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Cloning and Characterization of the Low-Affinity Cyclic AMP Phosphodiesterase Gene of *Saccharomyces cerevisiae*

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***Saccharomyces cerevisiae* contains two genes which encode cyclic AMP (cAMP) phosphodiesterases. We previously isolated and characterized *PDE2*, which encodes a high-affinity cAMP phosphodiesterase. We have now isolated the *PDE1* gene of *S. cerevisiae*, which encodes a low-affinity cAMP phosphodiesterase. These two genes represent highly divergent branches in the evolution of phosphodiesterases. High-copy-number plasmids containing either *PDE1* or *PDE2* can reverse the growth arrest defects of yeast cells carrying the *RAS2*$^{Val-19}$ mutation. *PDE1* and *PDE2* appear to account for the aggregate cAMP phosphodiesterase activity of *S. cerevisiae*. Disruption of both *PDE* genes results in a phenotype which resembles that induced by the *RAS2*$^{Val-19}$ mutation. *pde1*$^-$ *pde2*$^-$ *ras1*$^-$ *ras2*$^-$ cells are viable.**

We have been investigating the pathways of growth regulation in the yeast *Saccharomyces cerevisiae*, particularly those pathways which involve the RAS proteins. The *RAS1* and *RAS2* genes of *S. cerevisiae* are structurally and functionally closely related to the mammalian *ras* oncogenes (10, 11, 17, 29). In *S. cerevisiae*, RAS proteins modulate adenylate cyclase in a GTP-dependent manner (4, 37). Yeast cells have severe defects in growth control when they lack *RAS* genes or contain *RAS2* mutations analogous to those which activate the oncogenic properties of the mammalian *RAS* genes (18, 35). In particular, yeast cells containing the *RAS2*$^{Val-19}$ mutation are defective in their response to nutrient limitation; they fail to arrest in G1, fail to accumulate carbohydrate stores, cannot endure starvation, and are sensitive to heat shock (32, 37). To identify yeast proteins which are involved in the control of these responses, we have isolated genes which, when present in high copy numbers, can suppress the *RAS2*$^{Val-19}$ phenotypes. One such gene is *PDE2* (32). It encodes a high-affinity cyclic AMP (cAMP) phosphodiesterase (32, 34). In this report, we describe another such gene, *PDE1*, which encodes a low-affinity cAMP phosphodiesterase (21). We have examined the phenotype caused by disruption of the *PDE* genes and have found that *PDE1* does not appear to have any essential function. The *PDE1* and *PDE2* genes together account for the aggregate cAMP phosphodiesterase activity detectable in *S. cerevisiae*. Our studies also confirm previous conclusions that changes in the cAMP concentration mediate many of the effects of mutant RAS proteins (37). The relationship of the yeast phosphodiesterase genes to other known phosphodiesterases has been explored. Our studies indicate that at least two divergent branches of phosphodiesterase genes have evolved.

MATERIALS AND METHODS

Strains, media, and transformation. *S. cerevisiae* strains used in this study are shown in Table 1. The compositions of the rich medium (YPD), synthetic medium supplemented with appropriate auxotrophic supplements (SC), and nitrogen-depleted medium (YNB-N) have been previously described (37). General genetic manipulation of yeast cells was carried out as described previously (26). *Escherichia coli* HB101 (2) was used for plasmid propagation and isolation, and the MC1061 strain (5) was used for the construction of the yeast genomic library (see section below for details). *E. coli* cells were grown in Luria broth (23). Transformation of yeast cells was carried out with lithium acetate (15). *E. coli* transformation was performed by standard methods (23).

Nucleic acid manipulations. Purification of plasmid DNA from *E. coli* was carried out by the alkaline lysis method (23). Rapid preparation of yeast total genomic DNA was carried out as described by Nasmyth and Reed (27). The yeast-*E. coli* shuttle vector YEpM4 is an extrachromosomally replicating plasmid we constructed that contains the 2μm origin of replication (14), the *LEU2* gene (30) as a selectable marker, and parts of pUC18 (39), including the multicloning region. A yeast genomic library was constructed from the PS1-2 strain (Table 1) by partial cleavage with the restriction endonuclease *Sau*3A. Fragments, between 6 and 25 kilobases (kb) in length, were isolated and then cloned into the unique *Bam*HI site of the plasmid YEpM4. Nitrocellulose filter blot hybridization was performed as previously described by Southern (33). Filters were hybridized with appropriate DNA fragments ^{32}P labeled by nick translation (24). Plasmid p*pde1*::*LEU2* was constructed by the following method. The 2.7-kb *Eco*RI-*Sal*I fragment of pYT19 (Fig. 1) was isolated and inserted into pUC19, thereby creating p*PDE1*. This plasmid was linearized at the unique *Bal*I site that is in the *PDE1*-coding sequence, and the linear 2.3-kb *Hpa*I fragment containing the yeast *LEU2* gene was inserted. The *Xba*I fragment of p*pde1*::*LEU2* was used for the disruption of the *PDE1* gene.

Heat shock and starvation of yeast cells. Yeast cells were heat shocked and starved for nitrogen by a replica method (32). Heat shock was performed by replica plating cells to a plate that had been preheated for 30 min at 55°C. This plate was incubated for 10 min at 55°C and then transferred to a 30°C incubator for 2 days. Yeast cells were starved for nitrogen by replica plating cells that had grown to stationary phase to a plate (YNB-N) that lacked a source of nitrogen. This replica was incubated at room temperature for the indicated period, then replica plated back to a plate containing nitrogen sources, and incubated at 30°C.

DNA sequencing. Restriction endonuclease fragments were cloned into either M13mp8 or M13mp9 vectors (28, 39)

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TABLE 1. Strain descriptions

Strain	Genotype
TK161-R2V	*MATa leu2 his3 ura3 trp1 ade8 can1 RAS2*$^{Val-19}$
PS1-2[a]	*MATa leu2 his3 ura3 trp1 ade8 pde2::URA3 jun1::HIS3*
DJ13	*MATa/MATα leu1/leu2 his3/his3 ura3/ura3 trp1/trp1 ade8/ade8*
DJ1301[b]	*MATa/MATα leu2/leu2 his3/his3 ura3/ura3 trp1/trp1 ade8/ade8 PDE1/pde1::LEU2*
SP1	*MATa leu2 his3 ura3 trp1 ade8 can1*
J105[c]	*MATa leu2 his3 ura3 trp1 ade8 can1 pde1::LEU2*
DJ12-9D	*MATα leu2 his3 ura3 trp1 ade8 can1 pde2::URA3*
DJ23[d]	*MATa/MATα leu2/leu2 his3/his3 ura3/ura3 trp1/trp1 ade8/ade8 can1/can1 PDE1/pde1::LEU2 PDE2/pde2::URA3*
DJ23-3A, -5C, -6B[e]	*leu2 his3 ura3 trp1 ade8 can1*
DJ23-3B, -5D, -6C[e]	*leu2 his3 ura3 trp1 ade8 can1 pde2::URA3*
DJ23-3C, -5A, -6D[e]	*leu2 his3 ura3 trp1 ade8 can1 pde1::LEU2 pde2::URA3*
DJ23-3D, -5B, -6A[e]	*leu2 his3 ura3 trp1 ade8 can1 pde1::LEU2*
DJ32[f]	*MATa/MATα leu2/leu2 his3/his3 ura3/ura3 trp1/trp1 ade8/ade8 RAS1/ras1::HIS3 RAS2/ras2::ADE8 PDE1/pde1::LEU2 pde2::URA3/pde2::URA3*
DJ36[f]	*MATa/MATα leu2/leu2 his3/his3 ura3/ura3 trp1/trp1 ade8/ade8 RAS1/ras1::HIS3 RAS2/ras2::ADE8 pde1::LEU2/pde1::LEU2 PDE2/pde2::URA3*

[a] *JUN1* gene was isolated as a suppressor of the heat shock-sensitive *RAS2*$^{Val-19}$ strain, TK161-R2V. The *JUN1* gene has been disrupted by the *HIS3* gene (Nikawa et al., unpublished results). Disruption of *PDE2* was described previously (32).

[b] Transformant of DJ13 with 4.3-kb *Xba*I fragment of plasmid p*pde1::LEU2*.

[c] Transformant of SP1 with 4.3-kb *Xba*I fragment of plasmid p*pde::LEU2*.

[d] Diploid resulting from crossing J105 with DJ12-9D.

[e] Segregants from DJ23.

[f] Diploids created by crossing haploid strains derived by transformation of J105 and DJ12-9D with the indicated markers for disruption of *RAS1* and *RAS2*. The *ras2::ADE8* disruption will be described subsequently. It contains the *ADE8* gene replacing all coding sequences of *RAS2*. The *ras1::HIS3* construction is described by Kataoka et al. (18); the *pde2::URA3* is described by Sass et al. (32).

and then sequenced by a modification of the dideoxy chain termination method (1, 31).

Phosphodiesterase assay. Yeast cells were grown aerobically in rich medium (YPD) at 30°C. Exponentially growing cells (1.7×10^7 to 2.5×10^7 cells per ml) were harvested and then washed with a solution consisting of 50 mM Tris (pH 7.4), 1 mM EDTA, 1 mM β-mercaptoethanol, and 0.5 mM phenylmethylsulfonyl fluoride. Cells were resuspended in the same buffer and lysed by passage through a French press at 20,000 lb/in^2. Extracts were centrifuged at $1,600 \times g$ for 10 min, and the resulting supernatant was centrifuged at $18,000 \times g$ for 20 min. The supernatant was assayed for phosphodiesterase activity, with 0.5 mM [^{3}H]cAMP, by a modification (36) of the procedure of Kuo et al. (9, 19). Protein concentrations were determined by the method of Bradford (3) with reagents supplied by Bio-Rad Laboratories.

cAMP determination. The yeast strains were grown in YPD medium to a cell density of 5×10^7 to 1×10^8 cells per ml. Cells were harvested by centrifugation and suspended in 1 ml of 5% trichloroacetic acid. Cells were disrupted by vortexing in the presence of glass beads (1 ml, 0.3-mm diameter). The homogenized sample was centrifuged to remove insoluble material, and the supernatant was lyophilized. The lyophilized sample was dissolved in water and used for the cAMP determination by radioimmunoassay (13). Protein concentration of whole cells was determined by the method of Lowry et al. (22) after cells were boiled for 5 min in 1 N NaOH.

RESULTS

Isolation of the *PDE1* gene. In previous work, we identified one gene, *PDE2*, that could suppress the phenotype of *RAS2*$^{Val-19}$ when present on high-copy-number plasmids (32). Another such gene, *JUN1*, was found, which we have not yet described. To continue our search for such genes, we constructed new plasmid libraries in the yeast shuttle vector YEpM4 with genomic DNA from the yeast strain PS1-2 (Table 1). This strain lacks functional *PDE2* and *JUN1* genes because of disruption by prototrophic markers. The *RAS2*$^{Val-19}$ strain TK161-R2V was transformed with the plasmid library DNA, and about 12,000 Leu$^+$ transformants were selected and tested for heat shock sensitivity by a replica-plating method (32). Six independent heat shock-resistant transformants were obtained from these colonies. Segregation analysis showed that cells that lost the high-copy-number plasmid (i.e., were Leu$^-$) were heat shock sensitive, and cells were resistant to heat shock only if they retained the *LEU2* marker. The plasmids in these cells were transferred to *E. coli* by standard methods (23), and their DNA inserts were compared by restriction enzyme analysis. Three different genomic inserts were identified among these six transformants (Fig. 1A). These inserts ranged in size from 6.0 to 8.5 kb but contained overlapping DNA inserts from a locus we now call *PDE1*. Transformation of TK161-R2V with one *PDE1* plasmid, pYT22, clearly restored heat shock resistance to TK161-R2V, as well as the ability to survive prolonged starvation for nitrogen sources (Fig. 2).

To localize the functional region of the *PDE1* locus, we carried out subcloning experiments. We constructed several subclones by deleting various restriction fragments from plasmid pYT19 or pYT20 (Fig. 1B). Strain TK161-R2V (containing the *RAS2*$^{Val-19}$ mutation) was transformed with these plasmids and tested for heat shock resistance. The results clearly showed that *PDE1* was located within the 1.9-kb *Eco*RI-*Sma*I fragment. Both strands of this region were sequenced to define its coding potential.

The sequencing strategy is shown in Fig. 3A, along with the nucleotide sequence and predicted amino acid sequence (Fig. 3B). One large open reading frame of 369 codons, initiated by ATG, was found. This gave a calculated molecular mass of 42,056 daltons for the protein. A putative transcription start signal sequence TATAATA (12) was present in the 5′-flanking region beginning at nucleotide

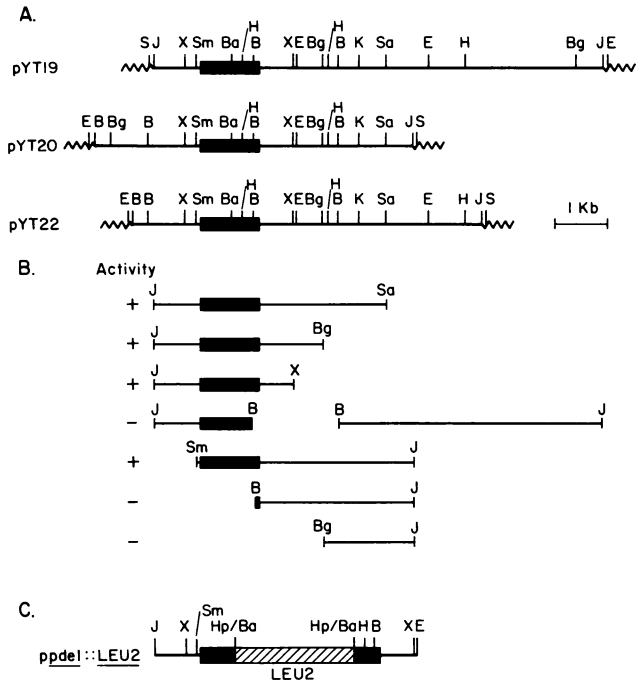


FIG. 1. Structure and disruption of the *PDE1* gene. (A) Restriction maps of the inserts in plasmids pYT19, pYT20, and pYT22 and the flanking regions of the vectors. Coding sequences (▬), determined subsequently, and vector sequences (〰) are indicated. (B) Subcloning strategy used to locate the *PDE1* gene. Restriction fragments which were cloned into the vector YEpM4 are indicated. The ability to suppress the heat shock-sensitive phenotype of the $RAS2^{Val-19}$ mutation is indicated for each plasmid (+, active fragment; −, inactive fragment). (C) Structure of *ppde1::LEU2* disruption plasmid. The *PDE1* gene was disrupted by inserting the fragment of *LEU2* gene (▥) at a unique *Bal*I site. See Materials and Methods for the construction of the plasmid. Abbreviations used: B, *Bam*HI; Ba, *Bal*I; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; K, *Kpn*I; S, *Sal*I; Sa, *Sac*I; Sm, *Sma*I; X, *Xba*I; J, junction between an insert DNA and a *Bam*HI cleavage site of the vector YEpM4.

−238. In our previous paper, comparison of the amino acid composition of *PDE2* with the known amino acid composition of yeast phosphodiesterases indicated that *PDE2* was likely to encode the high-affinity cAMP phosphodiesterase (32, 34). Similar comparisons indicate that *PDE1* might encode the low-affinity cAMP phosphodiesterase (21), since the *PDE1* gene product is predicted to have a molecular weight and amino acid composition similar to those of the low-affinity enzyme (Table 2). This conclusion is confirmed by biochemical data described below.

Gene disruption of *PDE1*. To continue our analysis, we constructed a plasmid containing the *LEU2* marker inserted into *PDE1*-coding sequences (Fig. 1C). The 4.3-kb *Xba*I fragment of the plasmid *ppde1::LEU2* was used to disrupt one *PDE1* locus of the diploid yeast strain DJ13. Tetrad analysis indicated that $pde1^-$ haploid progeny were viable. This conclusion was confirmed by Southern blot hybridization (data not shown). We also obtained Leu^+ transformants at the expected frequency by transforming the haploid strain SP1 with the same fragment of the *PDE1* gene disrupted by *LEU2*. Thus, like *PDE2*, *PDE1* is not by itself an essential gene.

To test if haploids containing disruptions of both *PDE1* and *PDE2* were viable, we mated a $pde1^-$ haploid with a $pde2^-$ haploid such that the resulting diploids were heterozygous at each *PDE* locus with distinct prototrophic markers at each disrupted allele. Upon sporulation and tetrad analysis, we found that the $pde1^-$ $pde2^-$ progeny were, in fact, viable. These results enabled us to perform biochemical analysis of yeast strains deficient for either *PDE1*, *PDE2*, or both.

Two forms of cAMP phosphodiesterase have been reported in *S. cerevisiae*: a high-affinity form of narrow specificity and a low-affinity form of broad specificity (21, 34). We have measured the presence of cAMP phosphodiesterase in strain DJ12-9D, which is $pde2^-$, and in strain DJ23-3C, which is both $pde1^-$ $pde2^-$. We used strains lacking *PDE2* to avoid interference in the assay by the high-affinity activity encoded by this gene. Previous enzymatic analysis indicated that $pde2^-$ strains lack any activity of the high-affinity form but contain a low-affinity form of phosphodiesterase (32). The assay, using high concentrations of [^{3}H]cAMP, has been previously described (32). The results shown in Fig. 4

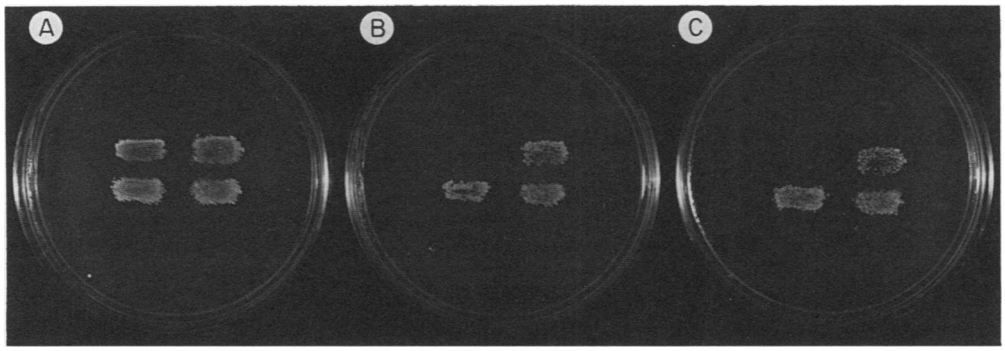
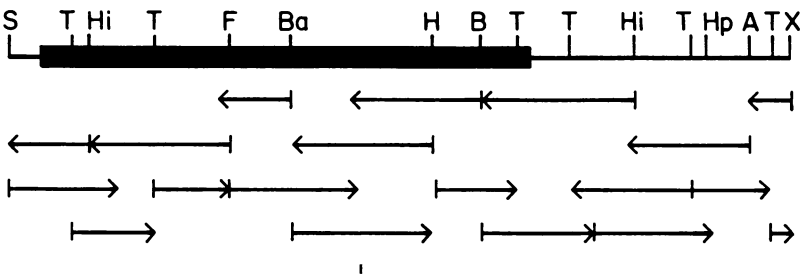


FIG. 2. Heat shock and starvation phenotypes. (A) The master plate (SC-leucine) was incubated with patches from four strains: $RAS2^{Val-19}$ strain TK161-R2V carrying a *LEU2* plasmid (YEpM4), upper left; wild-type strain SP1 carrying a *LEU2* plasmid (YEpM4), lower left; $RAS2^{Val-19}$ strain TK161-R2V carrying the plasmid pYT22 which contains *PDE1* and *LEU2* genes, upper right; and $RAS2^{Val-19}$ strain TK161-R2V carrying the plasmid YEp*PDE2-1* (32) which contains *PDE2* and *LEU2* genes, lower right. (B) The master plate was replica plated to a plate (SC-leucine) that had been preheated at 50°C for 30 min. The replica was incubated at 55°C for 10 min and then at 30°C for 2 days. (C) The same master plate used above was replica plated to YNB-N (a plate that lacks a source of nitrogen). The replica was kept at room temperature for 5 days and then replica plated to a SC-leucine plate. This plate was incubated at 30°C for 2 days.

A.



B.

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-605                                                                TCTAGATTTGGGATTTA
     ACATCCTCTTAGTACGGAAAATTTTCCAGACATATAGTCTCGAAGAATTTTTTATGGCAACTAATAA
-521 AGGAAGAAAGCAAAGCTGTTAAAATGCTTACGGTTTCACAAATGACTCGCCTGAAGAAGTTATTAAC
     CTTAGAATGGTAAATCCAGCAAGGATCGTTACCCGGTAAACTATTTATACTACGCCTGCCTCTTTCG
-387 TTCTTCGACTACATGCTACAGGTTAGTAAATTCAACCAAGGAAAATGTATTTATTAACATGAACTAG
     CCACCTAATTAACCCTTACCTTATTTTTTATACCCTTGGATCATCGGAGTATCTGTAGATCATATGT
-253 TAACGTATGTATAATATTACATCTGTCACAATAAACAACCCGAAAAAAATACCGCACGGAAAATGAG
     TTACTGATTAAGATGCACTTTTTGGTGAGGGTGCGTGACTTGAAACTAAAAATTACCAATAGTCAAA
-119 CCCTTTCCATTTTCGAGGGAAGCAATATCTGAAATTTATATTTTTAGAAATTTCCAAGAGAAAGTGG
          •••
 -52 CTTTTAA CGA GGT TCT TCT TCT TCA TCC CCT TTT TTA CCA ATA TTC CTT TTT

   1 Met Val Val Phe Glu Ile Thr Ile Leu Gly Ala Asn Gly Gly Pro Thr Glu
   1 ATG GTT GTA TTC GAA ATA ACT ATA CTT GGG GCC AAT GGA GGA CCC ACC GAA

  18 Tyr Gly Thr Gln Cys Phe Ile Leu Lys Pro Ala Arg Thr Glu Asp Pro Glu
  52 TAC GGA ACA CAG TGT TTT ATA CTT AAG CCT GCT AGA ACA GAG GAT CCA GAA

  35 Leu Ile Ala Val Asp Gly Gly Ala Gly Met Tyr Gln Leu Arg Glu Met Leu
 103 TTA ATA GCT GTA GAC GGT GGT GCA GGA ATG TAC CAG CTA CGG GAG ATG TTG

  52 Val Gln Gly Arg Asn Glu Asn Glu Gly Asp Asp Glu Leu Val Pro Ser Phe
 154 GTC CAA GGG CGA AAT GAA AAT GAA GGT GAT GAT GAG CTT GTT CCA AGC TTT

  69 Tyr Glu His Asp Arg Glu Pro Ile Glu Phe Phe Ile Asp Ser Lys Leu Asn
 205 TAT GAA CAT GAT CGA GAG CCA ATA GAG TTT TTT ATT GAT TCC AAA CTG AAC

  86 Ile Gln Lys Gly Leu Ser Lys Ser Phe Leu Gln Ser Leu Lys Arg Gln Gly
 256 ATA CAA AAG GGA TTA TCC AAG TCC TTC CTA CAA TCA TTA AAG AGG CAG GGA

 103 Glu His Phe Glu Ser Ala Asn Thr Met Lys Lys Thr Tyr Glu Val Phe Gln
 307 GAG CAT TTT GAA AGC GCC AAT ACA ATG AAG AAA ACT TAC GAG GTT TTC CAA

 120 Gly Ile Thr Asp Tyr Tyr Ile Thr His Pro His Leu Asp His Ile Ser Gly
 358 GGA ATT ACC GAC TAC TAC ATT ACC CAT CCC CAT CTG GAC CAT ATT AGT GGG

 137 Leu Val Val Asn Ser Pro Ser Ile Tyr Glu Gln Glu Asn Ser Lys Lys Lys
 409 CTG GTA GTA AAC TCC CCC TCA ATA TAT GAG CAG GAA AAC AGT AAA AAG AAA

 154 Thr Ile Trp Gly Leu Pro His Thr Ile Asp Val Leu Gln Lys His Val Phe
 460 ACT ATT TGG GGA TTA CCG CAT ACT ATT GAC GTT TTG CAA AAG CAT GTC TTT

 171 Asn Asp Leu Ile Trp Pro Asp Leu Thr Ala Glu Arg Ser Arg Lys Leu Lys
 511 AAT GAC TTA ATA TGG CCA GAT TTA ACG GCA GAA CGT TCA AGA AAA TTG AAG

 188 Leu Lys Cys Leu Asn Pro Lys Glu Val Gln Lys Cys Thr Ile Phe Pro Trp
 562 CTA AAA TGC TTG AAT CCG AAG GAA GTT CAA AAG TGC ACT ATC TTT CCT TGG

 205 Asp Val Ile Pro Phe Lys Val His His Gly Ile Gly Val Lys Thr Gly Ala
 613 GAT GTA ATA CCG TTC AAA GTT CAT CAT GGG ATA GGC GTC AAG ACT GGC GCG

 222 Pro Val Tyr Ser Thr Phe Tyr Ile Phe Arg Asp Arg Lys Ser Lys Asp Cys
 664 CCG GTA TAC AGC ACC TTC TAT ATC TTC AGA GAC AGG AAA AGC AAA GAC TGT

 239 Ile Ile Val Cys Gly Asp Val Glu Gln Asp Arg Arg Glu Ser Glu Glu Ser
 715 ATA ATA GTT TGC GGA GAT GTT GAG CAG GAC CGC AGA GAA TCT GAA GAA TCT

 256 Leu Leu Glu Glu Phe Trp Ser Tyr Val Ala Glu Asn Ile Pro Leu Val His
 766 CTA CTT GAA GAA TTT TGG TCT TAC GTT GCT GAA AAT ATA CCG CTT GTG CAT

 273 Leu Lys Gly Ile Leu Val Glu Cys Ser Cys Pro Leu Ser Ser Lys Pro Glu
 817 CTC AAG GGT ATA TTA GTC GAA TGT TCA TGC CCA TTA TCT TCC AAG CCC GAG

 290 Gln Leu Tyr Gly His Leu Ser Pro Ile Tyr Leu Ile Asn Glu Leu Ser Asn
 868 CAA TTG TAT GGT CAT CTA TCT CCA ATA TAT TTA ATC AAC GAA TTA TCC AAT

 307 Leu Asn Thr Leu Tyr Asn Ser Ser Lys Gly Leu Ser Gly Leu Asn Val Ile
 919 TTG AAC ACT TTA TAC AAC AGT AGT AAG GGA TTA AGC GGT TTG AAT GTT ATC

 324 Val Thr His Val Lys Ser Thr Pro Ala Lys Arg Asp Pro Arg Leu Thr Ile
 970 GTT ACT CAC GTT AAG TCA ACA CCT GCT AAA AGA GAC CCA AGA CTA ACC ATA

 341 Leu Glu Glu Leu Arg Phe Leu Ala Glu Glu Arg Asn Leu Gly Asp Leu Arg
1021 CTC GAA GAG TTA CGA TTT TTA GCT GAG GAG AGG AAC TTA GGA GAC TTG AGA

 358 Ile Ser Ile Ala Leu Glu Gly His Thr Leu Phe Leu •••
1072 ATA TCT ATT GCG CTA GAA GGC CAC ACT TTG TTT CTA TAATGTACACAACGTCAAT

1127 AACATAATACTACGTAGATGAAAAGCGGAAAAATTCATTACCCGGG
```

TABLE 2. Comparison of the amino acid composition of the high-K_m cAMP phosphodiesterase and *PDE1* gene product[a]

Amino acid	No. of residues	
	PDE1 gene product	High-K_m cAMP phosphodiesterase
Ala	11	13
Arg	16	16
Asx	34	39
Cys	7	ND[b]
Glx	45	47
Gly	24	23
His	13	13
Ile	28	26
Leu	42	44
Lys	25	25
Met	4	1
Phe	16	15
Pro	19	21
Ser	26	28
Thr	19	20
Trp	4	6
Tyr	13	13
Val	23	21

[a] Data for the high-K_m phosphodiesterase were derived from purified protein (21), whereas the data for *PDE1* were deduced from its nucleotide sequence. The molecular weight was 42,056 for the *PDE1* gene product and 43,000 for the high-K_m cAMP phosphodiesterase.
[b] ND, Not determined.

indicate that the DJ12-9D strain contained appreciable cAMP phosphodiesterase activity, while DJ23-3C strain had none. These results are consistent with the conclusion that *PDE1* does indeed encode a low-affinity phosphodiesterase and suggest that *PDE1* and *PDE2* together encode the cAMP phosphodiesterase activity detectable in total extracts of *S. cerevisiae*.

Phenotypic and genotypic consequences of perturbation of *PDE1* and *PDE2*. Measurements of cAMP in mutant cells were undertaken to test the above conclusions (Table 3). The results are somewhat surprising. cAMP levels were slightly elevated in the *pde2*$^-$ strain DJ23-3B and elevated two- to threefold in *pde1*$^-$ *pde2*$^-$ strain DJ23-3C. These changes were small but highly reproducible and confirm the conclusions that both *PDE* genes encode cAMP phosphodiesterases and that cAMP levels are jointly controlled. Nevertheless, it is very puzzling that cAMP levels were raised so modestly in cAMP phosphodiesterase-deficient strains. Explanations for this observation are discussed below.

Although elevations in cAMP were modest in *pde1*$^-$ *pde2*$^-$ strains, the phenotypic effects of such disruption are severe and resemble those induced by the presence of the *RAS2*$^{Val-19}$ mutation (18, 37). This mutation also modestly elevates cAMP levels (37). As is seen in Fig. 5, *pde1*$^-$ *pde2*$^-$ strains were heat shock sensitive, did not endure starvation, and did not stain with iodine (8), i.e., did not accumulate storage carbohydrates. Elimination of either *PDE* gene alone had only a minimal effect on phenotype, although some *pde2*$^-$ strains showed less accumulation of storage carbohydrates than the wild type did. The *pde1*$^-$ *pde2*$^-$ strains also could not grow on an acetate plate (2% acetate instead of glucose of YPD), a severe phenotype seen previously in *bcy1*$^-$ strains which lack the regulatory subunit of cAMP-dependent protein kinase (36a). The assignment of these phenotypes to the *pde* disruptions was confirmed by plasmid transformation and plasmid segregation experiments (data not shown).

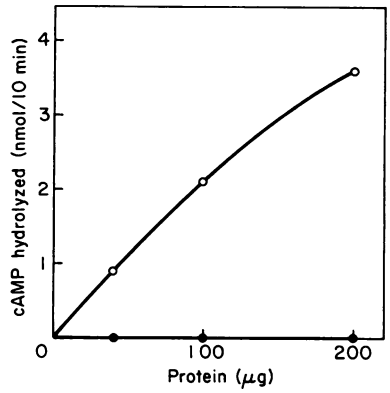

FIG. 4. Determination of phosphodiesterase activity in extracts from *PDE1* and *pde1::LEU2* strains. Crude extracts from the strains DJ12-9D (*PDE1 pde2::URA3*) (○) and DJ23-3C (*pde1::LEU2 pde2::URA3*) (●) were prepared and used for the determination of cAMP phosphodiesterase activity as described in Materials and Methods. The concentration of protein in the assay mixture was varied as indicated.

We next tested if disruption of the phosphodiesterase genes could suppress the lethality which ordinarily results from the disruption of both the *RAS1* and *RAS2* genes. Diploid strains DJ32 (+/*ras1* +/*ras2* +/*pde1 pde2*/*pde2*) and DJ36 (+/*ras1* +/*ras2 pde1*/*pde1* +/*pde2*) were constructed with auxotrophic disruptions at the indicated loci. Tetrad analysis, shown in Table 4, shows that cells lacking *RAS1* and *RAS2* were viable when they also lacked *PDE1* and *PDE2*. Some spores lacking *RAS1*, *RAS2*, and *PDE2* germinated and underwent a limited number of doublings. This result is consistent with the previous findings that disruption of *BCY1* (36a, 38), overexpression of the cAMP-dependent protein kinase catalytic subunits (36b), or overexpression of adenylate cyclase (16) can suppress the essential requirements for *RAS* function in the yeast *S. cerevisiae*. Moreover, this finding implies that adenylate cyclase must have some activity even in the absence of *RAS* function.

DISCUSSION

We have sought genes which in high copy number can reverse the phenotype of the *RAS2*$^{Val-19}$ mutation. Two *S. cerevisiae* genes encoding cAMP phosphodiesterases, *PDE1* and *PDE2*, were capable of this. Two other genes, *JUN1* and *JUN2*, of unknown function, have also been found to suppress the phenotype of *RAS2*$^{Val-19}$ (unpublished results). Disruption of both phosphodiesterases led to a phenotype similar to that induced by the *RAS2*$^{Val-19}$ mutation. Moreover, disruption of both phosphodiesterase genes suppressed the lethality which otherwise results from disruption of both yeast *RAS* genes. These results support in a general

FIG. 3. Sequencing strategy and the nucleotide sequence of the *PDE1* gene. (A) Strategy used for sequencing the *PDE1* gene. The thick line represents the coding region. The directions and approximate extents of the sequences obtained are indicated by the arrows. Abbreviations used: A, *Alu*I; B, *Bam*HI; Ba, *Bal*I; F, *Fnu*DII; H, *Hin*dIII, Hi, *Hin*cII; Hp, *Hpa*I; S, *Sma*I; T, *Taq*I; X, *Xba*I. (B) Nucleotide sequence is presented together with the predicted amino acid sequence of the open reading frame. Coordinates on the left indicate nucleotide and amino acid positions. Asterisks indicate the termination codons.

TABLE 3. Determination of the level of cAMP in *PDE* mutants

Strain	Genotype[a]		cAMP level[b] (pmol/mg of protein)
	PDE1	*PDE2*	
DJ23-3A	+	+	2.1
DJ23-3B	+	−	3.0
DJ23-3C	−	−	4.4
DJ23-3D	−	+	2.1

[a] The full genotypes of the indicated strains are given in Table 1.
[b] Cells were grown in YPD medium and used for the determination of cAMP as described in Materials and Methods.

way our contention that the major effects of the *RAS*-encoded products are mediated through alterations in cAMP.

Two cAMP phosphodiesterases have been purified and characterized from yeasts (21, 34). The genes for both have now been cloned. Mutants in a low-affinity cAMP phosphodiesterase have been reported by Uno et al. (38), who called this mutant *pde1*. We presume that our *PDE1* is the same locus described by Uno et al., although we have not proven this. From our analysis, it seems that both *PDE1* and *PDE2* proteins are capable of regulating cAMP levels in cells. Redundancy in the growth regulatory pathways of *S. cerevisiae* seems to be a recurrent theme in this organism. There are two *RAS* genes with redundant function (10, 11, 18, 29), and recently we have discovered that three genes encode catalytic subunits of the cAMP-dependent protein kinase system (36b). The advantage of this redundancy to the organism is not clear. It is possible that these genes will prove to have distinguishable functions and may be under somewhat independent control. Alternatively, safety in numbers may apply, especially to an organism that spends part of its time in the haploid state.

TABLE 4. Tetrad dissection of DJ32 (+/*ras1* +/*ras2* +/*pde1* *pde2*/*pde2*) and DJ36 (+/*ras1* +/*ras2* *pde1*/*pde1* +/*pde2*) diploids[a]

Genotype of progeny				No. of large viable colonies/total spores for diploid strain	
RAS1	*RAS2*	*PDE1*	*PDE2*	DJ32	DJ36
+	+	+	−	10/10	
−	+	+	−	11/11	
+	−	+	−	9/9	
−	−	+	−	0/9[b]	
+	+	−	−	9/9	6/6
−	+	−	−	10/10	12/12
+	−	−	−	12/12	11/11
−	−	−	−	10/10	7/11
+	+	−	+		15/15
−	+	−	+		7/7
+	−	−	+		8/8
−	−	−	+		0/10[c]

[a] The full genotypes of the indicated strains and of the progeny, determined by the presence of disruption markers, are given in Table 1.
[b] No tiny colonies were observed.
[c] Some spores formed tiny colonies observable by microscopy.

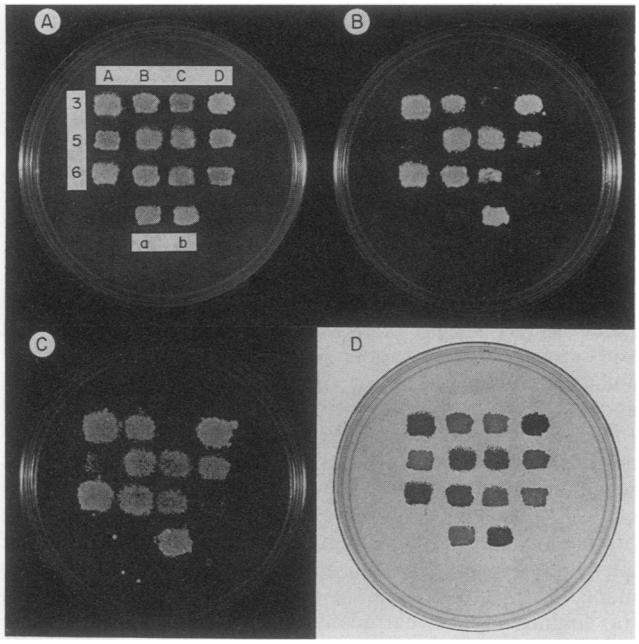

FIG. 5. Phenotypes of *PDE* disrupted strains. (A) The master plate (YPD) was incubated with the patches of tetrad segregants from diploid DJ23 (Table 1). Tetrads derived from the same asci are aligned on the horizontal. Genotypes of each segregant are as follows: *PDE1 PDE2* for DJ23-3A, DJ23-5C, and DJ23-6B; *PDE1 pde2*::*URA3* for DJ23-3B, DJ23-5D, and DJ23-6C; *pde1*::*LEU2 PDE2* for DJ23-3D, DJ23-5B, and DJ23-6A; and *pde1*::*LEU2 pde2*::*URA3* for DJ23-3C, DJ23-5A, and DJ23-6D. The control strains used for this experiment were (a) the *RAS2*$^{Val-19}$-containing strain, TK161-R2V, and (b) the wild-type strain, SP1. 3A to 3D, 5A to 5D, and 6A to 6D represent tetrad, segregants from the diploid strain DJ23. See Table 1 for a description of these strains. (B) Heat shock phenotype. The master plate was replica plated to a rich plate (YPD) that had been preheated at 55°C for 30 min. The replica was incubated at 55°C for 10 min and then at 30°C for 2 days. (C) Nitrogen starvation phenotype. The master plate was replica plated to a plate (YNB-N) that lacked a source of nitrogen. The replica was incubated at room temperature for 19 days and then replica plated to a rich plate (YPD). The YPD plate was then incubated at 30°C for 2 days. (D) The iodine-staining phenotype. The tetrad segregants from DJ23 were grown on a YPD plate at 30°C and stained by iodine (8) to detect the accumulation of glycogen and trehalose as described previously (37).

One puzzling aspect of our work is that cAMP levels were only modestly elevated in *pde1*$^-$ *pde2*$^-$ strains. Several possible explanations can be considered. First, there may be another cAMP phosphodiesterase in *S. cerevisiae* that has not yet been detected. We have examined the crude extract from a *pde1*$^-$ *pde2*$^-$ strain and found no phosphodiesterase activity. We cannot rule out the possibility that *S. cerevisiae* has another phosphodiesterase that is labile or requires an unknown cofactor for its activity. Second, cAMP may be secreted by cells. Indeed, cAMP-permeable strains of *S. cerevisiae* have been described (25). Third, there may be a feedback mechanism which diminishes cAMP production when either cAMP levels or cAMP-dependent protein kinase activity is elevated. Indeed, the latter possibility is actually correct, and data on this will be presented shortly.

We have compared the amino acid sequence of the *PDE1* gene product with those of other known phosphodiesterases. Previously, sequence conservation between *PDE2* and a *Drosophila* high-affinity cAMP phosphodiesterase, a calmodulin-stimulated phosphodiesterase from bovine brain, and a cyclic GMP-stimulated cAMP phosphodiesterase from bovine heart has been reported (6, 7). *PDE1* showed no apparent homology to *PDE2* or these other phosphodiesterases but, surprisingly, did show homology to the cyclic nucleotide phosphodiesterase of *Dictyostelium discoideum* (20), which is a secreted protein (Fig. 6). Amino acid stretches 124 to 172 and 259 to 297 in yeast *PDE1* show particularly strong homology to this phosphodiesterase.

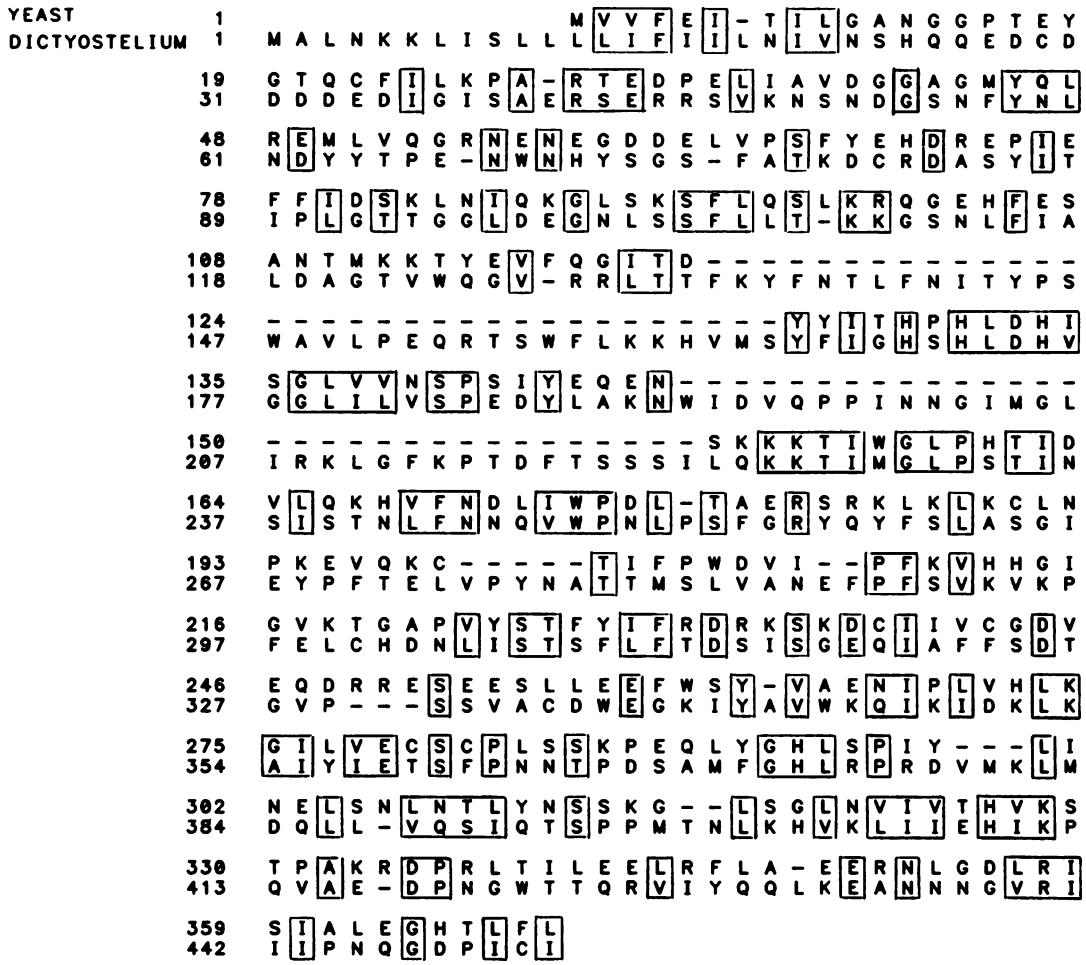

FIG. 6. Primary sequence homology of yeast low-affinity phosphodiesterase and *D. discoideum* cyclic nucleotide phosphodiesterase. The amino acid sequence of the yeast *PDE1* gene product was aligned with the *Dictyostelium* cyclic nucleotide phosphodiesterase (20). Identical or conservative amino acid substitutions are boxed. Conservative amino acid substitutions are grouped as follows: A = G, D = E, Q = N, S = T, K = R, I = L = V (where "=" means equivalent amino acid substitution).

Like the yeast *PDE1*, the *D. discoideum* phosphodiesterase shows no homology to the previously identified phosphodiesterases. It appears that at least two distinct branches of cAMP phosphodiesterases have arisen during evolution.

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