen, impaired growth on nonfermentable carbon sources, heat-shock resistance, impaired growth on low concentrations of glucose, and lithium sensitivity. Of these, impaired growth on low concentrations of glucose and sensitivity to lithium are new phenotypes, which have not been reported for mutants defective in the cAMP pathway.

THE roles of adenosine 3′,5′-monophosphate (cAMP) in the yeast *Saccharomyces cerevisiae* have been studied through the isolation and characterization of the mutations in the *CYR1* and *BCY1* genes, which encode adenylate cyclase and the regulatory subunit of cAMP-dependent protein kinase, respectively (reviewed by MATSUMOTO, UNO and ISHIKAWA 1985). The *cyr1* mutations cause the cell-cycle arrest in the G_1 phase under the restrictive conditions, and one of the *cyr1* mutations (*cyr1-2*) causes sporulation on rich media. The *bcy1* mutation, which was originally isolated as a suppressor of *cyr1-1*, results in inability to sporulate. Therefore cAMP acts as a positive regulator at the "start" of the cell division cycle and as a negative regulator of sporulation in diploids.

S. cerevisiae contains two genes, *RAS1* and *RAS2*, which are structurally and functionally homologous to the mammalian *ras* oncogenes (DEFEO-JONES *et al.* 1983, 1985; POWERS *et al.* 1984; KATAOKA *et al.* 1985). At least one functional *RAS* gene is required for spore germination and vegetative growth (KATAOKA *et al.* 1984, 1985; TATCHELL *et al.* 1984). Genetic and biochemical results have shown that the yeast *RAS* products stimulate adenylate cyclase activity (TODA *et al.* 1985). The activity of the *RAS* proteins is likely to be controlled by the product of the *CDC25* gene (BROEK *et al.* 1987; ROBINSON *et al.* 1987). The phenotypes of mutants in the cAMP pathway suggest that the activity of adenylate cyclase is modulated through the *RAS* proteins in response to nutritional conditions, although the nature of signals mediated by this pathway is still unknown.

Temperature-sensitive mutations are powerful tools for analyzing the functional roles of the a gene of interest. Furthermore, temperature-sensitive mutants provide hosts for the isolation of extragenic suppressors. A temperature-sensitive mutation in the *CYR1* gene has been isolated and characterized (MATSUMOTO, UNO and ISHIKAWA 1984). One of the previously isolated cell-division-cycle mutations, *cdc35*, has been shown to be allelic to *cyr1* (CASPERSON, WALKER and BOURNE 1985; BOUTELET, PETITJEAN and HILGER 1985). DE VENDITTIS *et al.* (1986) have reported the isolation and characterization of a temperature-sensitive mutation in the *RAS2* gene. Other groups have also used temperature-sensitive *ras2* mutants to study the cAMP pathway (TODA *et al.* 1987; NAKAFUKU *et al.* 1988).

In this paper we describe an efficient method for

The publication costs of this article were partly defrayed by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

TABLE 1

***S. cerevisiae* strains**

Strain[a]	Genotype or cross[b]
AM203-1B	*MATα his7 bcy1*
AM248-5B	*MAT***a** *his7 cyr1-2*
K393-35C	*MATα spo11 ura3 his2 leu1 lys1 met4 pet8*
K396-11A	*MAT***a** *spo11 ura3 ade1 his1 leu2 lys7 met3 trp5*
T3-28D	*MATα leu2 his3 ura3 trp1 ras1::HIS3*
T3-35D	*MATα leu2 his3 ura3 trp1 ade8 ras2::URA3*
T26-19C	*MATα leu2 his3 trp1 can1 bcy1 ras1::HIS3 ras2::LEU2*
HM3-6B	*MAT***a** *leu2 his3 ura3 met3 ras2::URA3*
HM6-8B	*MAT***a** *leu2 his3 ura3 met3*
HM21-10B	*MAT***a** *leu2 his3 ura3 trp1 ade8 met4 ras1::HIS3*
HM29-7B	*MAT***a** *leu2 his3 ura3 trp1 ade8 ras1::HIS3 ras2-125*
HM38-1A	*MATα leu2 his3 ura3 trp1 cyr1-230*
HM39-8D	*MAT***a** *leu2 his3 ura3 trp1 ade8 ras1::HIS3 ras2-125*
HM54-14A	*MAT***a** *leu2 his3 ura3 trp1 ras1::HIS3*
HM54-14B	*MATα leu2 his3 ura3 trp1 ade8 ras1::HIS3 ras2-125*
HM54-14C	*MATα leu2 his3 ura3 trp1 ade8 ras1::HIS3*
HM54-14D	*MAT***a** *leu2 his3 ura3 trp1 ras1::HIS3 ras2-125*
HM55-6A	*MATα leu2 his3 ura3 trp1 ras1::HIS3 ras2-23*
HM55-6B	*MAT***a** *leu2 his3 ura3 trp1 ade8 ras1::HIS3 ras2-23*
HM56-1A	*MAT***a** *leu2 his3 ura3 trp1 ade8 ras1::HIS3 ras2-47*
HM56-1C	*MATα leu2 his3 ura3 trp1 ras1::HIS3 ras2-47*
HM57-2B	*MATα leu2 his3 ura3 trp1 ras1::HIS3 cyr1-230*
HM57-2C	*MAT***a** *leu2 his3 ura3 trp1 met4 ras1::HIS3 cyr1-230*
HM58-1D	*MATα leu2 his3 ura3 trp1 ade8 cyr1-2 (his7?)*
HM65-24D	*MAT***a** *leu2 his3 ura3 met3 ras1::HIS3*
HM67-2C	*MATα leu2 his3 ura3 trp1 ras1::HIS3 ras2-35*
HM67-3C	*MAT***a** *leu2 his3 ura3 trp1 ade8 ras1::HIS3 ras2-35*
HM25	T3-28D × HM3-6B
HM32	2-30 × HM29-7B
HM54	T3-28D × HM39-8D
HM66	T3-35D × HM65-24D
HM68	HM54-14A × HM54-14C
HM69	HM54-14A × HM54-14B
HM70	HM54-14B × HM54-14D
HM71	HM55-6A × HM55-6B
HM72	HM67-2C × HM67-3C
HM73	HM56-1A × HM56-1C
HM74	HM57-2B × HM57-2C

[a] Source or reference is as follows: AM203-1B and AM248-5B, K. MATSUMOTO; K393-35C and K396-11A KLAPHOLZ and ESPOSITO (1982); T3-28D, T3-35D and T26-19C, TODA *et al.* (1985). All other strains were constructed during the course of this study.

[b] For diploids, haploid parents are shown.

isolating temperature-sensitive mutations in the *RAS2* gene. We recovered not only $ras2^{ts}$ mutations but also a $cyr1^{ts}$ mutation that can be suppressed by overexpression of *RAS2*; *ras1* $ras2^{ts}$ and *ras1* $cyr1^{ts}$ mutants with the same genetic background have become available as a result. We show that increased dosage of either *RAS2* or *CYR1* can suppress the temperature sensitivity caused by a mutation in the other. We also show that *ras1* $ras2^{ts}$ and *ras1* $cyr1^{ts}$ mutants have pleiotropic phenotypes even at the permissive temperature.

MATERIALS AND METHODS

Yeast strains: The yeast strains used in this study are listed in Table 1. K393-35C and K396-11A were used as sources of *met4* and *met3*, respectively. In this paper *ras1* and *ras2* represent null alleles constructed by inserting marker genes into the coding regions (KATAOKA *et al.* 1984). A *bcy1* mutant, 10-9, was isolated and identified as follows. Temperature-resistant revertants of a *ras1 cyr1-230* strain (HM57-2C) were screened for heat-shock sensitivity. The heat-shock sensitivity in a revertant (10-9) segregated 2:2 in crosses and was complemented by a *BCY1* plasmid (pSY2-1). Moreover, when crossed to a *bcy1* tester strain (AM203-1B), the revertant showed sporulation defect characteristic of *bcy1* diploid cells. The *bcy1* allele in the revertant is designated *bcy1-109*.

Media: Rich media contained 1% yeast extract, 2% polypepton and 2% glucose (YPD), 2% galactose (YPGal), 2% glycerol, 2% potassium acetate, 2% ethanol or 0.1% glucose. Synthetic medium contained 0.67% yeast nitrogen base without amino acids, 2% glucose and the supplements to satisfy auxotrophic requirements. Presporulation medium (YPA) contained 1% yeast extract, 2% polypepton and 1% potassium acetate. Sporulation medium (SPO) contained 1% potassium acetate. Solid media contained 2% agar.

Genetic methods and transformation. Standard yeast genetic procedures of crossing, sporulation and tetrad analysis were followed (SHERMAN, FINK and HICKS 1983). Diploids were isolated by prototrophic selection if possible. When prototrophic selection could not be employed, diploids were identified by testing for ability to sporulate after single colony isolation. To test for anaerobic growth on glucose, a GasPak disposable anaerobic system (BBL) was used. Ability to grow on various media was scored by spotting cell suspensions with a multipronged inoculating device. Conventional replica-plating procedure with velvet cloth was used for the primary screening of mutants. Yeast was transformed by the lithium acetate method (ITO *et al.* 1983).

Plasmids: Plasmid YCpLeCYR1 carries the entire coding region of *CYR1* (UNO *et al.* 1987). YCpLeGAPCYR1-ScaI(a) carries a truncated *CYR1* gene that encodes the C-terminal 432 amino acids of adenylate cyclase; the product has the catalytic activity independent of the *RAS* proteins (UNO *et al.* 1987). Plasmid pSY2-1, which was kindly provided by A. TOH-E, is a YCp19 derivative carrying the *BCY1* gene (YAMANO *et al.* 1987).

A 7.2-kb *Sal*I-*Bam*HI fragment containing the *GAL1* gene (ST. JOHN and DAVIS 1981) was isolated (T. OSHIMA, unpublished results). A 0.9-kb *Eco*RI-*Ava*I fragment containing the *GAL1* promoter was subcloned into pUC9. The *Eco*RI site was converted to a *Bam*HI site by addition of synthetic linkers, and an *Eco*RI site was constructed immediately before the *GAL1* translation initiation site by oligonucleotide-directed mutagenesis (MORINAGA *et al.* 1984). The plasmid was digested with *Bam*HI and *Eco*RI, and the resulting 0.8-kb *Bam*HI-*Eco*RI fragment was used as *GAL1* promoter. The *GAL1* promoter was fused to the *lacZ* gene of *Escherichia coli* to assess the promoter function by assaying for β-galactosidase. No detectable level of β-galactosidase activity was observed in cells carrying a *GAL1-lacZ* fusion plasmid when grown in YPD medium, whereas when grown in YPGal medium cells carrying the *GAL1-lacZ* fusion plasmid showed a 50-fold higher level of activity than did cells carrying a *RAS2-lacZ* fusion plasmid (data not shown). Plasmid placRAS2 (UNO *et al.* 1985) was digested with *Hin*dIII, treated with the Klenow fragment of *E. coli* DNA polymerase I to fill-in the cohesive ends, and then digested with *Eco*RI. The resulting 1.2-kb *Eco*RI-*Hin*dIII (filled-in) fragment containing a promoter-lacking *RAS2* gene and the 0.8-kb *Bam*HI-*Eco*RI *GAL1* promoter fragment described above were inserted into *Bam*HI-*Sal*I(filled-in) sites of YCpLe (UNO *et al.* 1987), yielding YCpLeGAL1RAS2. YEp13RAS2

was constructed by inserting a 3.0-kb *Eco*RI(filled-in)-*Hin*dIII fragment containing the *RAS2* gene (UNO *et al.* 1985) into *Bam*HI(filled-in)-*Hin*dIII sites of YEp13. YCp19RAS2 was constructed by inserting a 4.7-kb *Pvu*II-*Hin*dIII fragment containing the *RAS2* gene from YEp13RAS2 into *Pvu*II-*Hin*dIII sites of YCp19. Standard methods for recombinant DNA manipulation were followed (MANIATIS, FRITSCH and SAMBROOK 1982).

Isolation of mutants: Strain T3-28D transformed with YCpLeGAL1RAS2 was grown to stationary phase in synthetic medium lacking leucine at 30°. The cells were collected by centrifugation, resuspended in 0.1 M sodium phosphate buffer (pH 7.0), and treated with 3% ethyl methanesulfonate (EMS) at 30° for 60 min; 30–40% of the cells survived this treatment. To assure independent isolation of mutants, four single colonies of the parent strain were mutagenized independently. A portion of the suspension was then diluted 10-fold into 5% sodium thiosulfate to quench the reaction. These mutagenized cells were washed three times, inoculated into YPD medium at a density of about 4×10^7 cells/ml, and incubated at 25° for 2 days. A portion of the culture was diluted 50-fold into YPD medium again and incubated at 37° for 5 hr. The cells were then treated with 10 μg/ml nystatin (SNOW 1966) at 37° for 90 min; the frequency of survivors was about 5×10^{-5}. The cells were washed, plated on YPD, and incubated at 25° for 4 days. The colonies on each of the YPD plates were replica-plated to two YPD plates and one YPGal plate, and one YPD plate was incubated at 25° and the other YPD and the YPGal plates were at 37°. We picked colonies that showed galactose-dependent growth at 37°, *i.e.* colonies unable to grow on YPD at 37° but able to grow on YPD at 25° and on YPGal at 37°. These colonies were retested for galactose-dependent growth at 37° and tested for growth on synthetic medium lacking leucine at 25° by spotting cell suspensions. Potential *ras2^ts* mutants were tested for cosegregation of the galactose-dependent growth with the plasmid after growth without selection for the plasmid. Those mutants which showed galactose-dependent growth only in the presence of the plasmid were considered putative *ras2^ts* mutants. Mitotic segregants that had lost the plasmid were used in the subsequent experiments.

Heat shock experiments: For quantitative measurement of thermotolerance, cells growing exponentially in YPD at 25° (about 5×10^6 cells/ml) were exposed to 52° for 4 min in a water bath, plated on YPD after appropriate dilution, and incubated at 25° for 3–5 days. Percentage of survivors was determined by dividing by the number of colonies formed from a culture that was not subjected to heat shock. For qualitative measurements, cells spotted on YPD plates were incubated at 25° for 6 hr, exposed to 57° for 4–12 min on a water bath, and incubated at 25° for 2–5 days.

Glycogen measurement: Relative glycogen levels were measured by the iodine-staining method with a solution of 0.2% I_2 and 0.4% KI. This method stained colonies brown to varying degrees depending on the amounts of glycogen accumulated.

cAMP assay: Cells grown to early stationary phase in YPD at 25° were collected, resuspended in 5% trichloroacetic acid (TCA), and incubated at 4° for 1 hr. The TCA extract was used for cAMP assay after extraction with diethyl ether and neutralization with 0.5 M Tris-HCl (pH 7.5). cAMP was determined with the Amersham cAMP assay kit. The cells were boiled in 0.2 N NaOH for 10 min and then protein was determined with the Bio-Rad protein assay kit.

Adenylate cyclase assay: Yeast membrane extracts were prepared from cultures of cells grown to early stationary phase in YPD at 25° as described previously (MATSUMOTO, UNO and ISHIKAWA 1984), except that $MnCl_2$ was omitted from the buffers. Adenylate cyclase was assayed at 25° in the presence of either 5 mM $MnCl_2$ or 5 mM $MgCl_2$ with or without 0.1 mM Gpp(NH)p.

RESULTS

Strategy of mutant isolation: Yeast contains two *RAS* genes. *RAS1* and *RAS2*. Cells carrying a null allele of either gene are viable, although cells carrying null alleles of both genes are inviable. Thus a temperature-sensitive mutation in the *RAS2* gene would not cause temperature sensitivity for growth in the presence of *RAS1*. We therefore chose a strain carrying a null allele of *RAS1* as parent for mutant isolation. The *ras1* strain was transformed with a plasmid carrying a *RAS2* gene whose expression is under the control of the *GAL1* promoter, yielding a strain that carries a disrupted chromosomal copy of *RAS1* and two copies of *RAS2*, an intact chromosomal copy and a plasmid-borne copy whose expression is galactose-inducible. If a temperature-sensitive mutation occurred in the chromosomal copy of *RAS2*, the strain would be unable to grow on glucose-containing medium (YPD) at 37° but able to grow on galactose-containing medium (YPGal) at 37°. We thus expected to obtain *ras2^ts* mutants by screening mutagenized colonies for galactose-dependent growth at 37°. The procedure employed is depicted schematically in Figure 1.

Characterization of *GAL1-RAS2* plasmid: Plasmid YCpLeGAL1RAS2, on which the *RAS2* gene is fused to the *GAL1* promoter, was constructed. To determine whether the expression of the *RAS2* gene on the plasmid is galactose-inducible *in vivo*, a diploid heterozygous for *ras1* and *ras2* (HM25) was transformed with the plasmid, and then sporulated and subjected to tetrad analysis in which asci were dissected on YPD or YPGal. No viable *ras1 ras2* spores were recovered on YPD in 49 tetrads examined, although the viability of the spores of the other genotypes (*RAS1 RAS2*, *ras1 RAS2* and *RAS1 ras2*) was high (97%). In contrast, about half of *ras1 ras2* spores (28 out of 58 in 53 tetrads) were viable on YPGal, and all the viable *ras1 ras2* spores carried the *GAL1-RAS2* plasmid. When these *ras1 ras2* spores were transferred from YPGal to YPD, the cells arrested as unbudded cells after heavy residual growth. Thus the *GAL1-RAS2* plasmid could complement the *ras1 ras2* mutation in a galactose-dependent manner. We conclude that the expression of the *RAS2* gene on the plasmid is galactose-inducible *in vivo*. Since the expression of the *RAS2* gene under the control of the *GAL1* promoter was presumed to be higher than that of the normal *RAS2* gene (see MATERIALS AND METHODS), we examined the effect of the expression of the *GAL1-RAS2* gene on cell growth at a range of temperatures. Comparison of growth rates between *ras1* strains and *ras1 ras2* strains carrying *GAL1-RAS2* revealed no difference

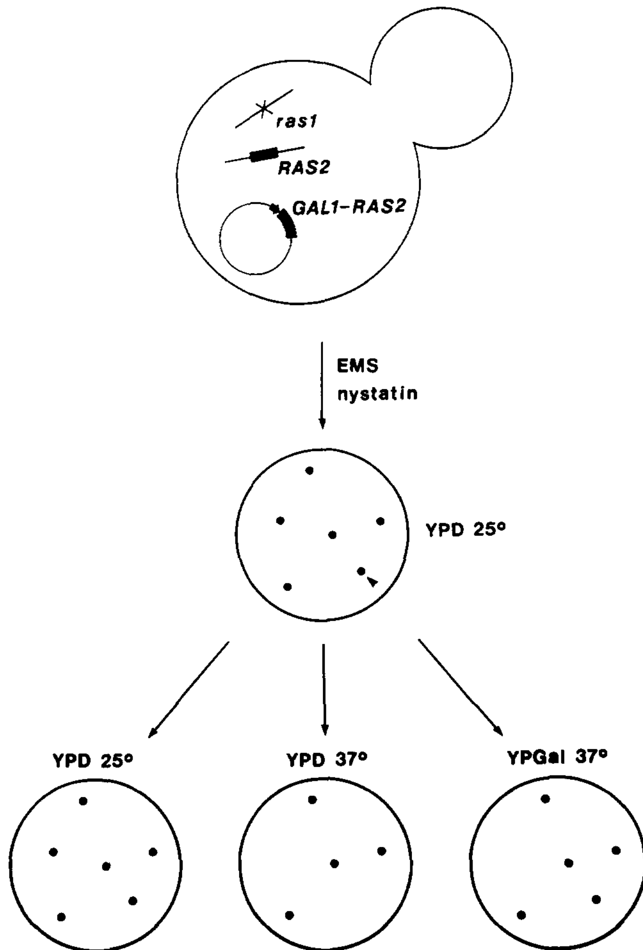

FIGURE 1.—Schematic representation of the mutant isolation strategy. A *ras1* strain carrying a *GAL1-RAS2* plasmid was subjected to EMS mutagenesis and nystatin enrichment and then screened for galactose-dependent growth at 37° by replica-plating. A potential *ras2*^{ts} mutant is indicated by an arrowhead. See text for details.

on YPGal at 25° and 30°; however, *ras1 ras2* strains carrying *GAL1-RAS2* grew more slowly than *ras1* strains at 37°.

Isolation of mutants: Cells of a *ras1* strain (T3-28D) transformed with the *GAL1-RAS2* plasmid were mutagenized with EMS. The mutagenized cells were then treated with nystatin to enrich *ras2*^{ts} mutants, because *ras2*^{ts} mutants would not continue growth under the restrictive condition and would thus be resistant to nystatin. About 17% of the replica-plated colonies were temperature sensitive for growth on YPD, indicating that the nystatin treatment was effective in enrichment of temperature-sensitive mutants. Screening of approximately 13,000 mutagenized colonies yielded seven putative *ras2*^{ts} mutants, which showed galactose-dependent growth at 37° only when the *GAL1-RAS2* plasmid was present. We also recovered two mutants that showed galactose-dependent growth at 37° even in the absence of the plasmid. These mutants may be defective in glycolytic pathway or glucose uptake. They were not studied further. We

TABLE 2

Complementation analysis

Allele	Original isolate[a]	Complementation[b]: *ras1 ras2-125*	*ras1 cyr1-230*	*ras1 ras2 bcy1*	*cyr1-2*
ras1 ras2-125	1-25	−	+	−	ND
ras1 ras2-23	2-3	−	+	−	ND
ras1 ras2-237	2-37	−	+	ND	ND
ras1 ras2-35	3-5	−	+	−	ND
ras1 ras2-47	4-7	−	+	−	ND
ras1 ras2-48	4-8	−	+	ND	ND
ras1 cyr1-230	2-30	+	−	+	−

[a] The number to the left of the hyphen refers to the single colony from which the mutant was derived. Thus mutants with different numbers are independent.

[b] Diploids were constructed and tested for growth on YPD at 37°. ND, not determined.

also attempted to isolate cold-sensitive *ras2* mutations by screening colonies grown on YPD at 30° for galactose-dependent growth at 17°; however, no cold-sensitive *ras2* mutations were recovered among approximately 12,000 colonies screened. The *RAS2* gene may not readily mutate to cold-sensitive alleles.

Identification of mutants: Each putative *ras2*^{ts} mutant was crossed to a *ras1 met4* strain (HM21-10B), yielding a diploid homozygous for *ras1* and heterozygous for the temperature-sensitive mutation and *met4*, which is tightly linked to *ras2* (KATAOKA *et al.* 1984). In each case the diploid was able to grow on YPD at 37°, indicating that the temperature sensitivity of the mutants is recessive. These diploids were sporulated and subjected to tetrad analysis. In each case temperature sensitivity segregated 2+:2−, indicating that this defect is due to a single nuclear mutation. Of the seven mutants, six (mutants 1-25, 2-3, 2-37, 3-5, 4-7 and 4-8) carried a mutation linked to *met4* (in the case of 1-25, for example, parental ditype (PD)/nonparental ditype (NPD)/tetratype (T); 24:0:2), suggesting that they are *ras2*^{ts} mutants; one (mutant 2-30) carried a mutation unlinked to *met4* (PD/NPD/T; 2:1:9), suggesting that the temperature sensitivity is due to a mutation in a gene other than *RAS2* and can be suppressed by overexpression of *RAS2*. Complementation analysis showed that the six mutants fall into the same complementation group and the mutant 2-30 defines another group, as expected (Table 2).

The mutations were tested for ability to complement a *ras2* null allele by crossing mutants to a *ras1 ras2 bcy1* strain (T26-19C); if a mutation was in *RAS2*, the resulting diploid would be unable to grow on YPD at 37° because *bcy1* is recessive. This was the case for the mutations linked to *met4*; these mutations failed to complement the *ras2* null allele (Table 2). Moreover, the temperature sensitivity of the mutant 1-25 was complemented by *RAS2* on a centromere plasmid,

YCp19RAS2. We conclude that the temperature-sensitive mutations linked to *met4* lie in the *RAS2* gene. Of the six *ras2*^{ts} mutations, four independent mutations (*ras2-125*, *ras2-23*, *ras2-35* and *ras2-47*) were studied further. On the other hand, the mutation unlinked to *met4* complemented the *ras2* null allele (Table 2). However, this mutation was found to fail to complement *cyr1-2* (Table 2), which is the previously isolated temperature-sensitive *cyr1* mutation (MATSUMOTO, UNO and ISHIKAWA 1984), indicating that the mutant 2-30 carries a mutation in the *CYR1* gene. Two observations confirm this conclusion. First, the mutation was tightly linked to *met3* (PD/NPD/T; 37:0:3); the previous mapping data had shown the tight linkage of *cyr1* to *met3* (BOUTELET, PETITJEAN and HILGER 1985; CANNON, GIBBS and TATCHELL 1986; MARSHALL *et al.* 1988). Second, the temperature sensitivity caused by the mutation was complemented by *CYR1* on a centromere plasmid, YCpLe-CYR1. The mutant 2-30 was crossed to a *RAS1* strain (HM6-8B). Tetrad analysis of the resulting diploid showed 2:2 segregation for temperature sensitivity, indicating, in conjunction with the complementation of the temperature sensitivity by *CYR1*, that this phenotype is independent of the *RAS1* genotype. Tetrad analysis of a diploid heterozygous for *ras2-125* and *cyr1-230* (HM32) showed that *ras1 ras2-125 cyr1-230* triple mutants are inviable.

Suppression of a temperature-sensitive mutation in either *RAS2* or *CYR1* by increased dosage of the other: As mentioned above, the temperature sensitivity caused by the *cyr1-230* mutation is expected to be suppressed by overexpression of *RAS2*. We thus examined a high or low copy number plasmid carrying *RAS2* for ability to suppress the temperature sensitivity caused by the *cyr1-230* mutation. As expected, the temperature sensitivity of a *cyr1-230* strain (HM38-1A) was suppressed by a high copy number plasmid, YEp13RAS2, although the *cyr1-230* strain carrying YEp13RAS2 grew more slowly at 37° than the *cyr1-230* strain carrying YCpLeCYR1. In contrast, YCp19RAS2 did not suppress the temperature sensitivity of the *cyr1-230* strain. To determine whether *cyr1-2* is suppressed by increased dosage of *RAS2*, a *cyr1-2* strain (HM58-1D), which was constructed by three serial backcrosses, was transformed with YEp13RAS2 or YCp19RAS2. The temperature sensitivity caused by *cyr1-2* was not suppressed by *RAS2* even in high copy number.

We examined whether the temperature sensitivity of *ras1 ras2*^{ts} mutants was suppressed by *CYR1*. The results are shown in Table 3. A truncated *CYR1* gene (*CYR1-ScaI*) that can suppress the lethality of *ras1 ras2* mutants (UNO *et al.* 1987) also suppressed the temperature sensitivity of the *ras1 ras2*^{ts} mutants, as expected. Surprisingly, one extra copy of *CYR1* on a centromere plasmid suppressed the temperature sensitivity of the *ras1 ras2-125*, *ras1 ras2-23* and *ras1 ras2-35* mutants. Interestingly, the same plasmid resulted in less efficient suppression in the homozygous diploids. The dosage of the wild-type *CYR1* allele and a *ras2*^{ts} allele is probably crucial for suppression. The lethality of *ras1 ras2* mutants is not suppressed by an extra copy of *CYR1* (UNO *et al.* 1987). Therefore suppression of the *ras2*^{ts} mutations by an extra copy of *CYR1* must rely upon the residual activity of the mutant *RAS2* protein at the restrictive temperature.

TABLE 3

Suppression of *ras2*^{ts} mutations by *CYR1* genes

Strain[a]	Relevant genotype[b]	Growth of transformant[c]		
		None	*CYR1-ScaI*	*CYR1*
HM54-14D	*ras2-125*	−	+	+
HM55-6B	*ras2-23*	−	+	+
HM67-3C	*ras2-35*	−	+	+
HM56-1A	*ras2-47*	−	+	−
HM70	*ras2-125/ras2-125*	−	+	±
HM71	*ras2-23/ras2-23*	−	+	±
HM72	*ras2-35/ras2-35*	−	+	−
HM73	*ras2-47/ras2-47*	−	+	−

[a] The strains used in this experiment were constructed as follows. The original isolates were crossed to HM21-10B, and segregants of these crosses were backcrossed two times to the parent strain T3-28D. From the resulting haploid strains, diploid strains were constructed. The same strains and strains constructed in the same manner were used in the subsequent experiments (Tables 4, 5, and 6).

[b] All haploid strains are *ras1* and all diploid strains are *ras1/ras1*.

[c] Cells of *ras1 ras2*^{ts} mutants carrying no plasmid (None), YCpLeGAPCYR1-ScaI(a) (*CYR1-ScaI*) or YCpLeCYR1 (*CYR1*) were spotted on YPD and incubated at 37° for 2 days.

Pleiotropic phenotypes of *ras1 ras2*^{ts} and *ras1 cyr1*^{ts} mutants: Table 4 shows the properties of diploids homozygous for *ras1* and either *ras2*^{ts} and *cyr1*^{ts}. The terminal phenotype of the mutants was examined by shifting exponentially growing cultures to the restrictive temperature. Both the *ras1 ras2*^{ts} and *ras1 cyr1*^{ts} mutants arrested as unbudded cells when shifted from 25° to 37°, indicating that the mutants arrested in the G_1 phase of the cell cycle. This result is consistent with the previous observations that the mutations either in *CYR1* or in *RAS1* and *RAS2* cause the G_1 arrest under the restrictive conditions (MATSUMOTO *et al.* 1982; MATSUMOTO, UNO and ISHIKAWA 1983a; KATAOKA *et al.* 1985; DE VENDITTIS *et al.* 1986). In all the mutants the number of cells increased less than twofold even after prolonged incubation at 37° (data not shown), indicating that cells arrested on the first cell cycle at the restrictive temperature. The increased fractions of unbudded cells at 25° in the *ras1 ras2-125*, *ras1 ras2-23* and *ras1 cyr1-230* mutants reflect the prolonged G_1 phases (see below).

A null mutation in *RAS2* causes several phenotypes, including sporulation on rich media in homozygous

TABLE 4

Properties of *ras1 ras2*^{ts} and *ras1 cyr1*^{ts} mutants

Strain	Relevant genotype[a]	Fraction of unbudded cells[b]		Doubling time[b] (hr)	Length of phase[c] (hr)		Sporulation efficiency[d] (%)			I_2-KI staining[e]	Growth on glycerol[f]	Survival after heat shock[g] (%)
		25°	37°		G_1	$S + G_2 + M$	YPD	YPA	SPO			
HM68	+	0.31	0.34	2.0	0.5	1.5	0.0	0.5	47.1	−	+	0.04
HM70	*ras2-125*	0.64	0.95	3.6	2.0	1.6	8.0	39.4	75.9	++	−	37.8
HM71	*ras2-23*	0.62	0.92	3.4	1.8	1.6	2.9	29.8	55.4	++	−	10.8
HM72	*ras2-35*	0.38	0.83	2.2	0.7	1.5	0.0	0.7	12.7	+	±	0.2
HM73	*ras2-47*	0.34	0.87	2.0	0.5	1.5	0.0	3.3	47.4	+	±	0.2
HM74	*cyr1-230*	0.54	0.92	3.0	1.4	1.6	0.0	1.2	58.2	+	±	28.8

[a] All strains are homozygous for the indicated allele and *ras1*.

[b] Cultures of cells growing exponentially in YPD at 25° (about 5 × 10^6 cells/ml) were divided into two parts. One was shifted to 37° and the fraction of unbudded cells was determined 4 hr after the temperature shift. The other was maintained at 25° and the fraction of unbudded cells (F) and the doubling time (D) were determined. At least 300 cells were counted by microscopy after fixation and brief sonication.

[c] The length of the G_1 phase (G_1) was calculated by the formula $G_1 = D(1 - \log(2-F)/\log 2)$ (Rivin and Fangman 1980). $S + M + G_2$ is the difference between D and G_1, and represents the combined length of the S, G_2 and M phases.

[d] Cells grown on YPD at 25° for 2 days were transferred to YPD, YPA and SPO plates. After 5 days at 25°, at least 200 cells and tetrads were counted by microscopy, and the percentage of sporulated cells was calculated. The average of three independent diploid isolates is given.

[e] For qualitative glycogen measurement, cells were grown on YPD at 25° for 5 days and stained with iodine solution: −, light brown; +, brown; ++, dark brown.

[f] Cells were spotted on rich medium containing 2% glycerol and incubated at 25° for 3 days.

[g] Cells growing exponentially in YPD at 25° were exposed to 52° for 4 min.

diploids, increased accumulation of storage carbohydrates and impaired growth on nonfermentable carbon sources (Toda *et al.* 1985; Tatchell, Robinson and Breitenbach 1985; Fraenkel 1985). The *cyr1-2* mutation causes sporulation on rich media and heat-shock resistance (Matsumoto, Uno and Ishikawa 1983b; Shin *et al.* 1987). We examined the phenotypes of the *ras1 ras2*^{ts} and *ras1 cyr1*^{ts} mutants at a temperature permissive for growth (Table 4). The *ras1 ras2*^{ts} mutants were divided into two groups on the basis of a set of phenotypes. The *ras1 ras2-125* and *ras1 ras2-23* mutants showed slow growth, sporulation on YPD, increased accumulation of glycogen, inability to grow on glycerol and heat-shock resistance. The increase in doubling time was due exclusively to the extension of the G_1 phase. These mutants sporulated efficiently on YPA. Inability of growth was observed on acetate and ethanol as well as glycerol. The *ras1 ras2-35* and *ras1 ras2-47* mutants showed neither slow growth nor sporulation on YPD, and showed less glycogen accumulation, growth defects on glycerol and heat-shock resistance than did the *ras1 ras2-125* and *ras1 ras2-23* mutants. The *ras1 cyr1-230* mutant also showed slow growth, increased accumulation of glycogen, impaired growth on glycerol and heat-shock resistance. For all the *ras2*^{ts} and *cyr1*^{ts} mutations, the same phenotypes except for sporulation on rich media were observed for haploid mutants. These phenotypes cosegregated completely with the temperature sensitivity for growth. A diploid homozygous for *ras1* and heterozygous for *ras2-125* (HM69) did not show slow growth, sporulation on YPD, increased accumulation of glycogen, inability to grow on glycerol and heat-shock resistance, indicating that *ras2-125* is recessive to the wild-type allele for all these phenotypes.

Many studies have established that addition of glucose to yeast cells causes an increase in intracellular cAMP level. Although the mechanism by which glucose causes the cAMP increase is not understood, one possibility is that glucose stimulates the activity of adenylate cyclase. The *ras2*^{ts} and *cyr1*^{ts} mutations may cause defects in growth on low concentrations of glucose. Thus *ras1 ras2*^{ts} and *ras1 cyr1*^{ts} mutants were tested for anaerobic growth on rich medium containing 2% or 0.1% glucose at 25°. *ras1 ras2-125* and *ras1 ras2-23* mutants showed impaired growth on 0.1% glucose. On the other hand, *ras1 ras2-35*, *ras1 ras2-47* and *ras1 cyr1-230* mutants did not show defects in growth on 0.1% glucose. Since *ras1 ras2-125* and *ras1 ras2-23* mutants are petite (defective in aerobic growth on nonfermentable carbon sources), ability to grow on 0.1% glucose could be scored clearly under aerobic as well as anaerobic conditions. As expected, these mutants showed impaired growth on 0.1% glucose even under aerobic condition. To determine whether a *ras2* null mutation causes growth defects on low concentrations of glucose, *ras2* mutants were tested for anaerobic growth on 2% and 0.1% glucose. *ras2* mutants did not show defects in growth on 0.1% glucose at 25°. However, *ras2* mutants were unable to grow on both 2% and 0.1% glucose at 37°.

Recently, it has been reported that lithium, a drug widely used to treat manic-depressive illness, perturbs the G protein function (Avissar *et al.* 1988). This

TABLE 5

Growth of *ras1 ras2*^{ts}, *ras1 cyr1*^{ts} and *ras1 cyr1*^{ts} *bcy1* mutants in the presence of lithium

Strain[a]	Relevant genotype[b]	Growth on LiCl[c] (mM)					
		1	3	6	10	15	20
T3-28D	+	+	+	+	–	–	–
HM54-14D	*ras2-125*	+	+	–	–	–	–
HM55-6B	*ras2-23*	+	+	–	–	–	–
HM67-3C	*ras2-35*	+	+	+	–	–	–
HM56-1A	*ras2-47*	+	+	+	–	–	–
HM57-2C	*cyr1-230*	+	–	–	–	–	–
HM57-2CC	*cyr1-230 CYR1*	+	+	+	–	–	–
10-9	*cyr1-230 bcy1-109*	+	+	+	+	+	–

[a] HM57-2CC is HM57-2C transformed with YCpLeCYR1. 10-9 is a *bcy1* mutant isolated from HM57-2C.
[b] All strains are *ras1*.
[c] Cells were spotted on YPD plates containing various concentrations of LiCl and incubated at 25° for 3 days.

finding prompted us to test the effect of lithium on growth of *ras1 ras2*^{ts} and *ras1 cyr1*^{ts} mutants. The results are shown in Table 5. The *ras1* parent strain T3-28D was resistant to LiCl up to a concentration of about 6 mM on YPD at 25°. The *ras1 ras2-125*, *ras1 ras2-23* and *ras1 cyr1-230* mutants were found to have increased sensitivity to lithium. On the other hand, the sensitivity to lithium of the *ras1 ras2-35* and *ras1 ras2-47* mutants was indistinguishable from that of the *ras1* parent. Similar results were obtained with lithium acetate. Association of the lithium-sensitive phenotype with the temperature-sensitive mutations was confirmed in two ways. In the first experiment meiotic segregation of this phenotype was examined with tetrads derived from a diploid homozygous for *ras1* and heterozygous for *ras2-125* (HM54). The lithium sensitivity cosegregated with the temperature sensitivity for growth. The second experiment involves the construction of a set of isogenic strains (Table 5): a *ras1 cyr1-230* strain (HM57-2C), the *ras1 cyr1-230* strain carrying an extra copy of *CYR1* (HM57-2CC) and the *ras1 cyr1-230* strain carrying a *bcy1* mutation (10-9). The *ras1 cyr1-230* strain carrying *CYR1* showed a 2.4-fold higher level of cAMP at 25° than did the *ras1 cyr1-230* parent (data not shown). The *ras1 cyr1-230 bcy1-109* mutant was isolated as a temperature-resistant revertant of the *ras1 cyr1-230* strain (see MATERIALS AND METHODS). In contrast to the *ras1 cyr1-230* strain sensitive to 3 mM LiCl, the *ras1 cyr1-230* strain carrying *CYR1* was resistant to LiCl at a concentration of 6 mM (Table 5). Moreover, the *ras1 cyr1-230 bcy1-109* strain was resistant up to 15 mM LiCl (Table 5).

To determine whether *ras2* strains are sensitive to lithium compared with *RAS1 RAS2* and *ras1 RAS2* strains, segregants from a diploid heterozygous for *ras1* and *ras2* (HM66) were tested for sensitivity to lithium. Surprisingly, about half of viable spores (34 out of 69 in 22 tetrads) were resistant to high concentrations of LiCl (30–50 mM) irrespective to the *RAS1* and *RAS2* genotypes. All tetrads with four viable spores showed 2+:2– segregation for lithium resistance, and all tetrads with three viable spores showed 2+:1– or 1+:2– segregation. The *ras2* parent strain T3-35D was resistant to the high concentrations of lithium, whereas the *ras1* parent strain HM65-24D was not. These results suggest that assuming that the strain HM65-24D represented wild type, the strain T3-35D would carry a mutation that confer resistance to high concentrations of lithium. Since the lithium-resistant phenotype was found to be recessive, the mutation may result in a defect in uptake of lithium. We tentatively designate the mutation *lit1*^{R}. *RAS1 RAS2 LIT1*, *ras1 RAS2 LIT1* and *RAS1 ras2 LIT1* segregants were tested for sensitivity to lithium. The *ras2* null mutation did not cause increased sensitivity to lithium at 25°.

The effects of other metal ions were examined both in tetrads derived from a diploid homozygous for *ras1* and heterozygous for *ras2-125* (HM54) and in the isogenic *ras1 cyr1-230*, *ras1 cyr1-230 CYR1* and *ras1 cyr1-230 bcy1-109* strains described above by testing for growth at 25° on YPD medium containing various concentrations of NaCl, KCl, CsCl, $MgCl_2$, $CaCl_2$, $MnCl_2$, $CuCl_2$ or $ZnCl_2$. The *ras1 ras2-125* strains were more sensitive to CsCl than the *ras1* strains (2:2 segregation was observed on 20–30 mM CsCl), and slightly more resistant to $MnCl_2$, $CuCl_2$ and $ZnCl_2$ than the *ras1* strains (2:2 segregation was observed on 8–10 mM $MnCl_2$, 12 mM $CuCl_2$ and 10–12 mM $ZnCl_2$). Similar phenotypes were observed in the *ras1 cyr1-230* strain; the *ras1 cyr1-230* strain was more sensitive to CsCl and slightly more resistant to $MnCl_2$, $CuCl_2$ and $ZnCl_2$ than the *ras1 cyr1-230 CYR1* strain. The *ras1 cyr1-230 bcy1-109* strain was more resistant to CsCl and slightly more sensitive to $MnCl_2$, $CuCl_2$ and $ZnCl_2$ than the *ras1 cyr1-230 CYR1* strain. In summary, the *ras2-125* and *cyr1-230* mutations caused increased sensitivity to Cs^+ and increased resistance to Mn^{2+}, Cu^{2+} and Zn^{2+}, whereas the *bcy1-109* mutation caused increased resistance to Cs^+ and increased sensitivity to Mn^{2+}, Cu^{2+} and Zn^{2+}.

Intracellular cAMP and adenylate cyclase activity of *ras1 ras2*^{ts} and *ras1 cyr1*^{ts} mutants: Recessive phenotypes of *ras1 ras2*^{ts} and *ras1 cyr1*^{ts} mutants suggest that the mutant products are not fully functional even at a temperature permissive for growth. Intracellular cAMP levels in *ras1 ras2*^{ts} and *ras1 cyr1*^{ts} mutants were measured to assess adenylate cyclase activity *in vivo* (Table 6). Both the *ras1 ras2*^{ts} and *ras1 cyr1*^{ts} mutants had reduced levels of cAMP at 25° compared with the *ras1* parent. The levels of cAMP appear to correlate with severity of the phenotypes; the levels of cAMP in the *ras1 ras2-125* and *ras1 ras2-*

TABLE 6

Intracellular cAMP levels of *ras1 ras2*^{ts} and *ras1 cyr1*^{ts} mutants

Strain	Relevant genotype[a]	cAMP level (pmol/mg protein)
T3-28D	+	2.1
HM54-14D	*ras2-125*	0.5
HM55-6B	*ras2-23*	0.5
HM67-3C	*ras2-35*	1.5
HM56-1A	*ras2-47*	1.2
HM57-2C	*cyr1-230*	0.8

[a] All strains are *ras1*.

23 mutants were lower than those in the *ras1 ras2-35* and *ras1 ras2-47* mutants. Assay for adenylate cyclase indicated that the membranes from the *ras1 ras2*^{ts} and *ras1 cyr1*^{ts} mutants showed reduced levels of activity in the presence of Mg^{2+} compared with those from the *ras1* parent (data not shown).

DISCUSSION

Using a plasmid carrying a galactose-inducible *RAS2* gene, we isolated at least four independent temperature-sensitive mutations in the *RAS2* gene. DE VENDITTIS *et al.* (1986) have isolated a temperature-sensitive mutation in the *RAS2* gene by another procedure, in which the *RAS2* gene was mutagenized *in vitro* and then introduced into the chromosome by the one-step replacement method (ROTHSTEIN 1983). A cloned gene has been used in several ways to construct conditional-lethal mutations either on the chromosome or on a plasmid. The integrative replacement/disruption strategy depends on the integration of a truncated gene that is mutagenized *in vitro* into the chromosome followed by a partial duplication of the gene (SHORTLE, NOVICK and BOTSTEIN 1984; HOLM *et al.* 1985; SEGEV and BOTSTEIN 1987; HIMMELFARB, SIMPSON and FRIESEN 1987; NAUMOVSKI and FRIEDBERG 1987; NONET *et al.*, 1987; GUDENUS *et al.* 1988). The plasmid shuffling strategy involves an exchange of a plasmid carrying the wild-type copy of a gene for a mutagenized plasmid by counterselection against the former plasmid (MANN *et al.* 1987; BUDD and CAMPBELL 1987; BOEKE *et al.* 1987). SERRANO, MONTESINOS and CID (1986) have used a gene fused to the *GAL1* promoter to isolate a temperature-sensitive mutation. Their approach is different from ours in that the wild-type gene on a plasmid was mutagenized and then introduced into a strain carrying the *GAL1* fusion gene on the chromosome. Our procedure described in this paper will provide an alternative approach for isolating conditional-lethal mutations of a cloned gene as long as its overexpression is not deleterious to yeast cells. A useful feature of our procedure is that it has a potential to yield mutations that can be suppressed by overexpression of a cloned gene. Indeed, we isolated a *cyr1* mutation that can be suppressed by overexpression or increased dosage of *RAS2* (see below). Enrichment procedure would, if possible, increase the opportunity to isolate mutations. Since the number of nonspecific, galactose-dependent mutants was small, the occurrence of such mutants seems not to be a serious problem.

The temperature sensitivity caused by the *cyr1-230* mutation was suppressed by either a *GAL1-RAS2* plasmid or a high copy number *RAS2* plasmid, but not by a low copy number *RAS2* plasmid. A large amount of the *RAS2* product is likely to be required for suppression. On the other hand, some *ras2*^{ts} mutations were shown to be suppressed by *CYR1*. In this case one extra copy of *CYR1* was sufficient for suppression. Such a phenomenon, suppression by duplication of a gene, has been reported for other genes (HAYLES, AVES and NURSE 1986; SALMINEN and NOVICK 1987). Thus increased dosage of either *RAS2* or *CYR1* can suppress the temperature sensitivity caused by a mutation in the other.

ras1 ras2^{ts} and *ras1 cyr1*^{ts} mutants showed the cell-division-cycle arrest in the G_1 phase at the restrictive temperature, and showed pleiotropic phenotypes to varying degrees at the permissive temperature, including slow growth, sporulation on rich media, increased accumulation of glycogen, impaired growth on nonfermentable carbon sources, heat-shock resistance, impaired growth on low concentrations of glucose, and lithium sensitivity. *ras1 ras2-125* and *ras1 ras2-23* mutants showed more severe phenotypes and lower levels of cAMP than did *ras1 ras2-35* and *ras1 ras2-47* mutants, suggesting that the *ras2-125* and *ras2-23* products are less active than the *ras2-35* and *ras2-47* products at 25°. The observation that *ras2* null mutants did not show impaired growth on low concentration of glucose and increased sensitivity to lithium at 25° suggests that the residual *RAS* activity in *ras2* mutants at 25° is higher than that in *ras1 ras2-125* and *ras1 ras2-23* mutants. All the phenotypes observed for *ras1 cyr1-230* mutants at the permissive temperature is likely to be independent of the *RAS1* genotype for two reasons. First, no phenotypic changes have yet been observed for *ras1* mutants. Second, the temperature sensitivity caused by the *cyr1-230* mutation was independent of the *RAS1* genotype. Indeed, the lithium sensitivity of a *ras1 cyr1-230* mutant was complemented by *CYR1*, indicating that the phenotype results from the mutation in *CYR1*. Among the pleiotropic phenotypes described above, impaired growth on low concentrations of glucose and sensitivity to lithium are new phenotypes, which have not been reported for mutants defective in the cAMP pathway.

It is known that glucose addition to yeast cells causes a rapid increase in cAMP level. It is possible that glucose not only serves as energy source but also

stimulates adenylate cyclase. The observation that some *ras1 ras2*^{ts} mutants is defective in growth on low concentrations of glucose is consistent with the hypothesis that glucose stimulates adenylate cyclase activity through the *RAS* proteins. According to this hypothesis, the growth defects of *ras1 ras2*^{ts} mutants on low concentrations of glucose would result from the inability to produce sufficient amount of cAMP. NAKAFUKU *et al.* (1988) have reported that glucose addition does not cause cAMP increase in a *ras2*^{ts} mutant. However, we cannot exclude the possibility that the defects in growth on low concentrations of glucose results from low levels of cAMP-dependent phosphorylation, which may be required for utilization or uptake of glucose. Further studies will be required to elucidate the action of glucose.

It was found that *ras1 ras2*^{ts} and *ras1 cyr1*^{ts} mutants were sensitive to lithium and, in contrast, a *ras1 cyr1*^{ts} *bcy1* mutant was resistant to lithium compared with the *ras1* parent strain. There are two possible explanations for the action of lithium in yeast. First, lithium may inhibit the production of cAMP. The growth of a mutant that had a impaired function of *RAS2* or *CYR1* could be inhibited by lower concentrations of lithium. A *bcy1* mutation, which relieves the requirement for the cAMP-generating system, could cause increased resistance. Alternatively, lithium may inhibit a function that requires cAMP-dependent phosphorylation. According to this model, reduced level of phosphorylation, which results from reduced level of cAMP, would cause increased sensitivity to lithium. Increased resistance of a *bcy1* mutant could be explained by high-level constitutive phosphorylation.

Site-directed mutagenesis has been used for analyzing the function of the yeast *RAS2* protein; specific structural variants can be created *in vitro* to examine the effects of particular amino acid substitutions on the biological activity of the *RAS2* protein (KATAOKA *et al.* 1984; DESCHENES and BROACH 1987; MARSHALL *et al.* 1988). Isolation and sequence analysis of the *ras2*^{ts} alleles obtained in this study will reveal amino acid substitutions of the mutant *RAS2* proteins and thus provide a complementary approach for exploring relationship between the structure and function of the yeast *RAS2* protein.

Finally, the *ras2*^{ts} mutations are useful in isolating extragenic suppressors. Analysis of extragenic suppressors may allow identification of new genes whose products interact with the *RAS2* product.

We thank KUNIHIRO MATSUMOTO and MICHAEL WIGLER for strains, and AKIO TOH-E for a plasmid.

LITERATURE CITED

AVISSAR, S., G. SCHREIBER, A. DANON and R. H. BELMAKER, 1988 Lithium inhibits adrenergic and cholinergic increases in GTP binding in rat cortex. Nature **331:** 440–442.

BOEKE, J. D., J. TRUEHEART, G. NATSOULIS and G. R. FINK, 1987 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. Methods Enzymol. **154:** 164–175.

BOUTELET, F., A. PETITJEAN and F. HILGER, 1985 Yeast *cdc35* mutants are defective in adenylate cyclase and are allelic with *cyr1* mutants while *CAS1*, a new gene, is involved in the regulation of adenylate cyclase. EMBO J. **4:** 2635–2641.

BROEK, D., T. TODA, T. MICHAELI, L. LEVIN, C. BIRCHMEIER, M. ZOLLER, S. POWERS and M. WIGLER, 1987 The *S. cerevisiae CDC25* gene product regulates the *RAS*/adenylate cyclase pathway. Cell **48:** 789–799.

BUDD, M., and J. L. CAMPBELL, 1987 Temperature-sensitive mutations in the yeast DNA polymerase I gene. Proc. Natl. Acad. Sci. USA **84:** 2838–2842.

CANNON, J. F., J. B. GIBBS and K. TATCHELL, 1986 Suppressors of the *ras2* mutation of *Saccharomyces cerevisiae.* Genetics **113:** 247–264.

CASPERSON, G. F., N. WALKER and H. R. BOURNE, 1985 Isolation of the gene encoding adenylate cyclase in *Saccharomyces cerevisiae.* Proc. Natl. Acad. Sci. USA **82:** 5060–5063.

DEFEO-JONES, D., E. M. SCOLNICK, R. KOLLER and R. DHAR, 1983 *ras*-related gene sequences identified and isolated from *Saccharomyces cerevisiae.* Nature **306:** 707–709.

DEFEO-JONES, D., K. TATCHELL, L. C. ROBINSON, I. S. SIGAL, W. C. VASS, D. R. LOWY and E. M. SCOLNICK, 1985 Mammalian and yeast *ras* gene products: biological function in their heterologous systems. Science **228:** 179–184.

DESCHENES, R. J., and J. R. BROACH, 1987 Fatty acylation is important but not essential for *Saccharomyces cerevisiae RAS* function. Mol. Cell. Biol. **7:** 2344–2351.

DE VENDITTIS, E., A. VITELLI, R. ZAHN and O. FASANO, 1986 Suppression of defective *RAS1* and *RAS2* functions in yeast by an adenylate cyclase activated by a single amino acid change. EMBO J. **5:** 3657–3663.

FRAENKEL, D. G., 1985 On *ras* gene function in yeast. Proc. Natl. Acad. Sci. USA **82:** 4740–4744.

GUDENUS, R., S. MARIOTTE, A. MOENNE, A. RUET, S. MEMET, J.-M. BUHLER, A. SENTENAC and P. THURIAUX, 1988 Conditional mutants of *RPC160*, the gene encoding the largest subunit of RNA polymerase C in *Saccharomyces cerevisiae.* Genetics **119:** 517–526.

HAYLES, J., S. AVES and P. NURSE, 1986 *suc1* is an essential gene involved in both the cell cycle and growth in fission yeast. EMBO J. **5:** 3373–3379.

HIMMELFARB, H. J., E. M. SIMPSON and J. D. FRIESEN, 1987 Isolation and characterization of temperature-sensitive RNA polymerase II mutants of *Saccharomyces cerevisiae.* Mol. Cell. Biol. **7:** 2155–2164.

HOLM, C., T. GOTO, J. C. WANG and D. BOTSTEIN, 1985 DNA topoisomerase II is required at the time of mitosis in yeast. Cell **41:** 553–563.

ITO, H., Y. FUKUDA, K. MURATA and A. KIMURA, 1983 Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153:** 163–168.

KATAOKA, T., S. POWERS, C. MCGILL, O. FASANO, J. STRATHERN, J. BROACH and M. WIGLER, 1984 Genetic analysis of yeast *RAS1* and *RAS2* genes. Cell **37:** 437–445.

KATAOKA, T., S. POWERS, S. CAMERON, O. FASANO, M. GOLDFARB, J. BROACH and M. WIGLER, 1985 Functional homology of mammalian and yeast *RAS* genes. Cell **40:** 19–26.

KLAPHOLZ, S., and R. E. ESPOSITO, 1982 A new mapping method employing a meiotic *rec*$^-$ mutant of yeast. Genetics **100:** 387–412.

MANIATIS, T., E. F. FRITSCH and J. SAMBROOK, 1982 *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

MANN, C., J.-M. BUHLER, I. TREICH and A. SENTENAC, 1987 *RPC40*, a unique gene for a subunit shared between

yeast RNA polymerases A and C. Cell **48:** 627–637.

MARSHALL, M. S., J. B. GIBBS, E. M. SCOLNICK and I. S. SIGAL, 1988 An adenylate cyclase from *Saccharomyces cerevisiae* that is stimulated by *RAS* proteins with effector mutations. Mol. Cell. Biol. **8:** 52–61.

MATSUMOTO, K., I. UNO and T. ISHIKAWA, 1983a Control of cell division in *Saccharomyces cerevisiae* mutants defective in adenylate cyclase and cAMP-dependent protein kinase. Exp. Cell Res. **146:** 151–161.

MATSUMOTO, K., I. UNO and T. ISHIKAWA, 1983b Initiation of meiosis in yeast mutants defective in adenylate cyclase and cyclic AMP-dependent protein kinase. Cell **32:** 417–423.

MATSUMOTO, K., I. UNO and T. ISHIKAWA, 1984 Identification of the structural gene and nonsense alleles for adenylate cyclase in *Saccharomyces cerevisiae*. J. Bacteriol. **157:** 277–282.

MATSUMOTO, K., I. UNO and T. ISHIKAWA, 1985 Genetic analysis of the role of cAMP in yeast. Yeast **1:** 15–24.

MATSUMOTO, K., I. UNO, Y. OSHIMA and T. ISHIKAWA, 1982 Isolation and characterization of yeast mutants deficient in adenylate cyclase and cAMP-dependent protein kinase. Proc. Natl. Acad. Sci. USA **79:** 2355–2359.

MORINAGA, Y., T. FRANCESCHINI, S. INOUYE and M. INOUYE, 1984 Improvement of oligonucleotide-directed site-specific mutagenesis using double-stranded plasmid DNA. Biotechnology **2:** 636–639.

NAKAFUKU, M., T. OBARA, K. KAIBUCHI, I. MIYAJIMA, A. MIYAJIMA, H. ITOH, S. NAKAMURA, K. ARAI, K. MATSUMOTO and Y. KAZIRO, 1988 Isolation of a second yeast *Saccharomyces cerevisiae* gene (*GPA2*) coding for guanine nucleotide-binding regulatory protein: studies on its structure and possible functions. Proc. Natl. Acad. Sci. USA **85:** 1374–1378.

NAUMOVSKI, L., and E. C. FRIEDBERG, 1987 The *RAD3* gene of *Saccharomyces cerevisiae*: isolation and characterization on a temperature-sensitive mutant in the essential function and of extragenic suppressors of this mutant. Mol. Gen. Genet. **209:** 458–466.

NONET, M., C. SCAFE, J. SEXTON and R. YOUNG, 1987 Eukaryotic polymerase conditional mutant that rapidly ceases mRNA synthesis. Mol. Cell. Biol. **7:** 1602–1611.

POWERS, S., T. KATAOKA, O. FASANO, M. GOLDFARB, J. STRATHERN, J. BROACH and M. WIGLER, 1984 Genes in *S. cerevisiae* encoding proteins with domains homologous to the mammalian *ras* proteins. Cell **36:** 607–612.

RIVIN, C. J., and W. L. FANGMAN, 1980 Cell cycle phase expansion in nitrogen-limited cultures of *Saccharomyces cerevisiae*. J. Cell Biol. **85:** 96–107.

ROBINSON, L. C., J. B. GIBBS, M. S. MARSHALL, I. S. SIGAL and K. TATCHELL, 1987 *CDC25*: a component of the *RAS*-adenylate cyclase pathway in *Saccharomyces cerevisiae*. Science **235:** 1218–1221.

ROTHSTEIN, 1983 One-step gene disruption in yeast. Methods Enzymol. **101:** 202–211.

SALMINEN, A., and P. J. NOVICK, 1987 A *ras*-like protein is required for a post-Golgi event in yeast secretion. Cell **49:** 527–538.

SEGEV, N., and D. BOTSTEIN, 1987 The *ras*-like yeast *YPT1* gene is itself essential for growth, sporulation, and starvation response. Mol. Cell. Biol. **7:** 2367–2377.

SERRANO, R., C. MONTESINOS and A. CID, 1986 A temperature-sensitive mutant of the yeast plasma membrane ATPase obtained by *in vitro* mutagenesis. FEBS Lett. **208:** 143–146.

SHERMAN, F., G. R. FINK and J. B. HICKS, 1983 *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

SHIN, D.-Y., K. MATSUMOTO, H. IIDA, I. UNO and T. ISHIKAWA, 1987 Heat shock response of *Saccharomyces cerevisiae* mutants altered in cyclic AMP-dependent protein phosphorylation. Mol. Cell. Biol. **7:** 244–250.

SHORTLE, D., P. NOVICK and D. BOTSTEIN, 1984 Construction and genetic characterization of temperature-sensitive mutant alleles of the yeast actin gene. Proc. Natl. Acad. Sci. USA **81:** 4889–4893.

SNOW, R., 1966 An enrichment method for auxotrophic yeast mutants using the antibiotic "nystatin." Nature **211:** 206–207.

ST. JOHN, T. P., and R. W. DAVIS, 1981 The organization and transcription of the galactose gene cluster of Saccharomyces. J. Mol. Biol. **152:** 285–315.

TATCHELL, K., D. T. CHALEFF, D. DEFEO-JONES and E. M. SCOLNICK, 1984 Requirement of either of a pair of *ras*-related genes of *Saccharomyces cerevisiae* for spore viability. Nature **309:** 523–527.

TATCHELL, K., L. C. ROBINSON and M. BREITENBACH, 1985 *RAS2* of *Saccharomyces cerevisiae* is required for gluconeogenic growth and proper response to nutrient limitation. Proc. Natl. Acad. Sci. USA **82:** 3785–3789.

TODA, T., S. CAMERON, P. SASS, M. ZOLLER and M. WIGLER, 1987 Three different genes in *S. cerevisiae* encode the catalytic subunits of the cAMP-dependent protein kinase. Cell **50:** 277–287.

TODA, T., I. UNO, T. ISHIKAWA, S. POWERS, T. KATAOKA, D. BROEK, S. CAMERON, J. BROACH, K. MATSUMOTO and M. WIGLER, 1985 In yeast, *RAS* proteins are controlling elements of adenylate cyclase. Cell **40:** 27–36.

UNO, I., H. MITSUZAWA, K. MATSUMOTO, K. TANAKA, T. OSHIMA and T. ISHIKAWA, 1985 Reconstitution of the GTP-dependent adenylate cyclase from products of the yeast *CYR1* and *RAS2* genes in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **82:** 7855–7859.

UNO, I., H. MITSUZAWA, K. TANAKA, T. OSHIMA and T. ISHIKAWA, 1987 Identification of the domain of *Saccharomyces cerevisiae* adenylate cyclase associated with the regulatory function of *RAS* products. Mol. Gen. Genet. **210:** 187–194.

YAMANO, S., K. TANAKA, K. MATSUMOTO and A. TOH-E, 1987 Mutant regulatory subunit of 3',5'-cAMP-dependent protein kinase of yeast *Saccharomyces cerevisiae*. Mol. Gen. Genet. **210:** 413–418.

Communicating editor: D. BOTSTEIN