

# Cloning and characterization of the high-affinity cAMP phosphodiesterase of *Saccharomyces cerevisiae*

(heat shock/yeast *RAS2*<sup>Val19</sup> gene/suppressor/*PDE2* gene)

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**ABSTRACT** A gene, *PDE2*, has been cloned from the yeast *Saccharomyces cerevisiae* that, when present in high copy, reverses the phenotypic effects of *RAS2*<sup>Val19</sup>, a mutant form of the *RAS2* gene that renders yeast cells sensitive to heat shock and starvation. It has previously been shown that the *RAS* proteins are potent activators of yeast adenylate cyclase. We report here that *PDE2* encodes a high-affinity cAMP phosphodiesterase that shares sequence homology with animal cell phosphodiesterases. These results therefore imply that the effects of *RAS2*<sup>Val19</sup> are mediated through its changes in cAMP concentration.

Our laboratory group has been studying the mechanism of growth control in the yeast *Saccharomyces cerevisiae* with particular concentration on the functions of the *RAS1* and *RAS2* genes, which are structurally and functionally homologous to the *ras* oncogenes of mammalian cells (1-4). At least one *RAS1* or *RAS2* gene is required for the continued growth of yeast cells (5, 6) and it has been shown that *RAS* genes are essential controlling elements for adenylate cyclase in yeast (2, 7, 8). A mutant *RAS2* gene has been constructed that encodes valine at the 19th codon position instead of glycine (5). This mutant (*RAS2*<sup>Val19</sup>) is analogous to the mutant and oncogenic human *Ha-ras* gene, which was first recognized in the T24/EJ bladder cell line (9-11). Yeast cells that express the mutant *RAS2*<sup>Val19</sup> gene fail to synthesize glycogen, show an abnormal sensitivity to starvation (8), show a defective ability to arrest in the G<sub>1</sub> phase of the cell cycle (8), and are sensitive to heat shock (unpublished results). To better understand the mechanism of these effects, we have searched for yeast genes that, when present in high copy, reverse these phenotypic effects. One such gene has been found, and it encodes the high-affinity cAMP phosphodiesterase (*PDEase*) of *S. cerevisiae*. We here present the nucleotide sequence of this gene and describe some of the phenotypic consequences of its perturbation.

## METHODS

**Yeast Strains, Growth Media, Transformation, Heat Shock, and Starvation.** Growth and general genetic manipulation of yeast cells was carried out as described (12). Tetrad dissections and assignment of auxotrophic markers were performed as described (1, 5). A genomic library that had been constructed in the plasmid vector YEp13 from yeast DNA partially digested with *Sau3A* restriction endonuclease has been described previously (13, 14). YEp13 is an extrachromosomally replicating plasmid that contains the 2- $\mu$ m origin of replication, the *LEU2* gene, and parts of pBR322 (14). Transformation into yeast cells was carried out using lithium acetate (15). Yeast cells were heat shocked and starved for

nitrogen by a replica plating method. Heat shock was performed by replica plating cells that had grown for 2 days at 30°C on selective medium to a medium that had been heated for 1 hr at 55°C. After a 30-min incubation at 55°C this replica was transferred to a 30°C incubator for 2 days and then scored for growth. Yeast were starved for nitrogen by replica plating cells that had been grown for 2 days on selective plates at 30°C to plates that lacked a source of nitrogen (8). This replica was incubated at 30°C for 7 days, then replica plated onto rich medium and grown for 2 days at 30°C, and then scored.

**Plasmid Constructions and DNA Sequencing.** The cloning and structure of the *PDE2* gene and *ppde2::URA3* disruption plasmids are described below and in Fig. 1. Yeast DNA was prepared as described (16). Restriction endonuclease fragments were cloned into M13mp18 or M13mp19 vectors (17) and sequenced by a modification of the dideoxy chain-termination method (18, 19).

**PDEase Assays.** Yeast cells were lysed by passage through a French press at 20,000 psi (1 psi = 6.89 kPa). Extracts were centrifuged at 1600  $\times$  *g* for 10 min and the resulting supernatant was centrifuged at 22,000  $\times$  *g* for 90 min. The crude yeast extract was loaded onto a DEAE-Sephacel column and eluted with a 200-ml linear gradient as described (20). cAMP PDEase was assayed by a modification (21) of the procedure of Kuo *et al.* (22, 23).

## RESULTS

**A Gene in High Copy That Reverts the *RAS2*<sup>Val19</sup> Phenotype.** Cell strains containing the *RAS2*<sup>Val19</sup> allele have been described (8). The strain TK161-R2V (see Table 1 for strain list) shows all the characteristics typical of strains containing the *RAS2*<sup>Val19</sup> allele. In particular, cells of this strain are sensitive to heat shock, lose viability on starvation, and fail to accumulate glycogen. This strain was transformed with pooled DNA from a library of *S. cerevisiae* chromosomal DNA carried on the YEp13 shuttle vector, which contains the *LEU2* gene and the  $\beta$ -lactamase gene (13, 14). *Leu*<sup>+</sup> transformants were selected and tested for heat shock sensitivity by a replica plate method. Several heat shock-resistant colonies were observed in the first screening, and cells from these colonies showed this phenotype only when they retained the *LEU2* marker. Plasmids from two of these strains were used to transform *Escherichia coli* to ampicillin resistance. Restriction endonuclease maps of both plasmids indicated that they contained inserts from the same locus (Fig. 1a). Subcloning experiments helped to delineate the functional gene (Fig. 1b), which was then sequenced on both strands by the dideoxy chain-termination method (Fig. 1c).

The nucleotide sequence of this gene is presented in Fig. 2 together with the predicted amino acid sequence of its encoded product. The largest open reading frame initiated by

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Abbreviations: PDEase, phosphodiesterase; kb, kilobase(s).

Table 1. Strains used

Strain	Genotype and derivation
TK161-R2V*	<i>MATa leu2 his3 ura3 trp1 ade8 can1 RAS2<sup>Val19</sup></i>
R2V-YEp13	Transformant of TK161-R2V that contains YEp13
R2V-PDE2	Transformant of TK161-R2V that contains YEpPDE2-1
SPD-12	<i>MATa/MATα leu2/leu2 his3/+ his4/+ ura3/ura3 trp1/trp1 ade8/ade8 can1/+</i>
PS1-1	Transformant of diploid strain SPD-12 with the 5.1-kb <i>Bam</i> HI fragment of <i>ppe2::URA3</i>
PS1-1B	<i>MATα leu2 his3 ura3 pde2::URA3 trp1 ade8</i> (segregant from PS1-1)
PS1-1BY	Transformant of PS1-1B that contains YEpPDE2-1
PS1-1BYL	Derived from PS1-1BY by loss of YEpPDE2-1
SP1*	<i>MATa leu2 his3 ura3 trp1 ade8 can1</i>
SP1-YEp13	Transformant of SP1 that contains YEp13
SP1-PDE2	Transformant of SP1 that contains YEpPDE2-1

\*From ref. 8.

ATG is 526 codons long. An in-frame stop codon appears nine nucleotides 5' to this ATG. This gene can thus encode a protein of 60,910 daltons. Computer-assisted sequence comparisons showed no similarities between this sequence and any other published sequence (24). However, since *RAS2<sup>Val19</sup>* strains have high levels of cAMP and since our gene reverses the *RAS2<sup>Val19</sup>* phenotype, we considered whether our gene

might encode a cAMP PDEase. Biochemical analysis had previously indicated that there are at least two cAMP PDEases in yeast, one with low affinity and broad specificity and one with high affinity and narrow specificity (25, 26). The amino acid compositions of these enzymes have been published. Table 2 shows a comparison of the amino acid composition of our predicted gene product with these PDEases. There is a striking similarity in amino acid composition between our protein and the high-affinity cAMP PDEase. The major differences are in those amino acids that are the most difficult to quantitate accurately, namely, methionine, cysteine, and proline. Moreover, the high-affinity cAMP PDEase of yeast is estimated to be 61,000 daltons, in excellent agreement with the predicted molecular mass of our gene product. For these reasons we tentatively named our gene *PDE2*. This nomenclature is justified by the experiments below.

***PDE2* Encodes a High-Affinity cAMP PDEase.** A direct biochemical approach was taken to test whether indeed *PDE2* encodes a high-affinity cAMP PDEase. For this purpose, we constructed yeast strains carrying multiple copies of the *PDE2* gene and strains with a disrupted *PDE2* gene. To disrupt the *PDE2* gene, we first inserted the *URA3* marker into the unique *Hpa* I restriction endonuclease site of plasmid pPDE2 (Fig. 1d). This causes a disruption between the 261st and 262nd codons of *PDE2*. The 5.1-kilobase (kb) *Bam*HI fragment was then used to transform the diploid strain SPD-12. (See Table 1 for a description of yeast strains.) Southern blotting indicated that one allele of *PDE2* had been disrupted in a resulting transformant called PS1-1. This diploid was sporulated, giving rise to viable haploid progeny containing the *URA3* marker. One of the resulting *URA3*<sup>+</sup> progeny was then transformed with plasmid YEpPDE2-1 (Fig. 1a) to provide the *PDE2* gene on a high-copy extrachromosomally replicating plasmid. One transformant strain, PS1-1BY, was used in subsequent experiments. To obtain isogenic *PDE2*<sup>+</sup> and *pde2*<sup>-</sup> strains, strain PS1-1BYL was obtained from PS1-1BY by removal of the high-copy plasmid. Strains PS1-1BY, PS1-1BYL, and the wild-type haploid strain SP1 were then analyzed biochemically.

Hydrolysis of cAMP was measured by a modification (21) of the method of Kuo *et al.* (22, 23). Assays for cAMP PDEase used cAMP concentrations in the micromolar range and hence measured predominantly, but not entirely, the low *K<sub>m</sub>* or high-affinity form of PDEase (26). Perhaps because of this, the crude yeast cell extracts showed variable levels of PDEase activity even in strains lacking *PDE2* (data not shown). We therefore fractionated the crude extracts to separate high-affinity and low-affinity forms of PDEase. The crude cellular extracts were chromatographed on DEAE-Sephacel columns, eluting with NaCl gradients according to the method of Uno *et al.* (20). The PDEase activity profile of the fractions obtained showed the following results: (i) a peak

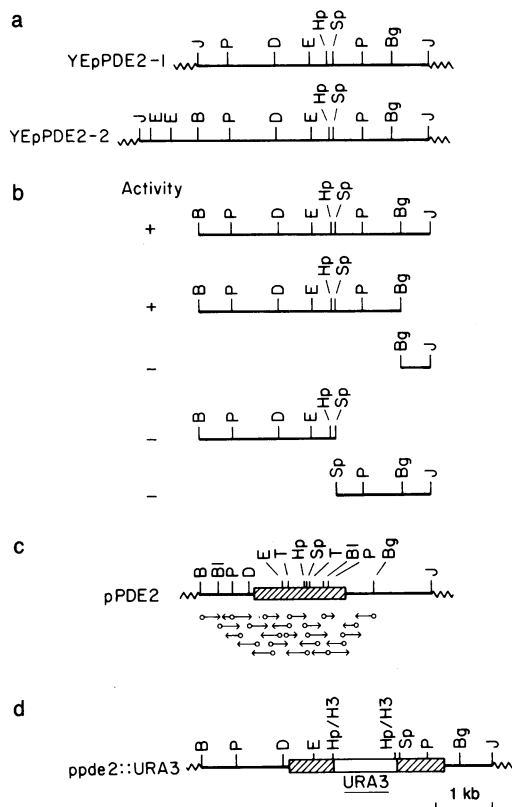


FIG. 1. Structure, sequencing strategy, and disruption of the PDEase gene. (a) Restriction cleavage maps of plasmids YEpPDE2-1 and YEpPDE2-2. (b) Subcloning strategy used to locate the *PDE2* gene. Restriction fragments that were active in suppressing the heat shock-sensitive phenotype of the *RAS2<sup>Val19</sup>* mutation are denoted by a +, inactive fragments, by a -. (c) Strategy used for sequencing the *PDE2* gene (hatched boxes represent coding sequences) using some of the restriction sites shown in a. Open circles and arrows indicate starting sites, directions, and ranges of sequencing by the M13 dideoxy nucleotide sequencing method (21, 22). (d) Disruption of the *PDE2* coding sequences by insertion of the 1.1-kb *URA3* gene into the unique *Hpa* I restriction endonuclease site. Abbreviations used: B, *Bam*HI; Bl, *Bal* I; Bg, *Bgl* II; D, *Dra* I; E, *Eco*RI; H3, *Hind*III; Hp, *Hpa* I; P, *Pst* I; Sp, *Sph* I; T, *Taq* I. J represents a junction between an insert DNA and a *Bam*HI cleavage site of the vector YEp13. The squiggly lines indicate vector sequences.

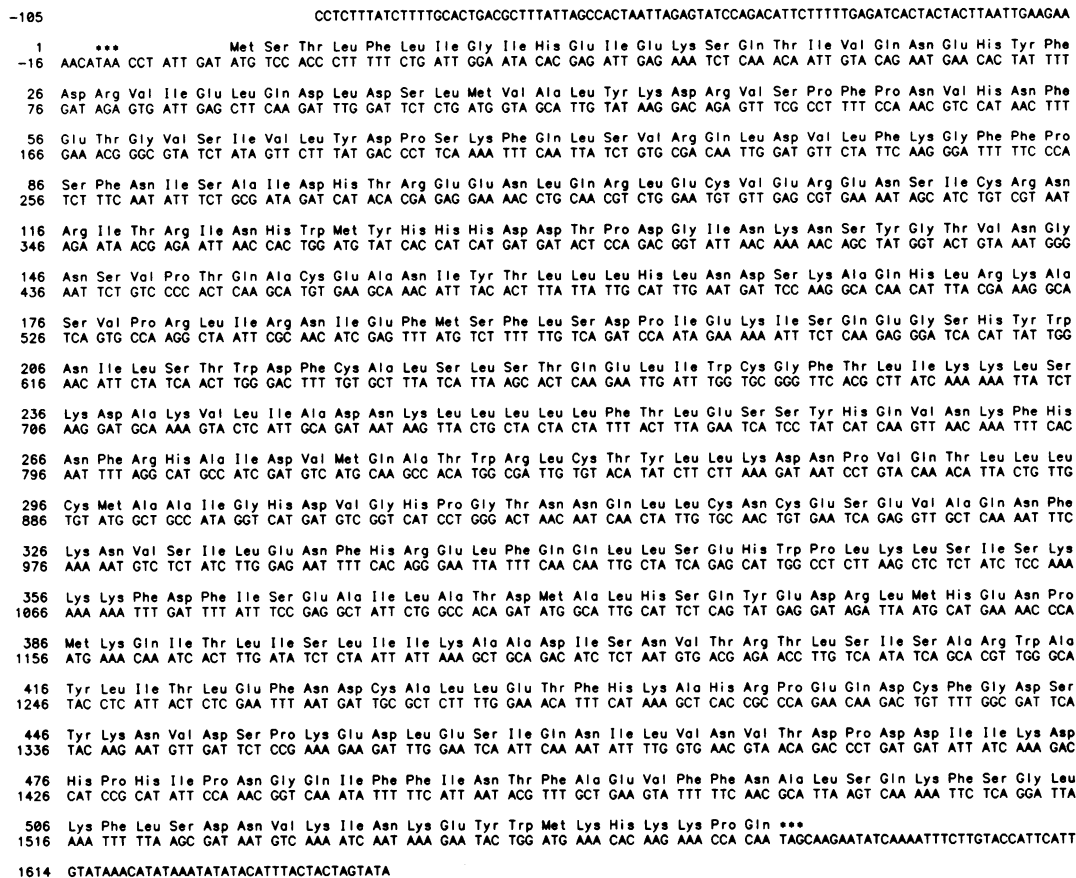


FIG. 2. Nucleotide sequence of the *PDE2* gene and deduced amino acid sequences of its gene product. The nucleotide sequence was obtained by the strategy shown in Fig. 1c. There is one open reading frame. Coordinates on the left indicate nucleotide and amino acid positions. Asterisks indicate termination codons.

of activity in extracts of wild-type cells that eluted at 0.090 M NaCl (Fig. 3A); and (ii) a larger peak of activity is strain PS1-1BY, which contains a high-copy plasmid carrying the

Table 2. Comparison of amino acid compositions of the low-affinity and the high-affinity cAMP PDEases and the heat shock suppressor of *RAS2*<sup>Val19</sup>

Amino acid	Low affinity*		High affinity†		<i>PDE2</i>	
	No.	Mol %	No.	Mol %	No.	Mol %
Asx	39	10.5	71.3	13.5	69	13.1
Thr	20	5.4	25.3	4.8	25	4.8
Ser	28	7.5	39.7	7.5	41	7.8
Glx	47	12.7	59.6	11.3	55	10.5
Pro	21	5.7	26	4.9	18	3.4
Gly	23	6.2	14.4	2.7	14	2.7
Ala	13	3.5	24	4.6	25	4.8
Val	21	5.7	26	4.9	26	4.9
Met	1	0.2	5.1	0.9	10	1.9
Cys	ND	—	5.1	0.9	11	2.1
Ile	26	7.0	39.7	7.5	42	8.0
Leu	44	11.9	61.7	11.7	61	11.6
Tyr	13	3.5	11.0	2.1	13	2.5
Phe	15	4.0	29.5	5.6	31	5.9
Lys	25	6.7	33.6	6.4	33	6.3
His	13	3.5	24	4.6	25	4.8
Trp	6	1.6	11	2.1	8	1.5
Arg	16	4.3	19.2	3.6	19	3.6

Data for the low- and high-affinity PDEases were derived from purified protein (25, 26) whereas data for the heat shock suppressor were deduced from the nucleotide sequence. ND, not determined.

\*From ref. 25.

†From ref. 26.

*PDE2* gene (Fig. 3B); but no peak of activity is seen in the isogenic strain PS1-1BYL, which lacks the *PDE2* gene (Fig. 3C). This result is consistent with the hypothesis that *PDE2* encodes a cAMP PDEase.

To demonstrate that *PDE2* encodes the PDEase with high affinity for cAMP the initial rate of cAMP hydrolysis was measured as a function of cAMP concentration using the peak fraction from the DEAE-Sephacel column. The Lineweaver-Burk plot of these data (Fig. 4) indicates a  $K_m$  of  $1.0 \times 10^{-6}$  M, in good agreement with the data of Suoranta and Londesborough (26), who analyzed the high-affinity cAMP PDEase of yeast.

In all of our strains, including those lacking *PDE2*, it was apparent that there was a residual PDEase activity present in high concentrations of cellular extracts. This was presumably due to the presence of large amounts of a low-affinity form of cAMP PDEase, which has been reported by Uno *et al.* (20) and must be encoded by a separate gene. We do not present any data on this type of activity.

**Phenotypic Consequences of Perturbation of *PDE2*.** As indicated above, the *PDE2* gene was selected by its ability to restore heat shock resistance to cells containing *RAS2*<sup>Val19</sup>. This effect is demonstrated in Fig. 5, in which four types of strains are compared. Strain SP1-YEp13 was a control strain derived from SP1 by transformation and contained the *LEU2* YEp13 vector alone. Strain SP1-PDE2 was another control strain derived from SP1 and contained the *PDE2* gene on the high-copy *LEU2* plasmid YEp13. Strain R2V-YEp13 was a control strain derived from the *RAS2*<sup>Val19</sup>-containing strain TK161-R2V by transformation with plasmid YEp13. Strain R2V-PDE2 was the experimental strain derived from TK161-R2V by transformation with the high-copy plasmid containing the *PDE2* gene, YEpPDE2-1. These strains were inocu-

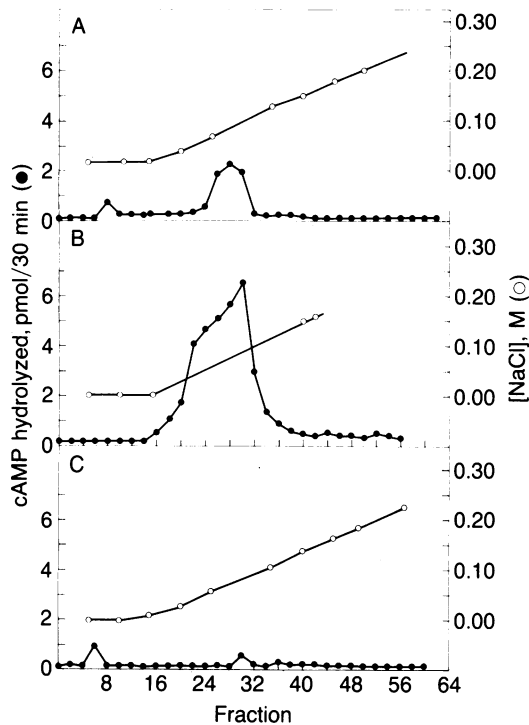


FIG. 3. DEAE-Sephacel column profiles of PDEase activity. (A) Wild-type strain, SP1 (75 mg of protein). (B) Strain PS1-1BY containing the high-copy *PDE2* plasmid (25 mg of protein). (C) Strain PS1-1BYL disrupted at the *PDE2* locus (100 mg of protein).

lated onto synthetic medium lacking leucine (Fig. 5A) and replica plated onto solid medium preheated to 55°C. They were maintained at this temperature for 30 min, allowed to cool, and then incubated at 30°C. In these experiments, the density of the cell patch that regrows indicates the degree of resistance to heat shock (Fig. 5B). The *RAS2<sup>Val19</sup>* strain was clearly sensitive to heat shock but the *RAS2<sup>Val19</sup>* cells with the high-copy *PDE2* appeared as resistant to heat shock as the wild-type cells.

Another phenotype of cells containing the *RAS2<sup>Val19</sup>* allele is sensitivity to starvation. To test whether *PDE2* reverses this phenotype, the strains described above were treated as follows. Cells were inoculated onto synthetic medium and then replica plated onto nitrogen-lacking synthetic medium. After 7 days, cells were replica plated back onto rich medium and allowed to grow at 30°C. The results indicate that the presence of the *PDE2* gene on a high-copy plasmid restores the ability of cells containing *RAS2<sup>Val19</sup>* to survive prolonged nitrogen starvation (Fig. 5C).

We examined the effect of loss of the *PDE2* gene on cell viability. For this purpose, we transformed a diploid cell with a fragment of the *PDE2* gene disrupted by the *URA3*

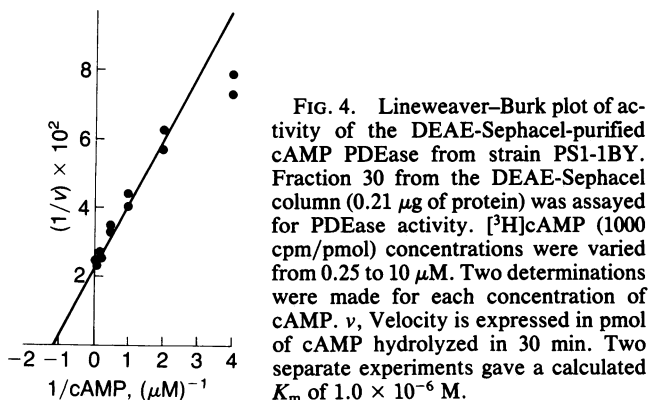


FIG. 4. Lineweaver-Burk plot of activity of the DEAE-Sephacel-purified cAMP PDEase from strain PS1-1BY. Fraction 30 from the DEAE-Sephacel column (0.21  $\mu$ g of protein) was assayed for PDEase activity. [ $^3$ H]cAMP (1000 cpm/pmol) concentrations were varied from 0.25 to 10  $\mu$ M. Two determinations were made for each concentration of cAMP.  $v$ , Velocity is expressed in pmol of cAMP hydrolyzed in 30 min. Two separate experiments gave a calculated  $K_m$  of  $1.0 \times 10^{-6}$  M.

prototrophic marker, as described above. Diploids were then examined by Southern blotting to determine whether the prototrophic marker had integrated within a single *PDE2* locus. Several diploid strains were thus obtained and one was subject to tetrad analysis. The results indicate that there is no loss of viability in spores lacking the *PDE2* gene (data not shown). Finally, we examined the effect of high copy of the *PDE2* gene on the phenotype of wild-type *S. cerevisiae* by transforming strain SP1 with plasmid YEpPDE2-1. Such transformants grow more slowly than the parental strain in synthetic medium but show no difference in heat shock sensitivity or glycogen accumulation (data not shown).

## DISCUSSION

Cells containing the *RAS2<sup>Val19</sup>* mutation fail to accumulate glycogen when they reach growth saturation, are unable to arrest properly in an unbudded state when starved, show an unusual sensitivity to starvation (8), and fail to become heat shock resistant when they reach stationary growth phase (unpublished data and this paper). We attribute these defects to an inability of *RAS2<sup>Val19</sup>* cells to enter a  $G_1$  growth arrest state. We have developed methods for cloning genes that in high copy reverse the phenotypes due to the *RAS2<sup>Val19</sup>* mutation. One such gene is *PDE2*, which encodes a cAMP PDEase. We have previously shown that the *RAS* genes are essential controlling components of yeast adenylate cyclase (7, 8) and that cells with the *RAS2<sup>Val19</sup>* allele have elevated cAMP levels (8). Overexpression of the cAMP effector pathway because of high level expression of adenylate cyclase (7), or because of overexpression of the catalytic subunit of cAMP-dependent protein kinase (our unpublished data), or because of deletion of the regulatory subunit of cAMP-dependent protein kinase leads to the same set of phenotypes as the *RAS2<sup>Val19</sup>* mutation (7, 27). These findings together strongly suggest that many, if not all, of the phenotypic abnormalities of *RAS2<sup>Val19</sup>* are mediated through its elevation of cAMP. The cloned cAMP PDEase gene may provide a useful experimental tool for exploring the role of cAMP in other eukaryotic cells.

A variety of forms of cAMP PDEases have been reported in eukaryotic cells, including high-affinity and low-affinity forms, forms having broad and narrow specificity, and forms that are stimulated by various cofactors such as calmodulin and cGMP (28, 29). Two cAMP PDEase activities have been described in yeast: a low-affinity form having broad specificity and a high-affinity form having narrow specificity (25, 26). The high-affinity form is a zinc-requiring enzyme, with a  $K_m$  for cAMP of  $1 \times 10^{-6}$  M, and fails to hydrolyze cGMP (26). It has an amino acid composition similar to that which we predict for the *PDE2*-encoded product. Indeed, we have shown that the *PDE2* gene of the yeast *S. cerevisiae* appears to encode a high-affinity cAMP PDEase. Gene disruption experiments indicate that the *PDE2* gene is not essential for viable haploid yeast. This, presumably, is because yeast contain a second, low-affinity cAMP PDEase. Indeed, we detect considerable cAMP hydrolysis in crude extracts of cells containing disrupted forms of the *PDE2* gene. We do not know whether yeast cells lacking both PDEases would be viable. Sequence data have been obtained for a *Drosophila* high-affinity cAMP PDEase, a calmodulin-stimulated PDEase from bovine brain, and a cGMP-stimulated cAMP PDEase from bovine heart (30, 31). Comparison of these sequences with the sequence predicted from the yeast *PDE2* gene reveals multiple regions of homology [these data will be discussed by Charbonneau *et al.* (31)]. However, comparison of the conserved sequences in the PDEase proteins with the mammalian regulatory subunits of cAMP-dependent protein kinase (32, 33), the regulatory subunit of the cAMP-dependent protein kinase of yeast (our unpublished data), the

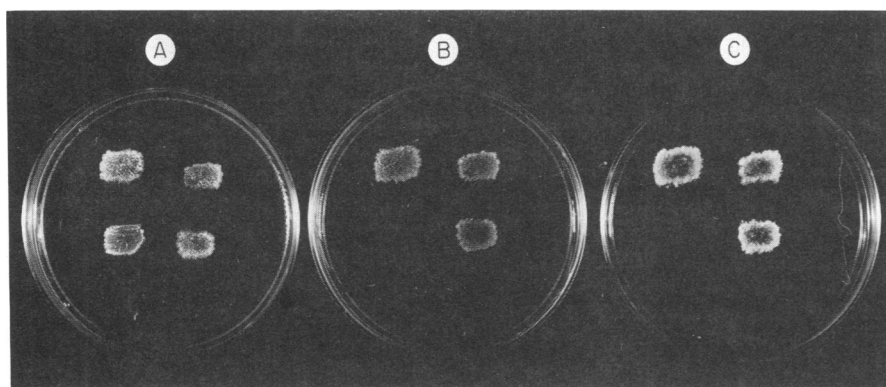


FIG. 5. Heat shock and starvation phenotypes. Four yeast strains were tested for sensitivity to heat shock (B) or to starvation (C) by a replica-plating method. (A) The master plate was inoculated with patches from four strains: SP1-YEp13, upper left; SP1-PDE2, upper right; R2V-YEp13 (*RAS2*<sup>Val19</sup> strain; lower left); and R2V-PDE2, lower right. (B) The plate was heated to 55°C for 1 hr, and then cells were replica plated to it from the master plate and incubated at 55°C for 30 min, and at 30°C for 48 hr. (C) The master plate was replica plated to a plate that lacked a source of nitrogen, and this plate was incubated at 30°C for 7 days, then replica plated to a rich plate, which was incubated for 2 days at 30°C.

cAMP binding protein of *E. coli* (34), and the yeast adenylate cyclase gene (7) show no regions of significant homology. Since these proteins, with the exception of the yeast adenylate cyclase gene, contain a region that is highly conserved and thought to be important in cAMP binding, it is interesting that the *PDE2* gene lacks this nucleotide binding region. Thus it is not clear which sequence of the *PDE2*-encoded protein can recognize cAMP.

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