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Three Different Genes in S. cerevisiae Encode the Catalytic Subunits of the cAMP-Dependent Protein Kinase

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Summary

We have isolated three genes (*TPK1*, *TPK2*, and *TPK3*) from the yeast S. cerevisiae that encode the catalytic subunits of the cAMP-dependent protein kinase. Gene disruption experiments demonstrated that no two of the three genes are essential by themselves but at least one *TPK* gene is required for a cell to grow normally. Comparison of the predicted amino acid sequences of the *TPK* genes indicates conserved and variable domains. The carboxy-terminal 320 amino acid residues have more than 75% homology to each other and more than 50% homology to the bovine catalytic subunit. The amino-terminal regions show no homology to each other and are heterogeneous in length. The *TPK1* gene carried on a multicopy plasmid can suppress both a temperature-sensitive *ras2* gene and adenylate cyclase gene.

Introduction

In eukaryotes, the "second messenger" cAMP is known to exert its effects by activating a cAMP-dependent protein kinase (Robinson et al., 1971). Holoenzyme of this kinase is a tetrameric protein consisting of two catalytic subunits and two regulatory subunits. cAMP activates the kinase by dissociating the inactive holoenzyme into two active monomeric catalytic subunits and the dimeric regulatory subunit (Krebs and Beavo, 1979). In the yeast Saccharomyces cerevisiae, cAMP plays a critical role in cell cycle progression (for review, see Matsumoto et al., 1985). We have found that yeast *RAS* proteins, which are structurally homologous to mammalian *RAS* oncoproteins, modulate adenylate cyclase (Toda et al., 1985; Broek et al., 1985). Yeast strains deficient in *RAS* function have low levels of cAMP and exhibit properties similar to those of adenylate cyclase–deficient yeast. In contrast, strains that have an activated form of *RAS2*, *RAS2*^{val19}, have elevated levels of cAMP and show very similar phenotypes to yeast strains that have activated cAMP-dependent protein kinase (Toda et al., 1985). To understand better how *RAS*/cAMP regulates cell growth, we have begun to isolate genes that are involved in this effector pathway. We have described the yeast adenylate cyclase gene, *CYR1* (Kataoka et al., 1985); the *RAM* gene, which is required for processing *RAS* proteins (Powers et al., 1986); and the *CDC25* gene, which, we propose, modulates the activity of *RAS* proteins (Broek et al., 1987). We also reported the cloning and characterization of *BCY1*, which encodes the regulatory subunit of the cAMP-dependent protein kinase (Toda et al., 1987), and the *PDE1* and *PDE2* genes, which encode cAMP phosphodiesterases (Sass et al., 1986; Nikawa et al., unpublished data). In this paper we present the nucleotide sequence of the genes for the cAMP-dependent protein kinase catalytic subunits, which are encoded by three similar but distinct genes (*TPK1*, *TPK2*, and *TPK3*). We also present biochemical and genetic analyses of the cAMP-dependent protein kinase system in yeast.

Results

Isolation of the *TPK1* Gene

We transformed a temperature-sensitive *cdc25* strain, TT25-6 (see Table 1), with pooled DNA from a S. cerevisiae genomic library that had been constructed in the centromere-containing *URA3* vector YCp50 (kindly provided by M. Rose and G. Fink). Transformants were directly incubated at 35°C on synthetic plates lacking uracil. Colonies that could grow at 35°C were picked and plasmid segregation analysis was performed. Transformants whose growth at 35°C was plasmid-dependent were grown, and their plasmids were recovered in E. coli. Two different suppressor plasmids were obtained. One of these plasmids was shown to be allelic to the *CDC25* locus by an integrative mapping method (Broek et al., 1987). The other suppressor (designated as *TPK1*) had a restriction endonuclease map which differed from that of *CDC25*. Integrative mapping indicated that its chromosomal locus is not linked to *CDC25* (see Experimental Procedures). Previous results had shown that plasmids containing either the S. cerevisiae adenylate cyclase gene *CYR1* (Kataoka et al., 1985) or the *RAS2* gene (Powers et al., 1984) could suppress the temperature-sensitive *cdc25* strain (Broek et al., 1987). We therefore expected it would be possible to clone additional new genes in the *RAS*/cAMP effector pathway by isolating a suppressor plasmid of the *cdc25*^{ts} strain.

To locate the coding region of *TPK1*, various restriction fragments were isolated and subcloned into the yeast shuttle vector, YEp13 or YEp213 (Sherman et al., 1982). These plasmids were transformed into the *cdc25*^{ts} strain TT25-6 and examined for complementing activity (Figure 1). A plasmid subclone that contained the 2.4 kb HindIII–SphI fragment was found to be the minimum complementing fragment. Restriction endonuclease mapping indicated that *TPK1* was not *CYR1*, *RAS1*, or *RAS2*.

Isolation of Genes Homologous to *TPK1*

To examine whether the *TPK1* gene is a member of a larger gene family, a genomic Southern blotting experiment was performed using the 1.0 kb XbaI fragment isolated from the *TPK1* clone as a probe (see Figure 1), under both high and low stringency conditions (Figure 2). This *TPK1* probe hybridized strongly to itself (>10 kb with EcoRI, 4.6 kb with HindIII, and 4.6 kb with EcoRI–HindIII), and weakly to two additional homologous fragments (3.4 kb and 2.2 kb bands with EcoRI, 2.7 kb and 2.0 kb bands

Table 1. Strain Description

Strain	Genotype Derivation	Source
25-1	*Matα leu2 ura1 ade2 cdc25-1*	Obtained from Dr. Johnston.
TT25-6	*Matα leu2 ura3 trp1 can1 cdc25-1*	
T3-28C	*Mata his3 leu2 ura3 trp1 ade8*	Toda et al. (1985).
SP1	*Mata his3 leu2 ura3 trp1 ade8 can1*	CSHL collection.
DC124	*Matα his4 leu2 ura3 trp1 ade8*	CSHL collection.
DC5	*Mata his3 leu2 can1 gal2 mal*	CSHL collection.
AB320	*HO ade2 lys2 trp5 leu2 can1 ura3*	Nasmyth and Reed (1980)
TT19	*Mata his3 leu2 ura3 trp1 ade8*	Segregant from cross between SP1 and DC124.
TT152	*Matα his3 leu2 ura3 trp1 ade8*	Segregant from cross between SP1 and DC124.
T162	A diploid from a cross between TT19 and TT152	
TTS162-1	*Mata/Matα his3/his3 leu2/leu2 ura3/ura3 trp1/trp1 ade8/ade8 tpk1::URA3/+*	Transformant of T162 with HindIII–SphI fragment of p*tpk1::URA3*.
TTS162-2	*Mata/Matα his3/his3 leu2/leu2 ura3/ura3 trp1/trp1 ade8/ade8 tpk1::URA3/+ tpk2::HIS3/+*	Transformant of TTS162-1 with EcoRI fragment of p*tpk2::HIS3*.
TTS162-3	*Mata/Matα his3/his3 leu2/leu2 ura3/ura3 trp1/trp1 ade8/ade8 tpk1::URA3/+ tpk2::HIS3/+ tpk3::TRP1/+*	Transformant of TTS162-2 with PvuII fragment of p*tpk3::TRP1*.
T162-1A	*Matα his3 leu2 ura3 trp1 ade8 tpk2::HIS3 tpk3::TRP1*	Segregant from the TTS162-3 diploid
TT162-1B	*Matα his3 leu2 ura3 trp1 ade8 tpk1::URA3 tpk3::TRP1*	Segregant from the TTS162-3 diploid.
T162-3C	*Mata his3 leu2 ura3 trp1 ade8 tpk1::URA3 tpk2::HIS3*	Segregant from the TTS162-3 diploid.
T162-1AB	Transformant of T162-1A with YEp24-*BCY1*	
T162-1ABT	Transformant of T162-1AB with YEp*TPK1*	
T162-1ABY	Transformant of T162-1AB with YEp13	
TTS3000-TF1	*Matα his3 leu2 ura3 trp1 ade8 tpk2::His3 tpk3::TRP1 bcy1::URA3*	Transformant of T162-1A with BamHI fragment of p*bcy1::URA3*.
TTS3100-TF2	*Matα his3 leu2 ura3 trp1 ade8 tpk1::URA3 tpk3::TRP1 bcy1::LEU2.*	Transformant of T162-1B with BamHI fragment of p*bcy1::LEU2*.
TTS3200-TF3	*Mata his3 leu2 ura3 trp1 ade8 tpk1::URA3 tpk2::HIS3 bcy1::LEU2*	Transformant of T162-3C with BamHI fragment of p*bcy1::LEU2*.
M76-3C	*Mata leu2 his3 cyr1-1*	Obtained from Dr. Szostak.
T50-3A	*Matα his3 leu2 ura3 trp1 cyr1-2*	Kataoka et al. (1985).

with HindIII, and 1.8 kb doublet bands with EcoRI–HindIII, respectively). These results suggested the existence of two homologous genes in S. cerevisiae. To clone these, colony filter hybridizations using the 1.0 kb XbaI fragment of *TPK1* as a probe were performed against two different yeast genomic libraries that had been constructed in the plasmids YEp13 and YEp213 (Sherman et al., 1982; see Experimental Procedures). Hybridization signals were very strong to some colonies and weaker to others. Both strongly and weakly hybridizing colonies were picked and the insert fragments were mapped with various restriction enzymes. Consistent with the genomic Southern hybridization, plasmid DNAs representing three different loci were isolated. All of the plasmids from strongly hybridizing colonies derived from the *TPK1* locus. However, the other plasmids from weakly hybridizing colonies contained inserts that were clearly different from *TPK1*, and derived from two loci we designated as *TPK2* and *TPK3* (Figure 3).

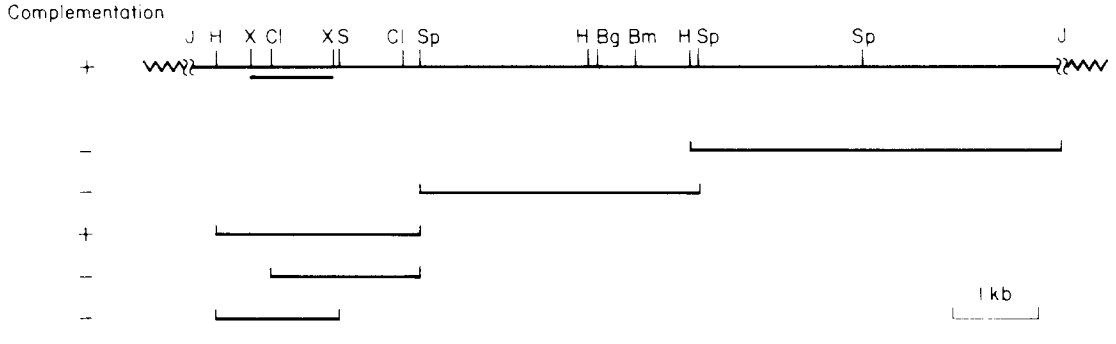


Figure 1. Restriction Map and Subcloning Analysis of *TPK1*

Structure and subcloning results of suppressor sequence *TPK1* are shown. Each fragment indicated in the figure was inserted into YEp13 (Broach et al., 1979) or YEp213 (Sherman et al., 1982). The resultant subclones were transformed into TT25-6 (temperature-sensitive *cdc25* strain; see Table 1) and suppression of temperature sensitivity was examined. "J" represents a junction between an insert yeast DNA and the vector. Abbreviations used are as follows: Bg, BglII; Bm, BamHI; Cl, ClaI; S, SalI; Sp, SphI; H, HindIII; X, XbaI. Only the 2.4 kb HindIII–SphI fragment (the left-most fragment in the figure) was mapped with ClaI, SalI, and XbaI. The 1.0 kb XbaI fragment that was used as a probe for genomic Southern hybridization is underlined (see the text and Figure 2).

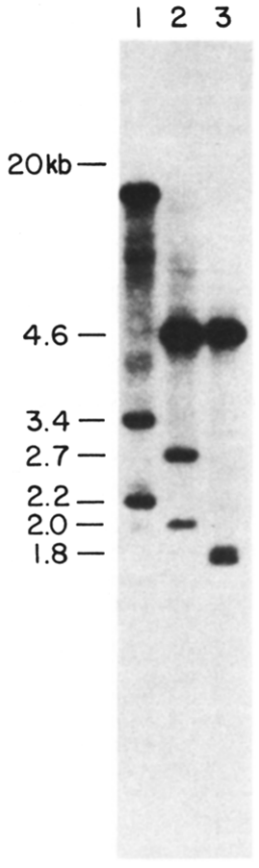


Figure 2. Genomic Southern Hybridization with *TPK1* Probe

Three micrograms of total yeast genomic DNA isolated from DC5 (Table 1) was digested with the indicated restriction endonucleases, electrophoresed on a 0.7% agarose gel, transferred to nitrocellulose, and hybridized with ^{32}P-labeled probe. Hybridization conditions were 55°C in 6× SSC/0.3% SDS with 10 μg/ml denatured salmon sperm DNA as carrier. The filter was washed in 2× SSC/0.2% SDS three times for 15 min each at room temperature. The 1.0 kb XbaI fragment of the *TPK1* gene that had been nick-translated to 1.0 × 10^8 cpm per microgram was used as a probe.

Nucleotide Sequence Analyses of the *TPK1*, *TPK2*, and *TPK3* Genes

The nucleotide sequence of the three *TPK* genes is presented in Figure 4 along with the predicted amino acid sequence (see Experimental Procedures for sequencing strategies). All of the three sequences have one long open reading frame (Figure 4). The largest open reading frame of the *TPK1* gene initiated by ATG can encode a protein 397 amino acid residues in length. An in-frame stop codon is found 30 nucleotides upstream from this ATG. The *TPK2* gene contains an open reading frame that can encode a protein 380 amino acid residues in length. An in-frame stop codon appears 96 nucleotides upstream from the ATG of *TPK2*. The *TPK3* gene contains an open reading frame that can encode a protein 398 amino acid residues in length. An in-frame stop codon appears 60 nucleotides upstream from the predicted initiator codon of *TPK3*. The three *TPK* genes clearly encode highly related proteins, with greater than 75% amino acid conservation over their carboxy-terminal 320 amino acid residues.

The deduced amino acid sequences of the three *TPK* genes reveals that they have a consensus sequence that has been found in all of the known protein kinases (Hunter and Cooper, 1986). That sequence is Gly-X-Gly-X-Gly (where X is any amino acid), followed 7–16 residues later by Lys, the putative ATP-binding site, shown with triangles in Figure 4 (Zoller et al., 1981; Kamps et al., 1984; Hannink and Donoghue, 1985). Another consensus sequence, Asp Phe Gly (shown with black dots in Figure 4) and Ala Pro Glu (shown with white dots in Figure 4), is found downstream of the ATP-binding site. Computer-assisted sequence comparisons (listed in GENEBANK and PIR databases) indicate that the three *TPK* genes share the strongest homology to the catalytic subunit of the cAMP-dependent protein kinase (Goad and Kanehisa, 1982; Keller et al., 1984).

Increased Activity of the cAMP-Dependent Protein Kinase in Strains Transformed with a TPK-Containing Plasmid

To test the possibility that the *TPK* genes encode catalytic subunits of the cAMP-dependent protein kinase, a direct biochemical approach was undertaken. We asked whether the amount of cAMP-dependent protein kinase is increased in yeast cells containing multicopy plasmids expressing the *TPK* genes. For this purpose, two different isogenic strains were made. One of these strains, T162-1ABT (Table 1), has only the *TPK1* gene intact. The *TPK2* and *TPK3* genes are disrupted in this strain (see the next section on gene disruption), and this strain carries two high copy plasmids, YEp*TPK1* and YEp24-*BCY1*, which express *TPK1* and *BCY1*, respectively. *BCY1* is the gene that codes for the regulatory subunit of the cAMP-dependent protein kinase (Toda et al., 1987). The other strain, T162-1ABY (Table 1), is different from T162-1ABT only in that it contains the vector plasmid YEp13 instead of YEp*TPK1*. YEp24-*BCY1* was introduced into both of these strains in the expectation that it would facilitate detection of the cAMP response of the cAMP-dependent protein kinase upon addition of cAMP.

Extracts were prepared from these two strains, applied to a DEAE-Sephacel column, and fractionated using a series of NaCl elution buffers that ranged in concentration from 50 mM to 300 mM. Each of the eluates was assayed for protein kinase activity with and without cAMP (Figure 5). Activity of the cAMP-dependent protein kinase was drastically increased, about 15-fold, in the strain T162-1ABT (Figure 5B), which has the *TPK1* gene carried on a multicopy plasmid, as compared with T162-1ABY (Figure 5A), which does not. Note that the kinase activity that is eluted at higher concentrations of NaCl (>200 mM) is not cAMP-dependent and does not differ in these two strains. This result clearly demonstrates that the *TPK1* gene encodes the catalytic subunit of the cAMP-dependent protein kinase and that introduction of this gene on a multicopy plasmid into yeast cells results in an approximately 15-fold overexpression of that kinase relative to a strain that contains only vector sequences. The same approach was taken with the *TPK3* gene, with results similar to those obtained with *TPK1* (data not shown).

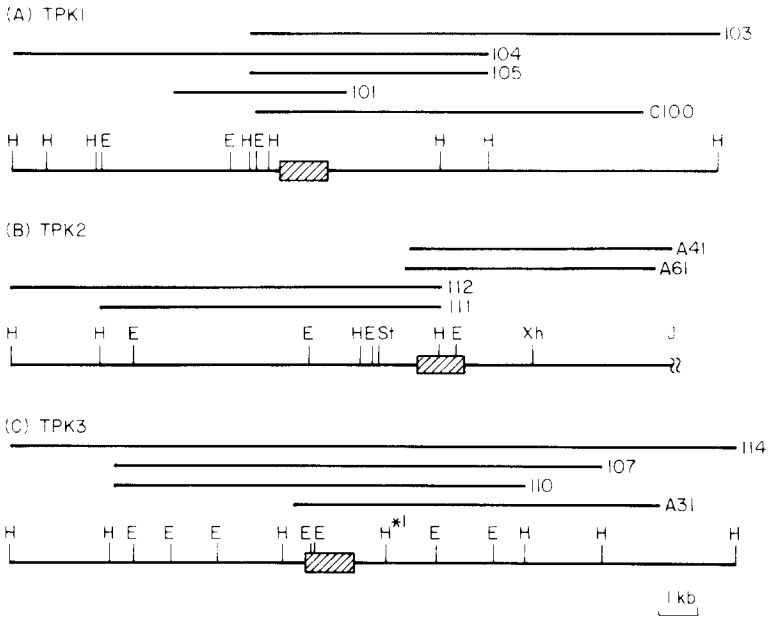


Figure 3. Physical Maps of *TPK1*, *TPK2*, and *TPK3*

Structures of *TPK1* (A), *TPK2* (B), and *TPK3* (C) are indicated. Two different yeast genomic libraries were used for isolation of the *TPK* genes. One library was constructed from yeast genomic DNA isolated from SP1 (see Table 1), partially digested with HindIII, and inserted into HindIII-digested YEp213 (Sherman et al., 1982; see Experimental Procedures). The other was made from DNA of strain AB320 (see Table 1), partially cleaved with Sau3A, and inserted into BamHI-digested YEp13 (Broach et al., 1979; Nasmyth and Reed, 1980).

The plasmid clones isolated from the yeast genomic libraries described above are shown as lines above the restriction maps. Inserts isolated from SP1 DNA are numbered starting with 100. Inserts from AB320 DNA are headed by "A." The clone "C100" in *TPK1* (A) represents the original *TPK1* sequence that was isolated from the YCp50 library as a suppressor plasmid of *cdc25*^{ts} (Figure 1). Hatched boxes represent the open reading frames. The direction of transcription of each gene is from the right to the left. Abbreviations used are as follows: E, EcoRI; H, HindIII; St, StuI; Xh, XhoI; J, Junction. Only the HindIII restriction sites have been completely mapped in these inserts. * 1: This HindIII site exists in DC5 and SP1 DNA but not in AB320 DNA, presumably due to strain polymorphism.

Disruption of *TPK1*, *TPK2*, and *TPK3* Genes

cAMP is thought to exert most, if not all, of its functions in eukaryotes through the cAMP-dependent protein kinase pathway (Robinson et al., 1971) and has been shown to play a crucial role in yeast (Matsumoto et al., 1982). It was therefore interesting to examine the effect of perturbation of the three *TPK* genes to see first, whether any of the *TPK*s have a distinct and unique function or, rather, share some overlapping roles; and second, whether disruption of all three genes, or any combination of the three, leads to a lethal effect. Therefore, the phenotypic consequences caused by disruption of the *TPK* genes were examined. To make a triply heterozygous diploid for the three kinase genes, three kinds of gene disruption plasmids having three different markers were constructed (see Figure 6 for details). The *TPK1* gene was inactivated by deleting almost the entire coding sequence (from the 15th to the 352nd amino acid) by digestion with the XbaI and insertion of the 1.1 kb *URA3* fragment (Figures 6A and 6D). The *TPK2* gene was disrupted by insertion of the 1.7 kb *HIS3* fragment into the unique BalI site. This causes an interruption of the *TPK2* gene in the 205th amino acid (Figures 6B and 6E). The *TPK3* gene was disrupted by insertion of the 1.4 kb *TRP1* fragment into the XbaI site. This causes an interruption of the *TPK3* gene in the 220th amino acid (Figures 6C and 6F). Both of the markers inserted into *TPK2* and *TPK3* separate a consensus ATP-binding sequence, Gly-X-Gly-X-X-Gly, from another consensus sequence, Glu Phe Gly. Both of these regions are thought to be important for kinase activity and therefore, we would predict that separation in that region would lead to an inactive kinase. Using these three disruption plasmids, diploid TTS162-3 was constructed that was heterozygous at each of the three *TPK* loci.

Tetrad analysis of the TTS162-3 diploid was carried out upon sporulation. Genotypes of viable spores were scored according to the auxotrophic requirements, and those of nonviable spores were predicted from those of the other spores in the same tetrad, assuming that normal Mendelian segregation occurred (Table 2). This genetic analysis gave the following result. First, cells that lack one or two *TPK* genes can grow as well as wild-type cells that have all three intact *TPK* genes. Second, such cells show no difference in growth characteristics at various temperatures, 23°C, 30°C, or 37°C, in comparison to wild-type cells. Third, haploid progenies that had no functional *TPK* genes were either nonviable (staying at the one cell stage after dissection) or formed very slowly growing colonies (see Discussion). From these observations, we concluded that none of the three *TPK* genes is essential by itself but at least one *TPK* gene is necessary for a cell to grow normally. Thus, in the yeast S. cerevisiae, genes coding for the catalytic subunits of the cAMP-dependent protein kinase consist of the three members *TPK1*, *TPK2*, and *TPK3*, each capable of complementing the other two genes.

Phenotypes of Single Kinase Strains Lacking the Regulatory Subunit of the cAMP-Dependent Protein Kinase

As previously reported, S. cerevisiae contains only one gene for the regulatory subunit of the cAMP-dependent protein kinase, namely, *BCY1* (Toda et al., 1987). Disruption

Table 2. Viability of Haploid Progeny from Heterozygous Diploids

Genotype			Viable Macro-colonies	Nonviable Spores/ Microcolonies
TPK1	*TPK2*	*TPK3*		
+	+	+	16	0/0
−	+	+	17	0/0
+	−	+	17	0/0
+	+	−	24	3/0
−	−	+	25	2/0
−	+	−	16	0/0
+	−	−	11	2/0
−	−	−	0	11/8

A triply heterozygous diploid strain, TTS162-3 (a/α *his3/his3 leu2/leu2 ura3/ura3 trp1/trp1 ade8/ade8 tpk1::URA3/+ tpk2::HIS3/+ tpk3::TRP1/+*), was sporulated and dissected. The genotypes of the resultant haploids were determined by checking auxotrophic requirements. For example, the *TPK1* locus is assigned to be wild type (+) if a segregant is uracil-requiring, and if a segregant is protrophic for uracil, its *TPK1* gene is assigned as disrupted (−). When possible, genotypes of nonviable or microcolonies were assigned based on the genotypes of the other viable spores in the same tetrad, assuming normal Mendelian segregation. Viability of spores was determined after 7 days of incubation at 30°C on YPD plates. Nonviable spores represent those that remained in the one cell stage under microscopic observation. Microcolonies mean those cells that divided and formed barely visible small colonies.

Table 4. Suppression Profile of Multicopy Plasmids Containing *TPK1*, *TPK2*, or *TPK3*

Multicopy Plasmid	Genotype[a]			
	cdc25^{ts}	*ras1*$^{-}$*ras2*^{ts}	*cyr1-1*	*cyr1-2*
YEp13	−	−	−	−
YEp*TPK1*[b]	+	+	+	+
YEp*TPK2*[c]	+	+/−	+	+
YEp*TPK3*[d]	+	+/−	+	+

[a] The *ras1*$^{-}$ *ras2*^{ts} strain was isolated by S. Powers (unpublished data). The cAMP-requiring mutant *cyr1-1* strain (M76-3C, kindly provided by Dr. Szostak; Table 1) was used. The temperature-sensitive *cyr1-2* strain (T50-3A, Table 1) was used.

[b] For this suppression experiment, a subclone containing the 2.4 kb HindIII–SphI fragment of *TPK1* in YEp213 (Sherman et al., 1982) was used (Figure 1).

[c] We could not isolate a complete and functional *TPK2* plasmid by colony hybridization. Therefore, a chimeric *TPK2* subclone was constructed from two overlapping isolates (Figure 3B). The 2.0 kb HindIII fragment that contains the carboxy-terminal and 3′-flanking sequences of the *TPK2* gene was isolated from clone 101 and then inserted into the HindIII-digested clone A61 which contains the amino-terminal and 5′-flanking sequences of the *TPK2* gene (see Figure 3B). This chimeric *TPK2* plasmid was used for the suppression studies described in this paper.

[d] Clone 107 (Figure 3C) was used as a *TPK3* plasmid.

of the *BCY1* gene causes the constitutive activation of the catalytic subunit of the cAMP-dependent protein kinase and has several peculiar phenotypes. These are failure to arrest properly in G1, extreme sensitivity to both nitrogen starvation and heat shock, and inability to grow on many different carbon sources other than glucose (Matsumoto et al., 1983; Sass et al., 1986; Toda et al., 1987). We tested whether these phenotypes were mediated by the kinase genes singly or in combination. Three kinds of strains, each with only one functional *TPK* gene, were obtained from tetrads of a triply heterozygous diploid, TTS162-3, as described above (Tables 1 and 2). These strains were then transformed with a disruption fragment of *BCY1* (Table 1), and their phenotypes were examined. Sensitivity to heat shock and growth on acetate were assayed in these strains. The results are summarized in Table 3. Any strain that contains only a single cAMP-dependent kinase, the other two having been destroyed, shows a sensitivity to heat shock when the *BCY1* gene is nonfunctional. There is, however, a difference in these strains regarding growth on acetate. *TPK1 tpk2*$^{-}$*tpk3*$^{-}$*bcy1*$^{-}$ and *tpk1*$^{-}$*tpk2*$^{-}$*TPK3 bcy1*$^{-}$ cells can grow on acetate medium, although their growth rate is slower than wild-type cells, whereas *tpk1*$^{-}$*TPK2 tpk3*$^{-}$*bcy1*$^{-}$ cells cannot grow on acetate. These results indicate that the *TPK* genes must have overlapping physiological roles, since they complement each other and are capable of blocking heat shock resistance, but they also may have distinguishing features, since only the presence of *TPK2* appears capable of blocking growth on acetate.

Table 3. Phenotypic Consequences of Disrupting *BCY1* in Various Kinase Backgrounds

Genotype				Heat Shock[a]	Growth on Acetate[b]
TPK1	*TPK2*	*TPK3*	*BCY1*		
+	+	+	+[c]	+ + +	+ + +
+	+	+	−	−	−
+	−	−	−	−	+
−	+	−	−	−	−
−	−	+	−	−	+ +

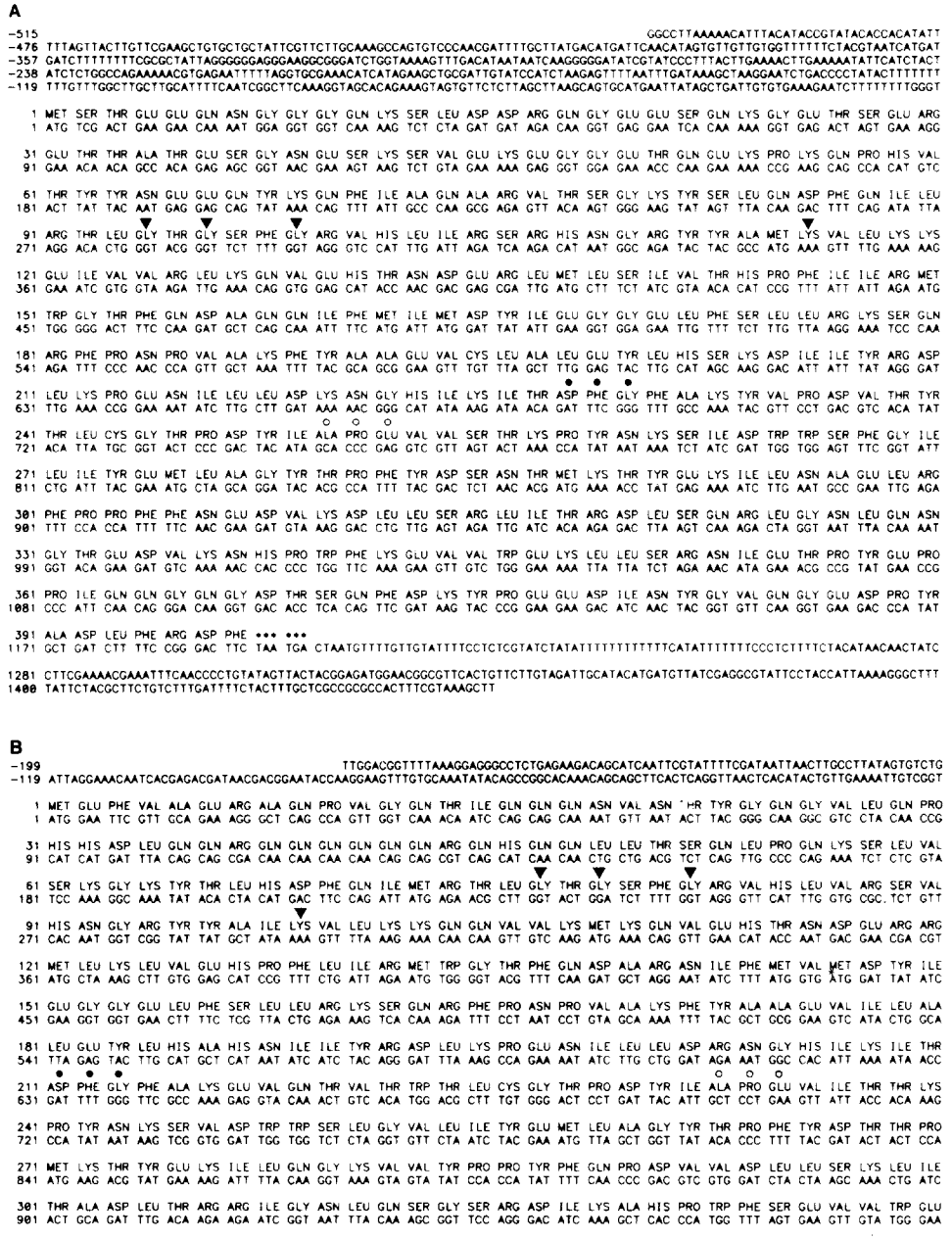
[a] Cells were patched onto YPD, grown for 2 days, and then replica-plated to a YPD plate. This replica plate was incubated for 45 min at 55°C and then transferred to 30°C for 2 days, after which it was scored. "+" means heat-shock resistant; "−" means heat-shock sensitive.

[b] Cells were patched onto YPD, grown for 2 days, replica-plated to a YPA plate (containing acetate as a carbon source instead of glucose), and then incubated at 30°C. Two days later, growth was scored.

[c] Strains that have a single kinase gene (the other two disrupted) and the *BCY1* gene intact show the same phenotype as wild-type cells (not shown here).

Suppression Profiles of *TPK* Genes on Multicopy Plasmids

As described above, *TPK1* was originally isolated as a suppressor plasmid of the temperature-sensitive mutant *cdc25* (see also Broek et al., 1987). We tested whether multicopy plasmids that contained *TPK2* or *TPK3* could suppress this same temperature-sensitive *cdc25* allele. We found that both *TPK2* and *TPK3* could suppress the *cdc25*^{ts}. Next, we examined suppression of other genes that are in the *RAS*/cAMP pathway. There are several mutant strains that are defective in the gene encoding adenylate cyclase (Matsumoto et al., 1982). We found that any of the *TPK* genes on multicopy plasmids can efficiently suppress a cAMP-requiring mutant of adenylate cyclase, *cyr1-1*, as well as the temperature-sensitive adenylate cyclase allele, *cyr1-2* (Table 4). We next asked whether the *TPK* genes can suppress strains with defective *RAS*



genes. Since all of the *TPK* genes can suppress mutant *cyr1* strains, we initially expected that all could suppress strains with defective *RAS* genes. Indeed, the *TPK1* gene was able to suppress the temperature-sensitive defect of a *ras1*$^{-}$ *ras2*^{ts} strain (isolated by S. Powers; unpublished data, Table 4). However, suppression of the *ras1*$^{-}$ *ras2*^{ts} with *TPK2* and *TPK3* genes was very weak (Table 4). Possible implications of this result are discussed below.

Discussion

We have cloned and sequenced three closely homologous genes (*TPK1*, *TPK2*, and *TPK3*) that appear to encode protein kinases. The *TPK1* gene was originally isolated as an extrachromosomal suppressor plasmid of *cdc25*^{ts}. *TPK2* and *TPK3* were isolated by colony filter hybridization using *TPK1* as a probe. It was predicted that amino acid sequences of these three genes would be very similar, and indeed they are (Figure 7). The *TPK*-encoded proteins are also all homologous to the bovine cAMP-dependent protein kinase catalytic subunit (Figure 7). Direct biochemical experiments indicate that *TPK1* and *TPK3* encode catalytic subunits of the S. cerevisiae cAMP-dependent protein kinase. Similar experiments with *TPK2* were not successful, probably for technical reasons such

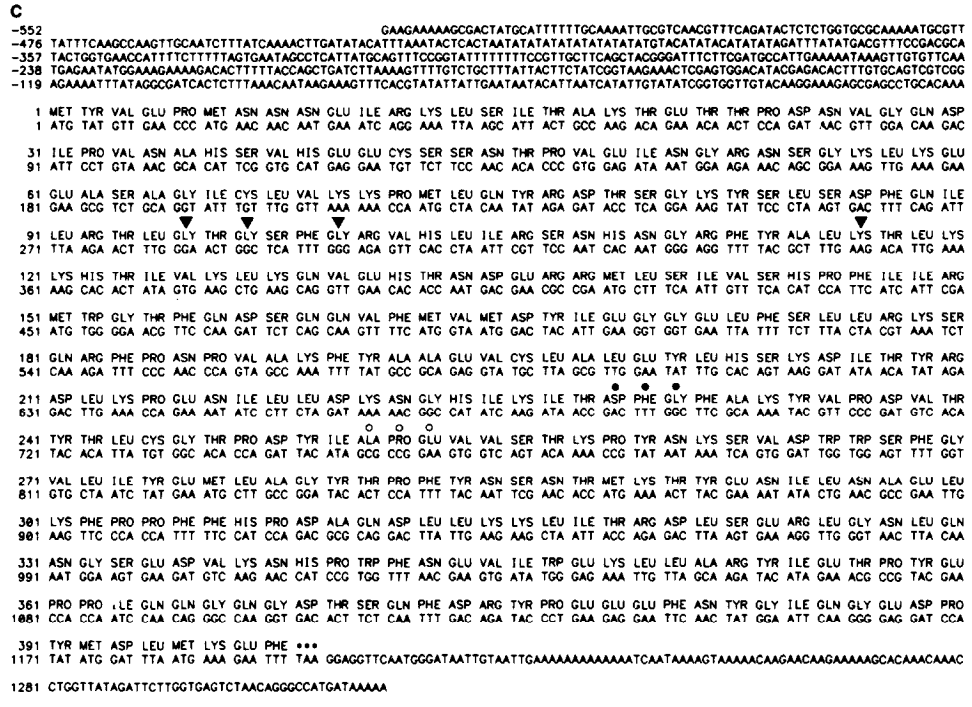


Figure 4. Nucleotide Sequences and Deduced Amino Acid Sequences of *TPK1*, *TPK2*, and *TPK3*

1974 nucleotides from *TPK1* (A), 1421 from *TPK2* (B), and 1976 from *TPK3* (C) are shown along with the deduced amino acid sequence for each. The consensus sequence for ATP-binding position (Glycines and Lysine; see text) is marked with triangles. Invariant sequences among known protein kinases are emphasized by black dots (Asp Phe Gly) and white dots (Ala Pro Glu). The 3′ termination codon is indicated by asterisks.

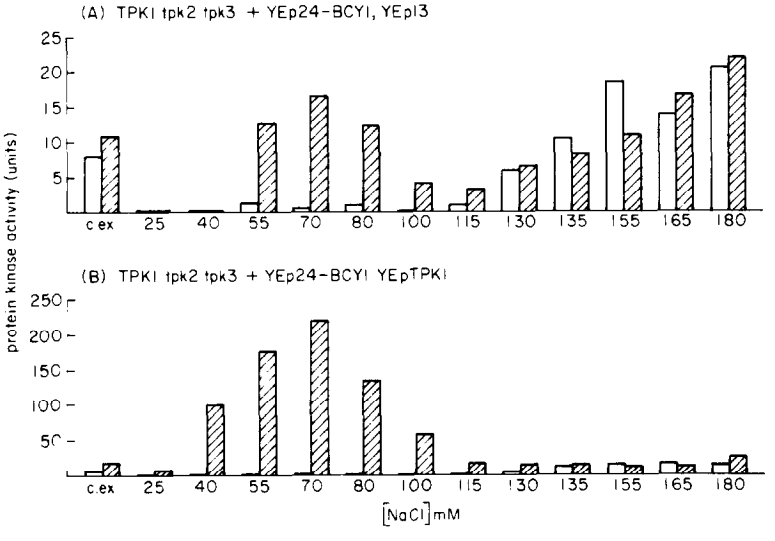


Figure 5. cAMP-Dependent Protein Kinase Activity in a Strain Carrying the *TPK1* Gene on a Multicopy Plasmid

Yeast cells were grown in SD medium supplemented with adenine, collected by centrifugation, and disrupted with a French press. Cell lysates were spun at 20,000 g for 1 hr and the supernatants were then fractionated on a DEAE-Sephacel column eluting with a NaCl step gradient. Each fraction was assayed for protein kinase activity with (hatched bars) and without (open bars) 10 μM cAMP. Histone IIa (Sigma) was used as a substrate. (A) T162-1ABY (*TPK1 tpk2⁻ tpk3⁻* containing YEp24-*BCY1*, YEp13). (B) T162-1ABT (*TPK1 tpk2⁻ tpk3⁻* containing YEp24-*BCY1*, YEp*TPK1*). "c.ex" means the crude extract. Note the different ordinate scale in (A) and (B). One unit of enzyme activity is defined as the amount of enzyme that transferred 1 pmol of [γ-^{32}P]ATP to substrate protein (histone) in 1 min at 30°C. Concentrations of NaCl (abscissa) in each fraction were determined by measuring the conductivities.

as instability of the kinase or failure to achieve high-level expression in multicopy plasmids. Nevertheless, we consider it very likely that *TPK2* also encodes a cAMP-dependent kinase because it is highly homologous to *TPK1* and *TPK3*, because it complements loss of *TPK1* and *TPK3*, and because, like *TPK1* and *TPK3*, it can suppress the growth defects resulting from loss of adenylate cyclase. Thus S. cerevisiae contains three genes that can encode cAMP-dependent protein kinase catalytic subunits. *TPK* gene disruption experiments (Table 2), low stringency hybridization experiments (Figure 2), and biochemical experiments (not shown) all fail to indicate the

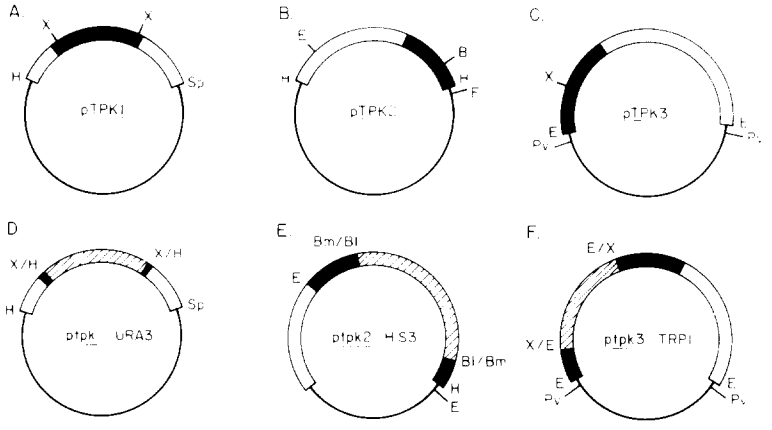


Figure 6. Plasmids Used for Gene Disruptions of *TPK* Genes

The 2.4 kb HindIII–SphI fragment from the plasmid C100 (Figures 1 and 3) was inserted into the 3.8 kb HindIII–SphI derivative of pBR322 (A; p*TPK1*). For construction of p*tpk1*::*URA3* (D), the 1.0 kb XbaI fragment of p*TPK1* was removed and the 1.1 kb HindIII fragment of *URA3* was inserted after the fragment was blunt-ended with large fragment of DNA polymerase I. The 2.5 kb HindIII–SphI fragment from p*tpk1*::*URA3* was used for the gene replacement experiment (Rothstein, 1983). The 2.0 kb HindIII fragment from plasmid 111 (Figure 3) was inserted into the HindIII site of pUC18 (B; p*TPK2*) (Yanisch-Perron et al., 1985). The 1.7 kb Klenow-filled HindIII fragment of *HIS3* was inserted into a unique BalI site in the coding region of the *TPK2* gene (E; p*tpk2*::*HIS3*). The 3.4 kb EcoRI fragment from p*tpk2*::*HIS3* was used for gene disruption of the *TPK2* gene. One of the two EcoRI sites in this fragment was from the polylinker sequence of pUC18. For fragment replacement, it is not essential that both ends of the restriction fragment be homologous to chromosomal sequences. The 3.2 kb EcoRI fragment from plasmid A31 (Figure 3) was inserted into the EcoRI site of pUC8 (C; p*TPK3*) (Vieira and Messing, 1982). The 1.4 kb EcoRI fragment of *TRP1* was inserted into a unique XbaI site in the coding region of the *TPK3* gene (F; p*tpk3*::*TRP1*). The 4.9 kb PvuII fragment from p*tpk3*::*TRP1* was used for disruption of the *TPK3* gene. Both of the PvuII sites are derived from the vector plasmid pUC8, but homologous recombination and fragment replacement could occur in the *TPK3* locus (confirmed by Southern blotting analysis; data not shown). Vector sequences are indicated by a thin line; coding sequences from *TPK* genes are indicated by a filled-in box; noncoding sequences, by empty boxes; DNAs from selectable amino acid markers, with slashes. Abbreviations used are as in Figure 1. BI and Pv represent BalI and PvuII, respectively.

existence of additional *TPK* genes. Thus S. cerevisiae probably encodes only three such genes.

We have compared the three S. cerevisiae cAMP-dependent protein kinase catalytic subunits with each other and with two from mammals (Figure 7). The *TPK* proteins are equidistant from each other. They show a variable domain in the N-terminal 60 to 80 amino acids. In this domain there is no homology and there is considerable heterogeneity in length. The *TPK2* gene encodes an unusual stretch of 14 glutamines in this region. On the other hand, there is greater than 75% homology in the carboxy-terminal 320 amino acids. Amino acid sequence of this region can be aligned perfectly without any shifts (Figure 7 and Table 5). Similarly, the two forms (Cα and Cβ) of mammalian catalytic subunits of the cAMP-dependent protein kinase are highly homologous to each other (Uhler et al., 1986a, 1986b; Showers and Mauver, 1986). Bovine kinase has a shorter amino-terminal sequence than those of *TPK* genes. The carboxy-terminal 310 amino acids of the bovine kinase are very homologous to those of *TPK* genes

Table 5. Homology of Amino Acid Sequences among the Catalytic Subunits of cAMP-Dependent Protein Kinase

	TPK2	*TPK3*	*cAPK*
TPK1	77	88	50
TPK2		75	53
TPK3			51

The numbers in this table represent the percentage of identity among the various cAMP-dependent protein kinases. The carboxy-terminal 321 amino acid residues were compared among *TPK1*, *TPK2*, and *TPK3*. When amino acid sequences of each of the *TPK* genes were compared with that of the catalytic subunit of the bovine cAMP-dependent protein kinase, cAPK (Shoji et al., 1983), only the carboxy-terminal 309 amino acid residues were considered.

Figure 7. Amino Acid Sequence Comparisons between the *TPK* Genes and the Catalytic Subunit of Bovine cAMP-Dependent Protein Kinase

The amino acid sequences of the three *TPK* genes and two forms (Cα and Cβ) of catalytic subunits of the bovine cAMP-dependent protein kinase (Shoji et al., 1983; Showers and Mauver, 1986) are shown. Shadowed amino acids show identity. An asterisk indicates a termination codon.

(more than 50%; Table 5), although deletions have to be postulated in three positions near the end of the protein (Figure 7). In contrast to the *TPK* genes, the amino-terminal regions of these mammalian proteins do not show variability. There is no homology to yeast protein in that region (Figure 7). Comparison of the two mammalian cAMP-dependent kinases indicates that they diverged from each other long after they diverged from the ancestor to the yeast cAMP-dependent kinases. However, it is clear that the class of cAMP-dependent kinases can undergo considerable divergence and still retain their biochemical function. Therefore, additional cAMP-dependent kinases may exist in mammals that are more highly diverged from the ones that have already been found.

A cAMP-requiring mutation, *cyr2*, was isolated by Matsumoto and coworkers (1982). A temperature-sensitive allele, *cyr2-1*, was reported to have an altered catalytic subunit of the cAMP-dependent protein kinase, showing a different elution profile on a DEAE-Sephacel column and lower affinity for ATP (Uno et al., 1984). Moreover, the cAMP-dependent kinase catalytic subunit of *cyr2-1* strains was found to be thermolabile. From these data, Uno and coworkers proposed that the *CYR2* gene was the structural gene for the catalytic subunit of the cAMP-dependent protein kinase (Uno et al., 1984). However, as we have shown in this paper, structural genes for the catalytic subunits of the cAMP-dependent protein kinase consist of three members (*TPK1*, *TPK2*, and *TPK3*) in yeast. In addition, since neither single nor even double disruptions of *TPK* genes have growth-defective phenotypes, *CYR2* is probably not one of the *TPK* genes. It is possible that *CYR2* might encode a gene product that modifies all three catalytic subunits.

The multiplicity of genes encoding catalytic subunits of the cAMP-dependent protein kinase in yeast and mammals raises the question of their functional divergence. No major differences in the function of the yeast kinases are immediately apparent. A single kinase is sufficient for apparently normal growth. Minor differences in the yeast kinases are seen in overexpression and gene disruption experiments (see Tables 3 and 4), but whether this reflects qualitative or quantitative differences is not yet clear. This question is under continued study in our laboratory. It is worth noting that there is considerable redundancy in genes encoding products of the *RAS*/cAMP effector pathway: two *RAS* genes (DeFeo-Jones et al., 1983; Powers et al., 1984), two phosphodiesterase genes (Sass et al., 1986; Nikawa et al., unpublished data), and three genes encoding kinase catalytic subunits. There appears to be only one gene each encoding adenylate cyclase (Kataoka et al., 1985) and the regulatory subunit of the cAMP-dependent protein kinase (Toda et al., 1987).

We have previously shown that the *bcy1* mutation can suppress lethality in *ras1*$^-$ *ras2*$^-$ yeast cells (Toda et al., 1985). *BCY1* was originally identified as a suppressor mutation of the adenylate cyclase–deficient *cyr1* mutant (Matsumoto et al., 1982), and it was shown later that it encodes the regulatory subunit of the cAMP-dependent protein kinase (Toda et al., 1987). In this study we show that a multicopy plasmid carrying the *TPK1* gene complements the temperature sensitivity of a *ras1*$^-$ *ras2*^{ts} strain as well as the cAMP-requiring phenotype of a *cyr1* mutant. These results are entirely consistent with the idea that activation of the cAMP-dependent protein kinase can overcome the requirement for adenylate cyclase and *RAS* proteins in yeast. Since *TPK2* and *TPK3* on a multicopy plasmid can suppress the *cyr1* mutation, one might well expect that these genes would also suppress the *ras1*$^-$ *ras2*^{ts} strain, as does the *TPK1* gene. It was therefore surprising to us that, in constrast to *TPK1*, *TPK2* and *TPK3* on multicopy plasmids failed to suppress the *ras1*$^-$ *ras2*^{ts} strain efficiently. A more detailed genetic analysis, which will be presented subsequently, indicates that the *TPK2* and *TPK3* genes carried on a multicopy plasmid can suppress the lethality of *ras1*$^-$ *ras2*$^-$ strains, but that such strains are somewhat temperature sensitive. This result may indicate that the *RAS* genes have a function in addition to the stimulation of adenylate cyclase. Further evidence for this comes from tetrad analysis of triple kinase disruptions, as discussed below.

Haploid spores lacking all three kinases are not completely inviable. Indeed, spores carrying no functional *TPK* genes can still germinate and grow, although the generation time of these cells is extremely long. We interpret this result to mean that the cAMP pathway is strongly required but not absolutely essential in yeast. However, *RAS1* and *RAS2* do have an absolutely essential function, and haploid spores carrying disruptions of both *RAS* genes either fail to germinate or germinate but arrest prior to bud emergence (Kataoka et al., 1984; Tatchell et al., 1984). These results support the suggestion from the results of *ras2*^{ts} suppression studies that *RAS* proteins may have another function in addition to stimulation of adenylate cyclase. A more detailed presentation of these findings will follow.

Experimental Procedures

Strains, Media, Genetic Procedures, and Nomenclature

Yeast strains used in this study are listed in Table 1. E. coli HB101 was used as a donor strain for plasmids. Rich yeast medium, YPD, contains 1% yeast extract, 2% Bacto-peptone, and 2% dextrose. Synthetic yeast medium, SD, contains 0.67% yeast nitrogen base without amino acids and 2% dextrose, and if necessary, appropriate amino acids were supplemented with a concentration of 80 μg/ml. All the other media used were previously described (Toda et al., 1985). Standard yeast genetic procedures were followed, as described by Sherman et al. (1982). The lithium acetate method was used for transformation of yeast cells (Ito et al., 1983). Gene disruptions are denoted by lowercase letters representing the disrupted gene followed by two colons and the wild-type gene marker used for disruption. In the text of this paper, gene disruptions are abbreviated by lowercase italicized letters representing the gene followed by a superscript minus sign, such as *tpk1*$^-$. A strain that has a temperature-sensitive recessive mutation is shown by lowercase italicized letters representing the gene followed by a superscript "ts," such as *cdc25*^{ts}.

DNA

Plasmid DNA was isolated from E. coli using the alkali-lysis method (Maniatis et al., 1982). Yeast DNA was prepared essentially as described by Nasmyth and Reed (1980). Restriction endonuclease, T4 DNA ligase, and large fragment of DNA polymerase I were used under conditions recommended by suppliers (New England BioLabs, Bethesda Research Labs, or Boehringer Mannheim Biochemicals). Stan-

dard molecular cloning techniques were as described by Maniatis et al. (1982).

Integrative Mapping

The 2.4 kb HindIII–SphI fragment of *TPK1* was inserted into those sites of pBR322. The 1.1 kb HindIII fragment of *URA3* was inserted into the HindIII site of this plasmid to generate YIp*TPK1*/*URA3*. YIp*TPK1*/*URA3* was linearized at the SalI site, which is located in the *TPK1* sequence (see Figure 1), and then transformed into TT25-6 (Table 1). A URA$^+$ transformant was picked, and integration of a single copy of YIp*TPK1*/*URA3* into the *TPK1* locus was checked by Southern hybridization (Orr-Weaver and Szostak, 1983; data not shown). This integrant was crossed with the mating strain T3-28C (Table 1). The resulting diploids were sporulated, and tetrad analysis was performed. The *TPK1* locus was followed by uracil requirement, and the *CDC25* locus was followed by its temperature-sensitive phenotype. Linkage between *TPK1* and *CDC25* was as follows: parental ditype, 3; tetratype, 12; and nonparental ditype, 3. We concluded that the *TPK1* gene is not linked to *CDC25*.

Yeast Genomic Library

A S. cerevisiae genomic library that had been constructed in the vector YCp50 was kindly provided by M. Rose and G. Fink. YCp50 carries a yeast centromere (*CEN4*), yeast replication origin (*ARS1*), and *URA3* as a selectable marker. This YCp50 library was used for isolation of suppressor plasmids of the temperature-sensitive *cdc25*.

Two other libraries were used to isolate *TPK2* and *TPK3* by colony filter hybridization with *TPK1* as a probe. One library was made from the yeast strain SP1 (see Table 1) using YEp213 (Sherman et al., 1982) as a vector. Genomic DNA from SP1 was partially digested with HindIII, and fragments ranging in size from 4 kb to 20 kb were gel-purified and ligated with HindIII-digested YEp213. Approximately 90,000 independent transformants were obtained after transformation to E. coli with this ligation mixture. Average insert size was 6 kb, estimated from gel analysis of 12 independent colonies. The other library used in this study has been described previously (Broach et al., 1979; Nasmyth and Reed, 1980).

Isolation of Genes That Encode *TPK2* and *TPK3*

Colony filter hybridization was performed to isolate *TPK2* and *TPK3* using the 1.0 kb XbaI fragment of *TPK1* as a probe (Figure 1; see text). Hybridization was carried out as described in Figure 3.

Protein Kinase Assay

One liter of yeast cells was grown in SD medium supplemented with adenine. Preparation of cell extracts and assay of protein kinase activity were performed as previously described (Toda et al., 1987). Histone IIa (Sigma) or Kemptide (Peninsula Laboratories, Inc.) was used for protein kinase assay as a substrate.

Nucleotide Sequence Determination

Various internal restriction fragments from the 2.4 kb HindIII–SphI fragment of *TPK1* were isolated and inserted into either M13mp18 or M13mp19 (Yanisch-Perron et al., 1985), and the nucleotide sequence was determined by the dideoxy method (Sanger et al., 1977) using [α-^{35}S] dATP as a substrate (Biggin et al., 1983).

For sequencing *TPK2* and *TPK3*, the unidirectional progressive deletion method (Henikoff, 1984) was used. The 4.1 kb XhoI–StuI fragment of *TPK2* was blunt-ended and inserted into the HincII site of pUC19 (Vieira and Messing, 1982; Yanisch-Perron et al., 1985) in both orientations. The resultant plasmids were digested with BamHI and SstI before deletion by ExoIII and ExoVII. The 2.7 kb HindIII fragment of *TPK3* was inserted into the HindIII site of pUC19 in both orientations. The resultant plasmids were digested with SalI and SstI. Both strands of *TPK1*, *TPK2*, and *TPK3* were completely sequenced in the coding regions.

Heat Shock

Heat shock experiments were performed basically as described by Sass et al. (1986).

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References

Biggin, M. D., Gibson, T. J., and Hong, G. F. (1983). Buffer gradient gels and ^{35}S label as an aid to rapid DNA sequence determination. Proc. Natl. Acad. Sci. USA *80*, 3963–3965.

Broach, J. R., Strathern, J. N., and Hicks, J. B. (1979). Transformation in yeast: development of a hybrid cloning vector and isolation of the *CAN1* gene. Gene *8*, 121–133.

Broek, D., Samiy, N., Fasano, O., Fujiyama, A., Tamanoi, F., Northup, J., and Wigler, M. (1985). Differential activation of yeast adenylate cyclase by wild-type and mutant *RAS* proteins. Cell *41*, 763–769.

Broek, D., Toda, T., Michaeli, T., Levin, L., Birchmeier, C., Zoller, M., Powers, S., and Wigler, M. (1987). The S. cerevisiae *CDC25* gene product regulates the *RAS*/adenylate cyclase pathway. Cell *48*, 789–799.

DeFeo-Jones, D., Scolnick, E. M., Koller, R., and Dhar, R. (1983). *ras*-Related gene sequences identified and isolated from *Saccharomyces cerevisiae*. Nature *306*, 707–709.

Goad, W. B., and Kanehisa, M. I. (1982). Pattern recognition in nucleic acid sequences. I. A general method for finding local homologies and symmetries. Nucl. Acids Res. *10*, 247–263.

Hannink, M., and Donoghue, D. J. (1985). Lysine residue 121 in the proposed ATP-binding site of the v-mos protein is required for transformation. Proc. Natl. Acad. Sci. USA *82*, 7894–7898.

Henikoff, S. (1984). Unidirectional digestion with Exonuclease III creates targeted breakpoints for DNA sequencing. Gene *28*, 351–359.

Hunter, T., and Cooper, J. A. (1986). Viral oncogenes and tyrosine phosphorylation. Ann. Rev. Biochem. *54*, 897–930.

Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. *153*, 163–168.

Kamps, M. P., Taylor, S. S., and Sefton, B. M. (1984). Direct evidence that oncogenic tyrosine protein kinases and cAMP-dependent protein kinase have homologous ATP-binding sites. Nature *310*, 589–592.

Kataoka, T., Powers, S., McGill, C., Fasano, O., Strathern, J., Broach, J., and Wigler, M. (1984). Genetic analysis of yeast *RAS1* and *RAS2* genes. Cell *37*, 437–445.

Kataoka, T., Broek, D., and Wigler, M. (1985). DNA sequence and characterization of the S. cerevisiae gene encoding adenylate cyclase. Cell *43*, 493–505.

Keller, C., Corcoran, M., and Roberts, R. J. (1984). Computer programs for handling nucleic acid sequences. Nucl. Acids Res. *12*, 379–384.

Krebs, E. G., and Beavo, J. A. (1979). Phosphorylation-dephosphorylation of enzymes. Ann. Rev. Biochem. *48*, 923–959.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

Matsumoto, K., Uno, I., Oshima, Y., and Ishikawa, T. (1982). Isolation and characterization of yeast mutants deficient in adenylate cyclase and cAMP-dependent protein kinase. Proc. Natl. Acad. Sci. USA *79*, 2355–2359.

Matsumoto, K., Uno, I., and Ishikawa, T. (1983). 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., Uno, I., and Ishikawa, T. (1985). Genetic analysis of the role of cAMP in yeast. Yeast *1*, 15–24.

Nasmyth, K. A., and Reed, S. I. (1980). Isolation of genes by complementation in yeast: molecular cloning of a cell-cycle gene. Proc. Natl. Acad. Sci. USA *77*, 2119–2123.

Orr-Weaver, T. L., and Szostak, J. W. (1983). Yeast recombination: the association between double-strand gap repair and crossing-over. Proc. Natl. Acad. Sci. USA *80*, 4417–4421.

Powers, S., Kataoka, T., Fasano, O., Goldfarb, M., Strathern, J., Broach, J., and Wigler, M. (1984). Genes in S. cerevisiae encoding proteins with domains homologous to the mammalian *ras* proteins. Cell *36*, 607–612.

Powers, S., Michaelis, S., Broek, D., Anna-A., S. S., Field, J., Herskowitz, I., and Wigler, M. (1986). *RAM*, a gene of yeast required for a functional modification of *RAS* proteins and for production of mating pheromone a-factor. Cell *47*, 413–422.

Robinson, G. A., Butcher, R., and Sutherland, E. W. (1971). Cyclic AMP (Academic Press: New York).

Rothstein, R. J. (1983). One-step gene disruption in yeast. Meth. Enzymol. *101*, 202–211.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA *74*, 5463–5467.

Sass, P., Field, J., Nikawa, J., Toda, T., and Wigler, M. (1986). Cloning and characterization of the high affinity cAMP phosphodiesterase of *S. cerevisiae*. Proc. Natl. Acad. Sci. USA *83*, 9303–9307.

Sherman, F., Fink, G. R., and Hicks, J. B. (1982). Methods in Yeast Genetics, F. Sherman, G. R. Fink, and J. B. Hicks, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

Shoji, S., Ericsson, L. H., Walsh, K. A., Fischer, E. H., and Titani, K. (1983). Amino acid sequence of the catalytic subunit of bovine typeII adenosine cyclic 3′, 5′-phosphate dependent protein kinase. Biochemistry *22*, 3702–3709.

Showers, M. O., and Mauver, R. A. (1986). A cloned bovine cDNA encodes an alternate form of the catalytic subunit of cAMP-dependent protein kinase. J. Biol. Chem. *261*, 16288–16291.

Tatchell, K., Chaleff, D., Defeo-Jones, D., and Scolnick, E. (1984). Requirement of either of a pair of *RAS* related genes of *Saccharomyces cerevisiae* for spore viability. Nature *309*, 523–527.

Toda, T., Uno, I., Ishikawa, T., Powers, S., Kataoka, T., Broek, D., Cameron, S., Broach, J., Matsumoto, K., and Wigler, M. (1985). In yeast, *RAS* proteins are controlling elements of adenylate cyclase. Cell *40*, 27–36.

Toda, T., Cameron, S., Sass, P., Zoller, M., Scott, J. D., McMullen, B., Hurwitz, M., Krebs, E. G., and Wigler, M. (1987). Cloning and characterization of *BCY1*, a locus encoding a regulatory subunit of the cAMP dependent protein kinase in yeast. Mol. Cell. Biol. *7*, 1371–1377.

Uhler, M. D., Carmichael, D. F., Lee, D. C., Chrivia, J. C., Krebs, E. G., and McKnight, G. S. (1986a). Isolation of cDNA clones coding for the catalytic subunit of mouse cAMP-dependent protein kinase. Proc. Natl. Acad. Sci. USA *83*, 1300–1304.

Uhler, M. D., Chrivia, J. C., and McKnight, G. S. (1986b). Evidence for a second isoform of the catalytic subunit of cAMP-dependent protein kinase. J. Biol. Chem. *261*, 15360–15363.

Uno, I., Matsumoto, K., Adachi, K., and Ishikawa, T. (1984). Characterization of cAMP-requiring mutants altered in the catalytic subunit of protein kinase. J. Biol. Chem. *259*, 12508–12513.

Vieira, J., and Messing, J. (1982). The pUC plasmids, an M13 mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene *19*, 259–268.

Yanisch-Perron, C., Vieira, J., and Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene *33*, 109–119.

Zoller, M. J., Nelson, N. C., and Taylor, S. S. (1981). Affinity labeling of cAMP-dependent protein kinase with p-fluorosulfonylbenzoyl adenosine. Covalent modification of lysine 71. J. Biol. Chem. *256*, 10837–10842.