cAMP-Independent Control of Sporulation, Glycogen Metabolism, and Heat Shock Resistance in S. cerevisiae

Scott Cameron, Lonny Levin, Mark Zoller, and Michael Wigler
Cold Spring Harbor Laboratory
P. O. Box 100
Cold Spring Harbor, New York 11724

Summary

Genes encoding the regulatory (*BCY1*) and catalytic (*TPK1*, *TPK2*, and *TPK3*) subunits of the cAMP-dependent protein kinase (cAPK) are found in S. cerevisiae. *bcy1⁻* yeast strains do not respond properly to nutrient conditions. Unlike wild type, *bcy1⁻* strains do not accumulate glycogen, form spores, or become resistant to heat shock when nutrient limited. We have isolated mutant *TPK* genes that suppress all of the *bcy1⁻* defects. The mutant *TPK* genes appear to encode functionally attenuated catalytic subunits of the cAPK. *bcy1⁻* yeast strains containing the mutant *TPK* genes respond appropriately to nutrient conditions, even in the absence of *CDC25*, both *RAS* genes, or *CYR1*. Together, these genes encode the known components of the cAMP-generating machinery. The results indicate that cAMP-independent mechanisms must exist for regulating glycogen accumulation, sporulation, and the acquisition of thermotolerance in S. cerevisiae.

Introduction

Eukaryotic cells contain cAMP-dependent protein kinases (cAPKs). In most eukaryotes, this kinase consists of two catalytic subunits complexed with a dimer of regulatory subunits (Beebe and Corbin, 1986; Edelman et al., 1987). When the regulatory subunits bind cAMP, two active catalytic subunits are released (Beebe and Corbin, 1986; Edelman et al., 1987). There is excellent evidence that the action of the catalytic subunits is responsible for mediating the effects of cAMP (Beebe and Corbin, 1986). The yeast Saccharomyces cerevisiae contains a cAMP-responsive kinase activity, and genes encoding a regulatory subunit (*BCY1*) (Toda et al., 1987b; Johnson et al., 1987; Cannon and Tatchell, 1987; Kunisawa et al., 1987) and three catalytic subunits (*TPK1*, *TPK2*, and *TPK3*) (Toda et al., 1987c; Cannon and Tatchell, 1987; Lisziewicz et al., 1987) have been isolated. Disruption of the *BCY1* gene results in several very severe phenotypes (Toda et al., 1987b; Cannon and Tatchell, 1987). We have characterized the role of the cAPK catalytic subunit genes in producing the *bcy1⁻* phenotypes, and in the process we generated mutant cAPK catalytic subunit genes that suppress the *bcy1⁻* defects. We have used these mutants to assess the physiologic function of the cAMP pathway in controlling the nutrient response in yeast.

Yeast cells lacking a functional regulatory subunit of the cAPK do not respond properly to nutrients. When sufficiently limited for nutrients, normal cells accumulate storage carbohydrates, arrest in the G1 phase of the cell cycle, and enter an altered physiologic state. In this altered state, yeast can survive heat shock treatment or long periods of nutrient deprivation (Pringle and Hartwell, 1981). *bcy1⁻* cells fail to do any of these things (Toda et al., 1987b; Cannon and Tatchell, 1987; Matsumoto et al., 1983a, 1983b). Similar phenotypes can be produced by mutation of other genes encoding components of the cAMP pathway. For example, activation of the *RAS* proteins (Toda et al., 1985; Kataoka et al., 1985; Marshall et al., 1987), which stimulate adenylyl cyclase (Toda et al., 1985; Broek et al., 1985; Cannon et al., 1986; Sigal et al., 1986), or deletion of the genes encoding the cAMP phosphodiesterases (Nikawa et al., 1987b) results in phenotypes very similar to those found in strains lacking the cAPK regulatory subunit. These observations support the ideas that the regulatory subunit of the cAPK mediates the effects of cAMP by controlling cAPK catalytic activity, and that the activity of the cAPK affects the regulation of responses to nutrient stress.

We have used the genetics available in yeast to examine these ideas more directly. We find that the defects apparent in a *bcy1⁻* strain are completely dependent upon the presence of wild-type genes encoding cAPK catalytic subunits. Mutant alleles of the *TPK* genes (called *tpkᵂ* alleles) have been isolated that completely suppress the *bcy1⁻* phenotypes. This suggests that modulation of cAPK activity is the major and perhaps sole function for the regulatory subunit of the cAPK in yeast. We present evidence that the *tpkᵂ* alleles encode functionally attenuated cAPK catalytic subunits. Cells without the regulatory subunit of the cAPK and containing only an attenuated cAPK catalytic subunit gene have been tested for their ability to regulate responses to nutritional stress. Such cells can regulate sporulation, heat shock resistance, and glycogen accumulation appropriately, even when the components of the cAMP-generating machinery are absent. These results argue that cAMP-independent pathways exist for controlling these responses in yeast.

Results

Disruption of *TPK* Genes Diminishes the Phenotype of *bcy1⁻* Mutants

Yeast strains lacking the regulatory subunit of the cAPK do not accumulate storage carbohydrates when nutrient limited, do not utilize many nonglucose carbon sources, and do not survive heat shock treatment or long periods of starvation for nitrogen (Toda et al., 1987b; Cannon and Tatchell, 1987). Diploid strains lacking the cAPK regulatory subunit do not sporulate (Matsumoto et al., 1983b; Cannon et al., 1986; Toda et al., 1987b). To characterize the relationship of the three cAPK catalytic subunit genes to the phenotype of strains lacking the gene for the regulatory subunit of the cAPK, we constructed a diploid strain, TF5.4, heterozygous for disruptions of the *TPK1*, *TPK2*,

Table 1. Genotype of Yeast Strains Used in This Work

Strain	Genotype
SP1[a]	*MATa his3 leu2 ura3 trp1 ade8 can1*
DC124[a]	*MATα his4 leu2 ura3 trp1 ade8*
S7 (TTS162-3)[b]	*MATa/α his3/his3 leu2/leu2 ura3/ura3 trp1/trp1 ade8/ade8* +/*tpk1::URA3* +/*tpk2::HIS3* +/*tpk3::TRP1*
TF5.4[c]	*MATa/α his3/his3 leu2/leu2 ura3/ura3 trp1/trp1 ade8/ade8* +/*tpk1::URA3* +/*tpk2::HIS3* +/*tpk3::TRP1* +/*bcy1::LEU2*
S7-1A[d]	*MATa his3 leu2 ura3 trp1 ade8 tpk2::HIS3 tpk3::TRP1*
S7-1B[d]	*MATα his3 leu2 ura3 trp1 ade8 tpk1::URA3 tpk3::TRP1*
S7-3C[d]	*MATa his3 leu2 ura3 trp1 ade8 tpk1::URA3 tpk2::HIS3*
S7-4B[d]	*MATα his3 leu2 ura3 trp1 ade8 tpk1::URA3 tpk3::TRP1*
S7-6C[d]	*MATa his3 leu2 ura3 trp1 ade8 tpk1::URA3 tpk2::HIS3*
S7-7A[d]	*MATa his3 leu2 ura3 trp1 ade8 tpk2::HIS3 tpk3::TRP1*
S7-21B[d]	*MATα his3 leu2 ura3 trp1 ade8 tpk1::URA3 tpk2::HIS3*
S13-7C[e]	*MATa his3 leu2 ura3 trp1 ade8 tpk1::URA3 tpk3::TRP1 bcy1::LEU2*
S13-29D[e]	*MATα his3 leu2 ura3 trp1 ade8 tpk1::URA3 tpk2::HIS3 bcy1::LEU2*
S13-58A[e]	*MATa his3 leu2 ura3 trp1 ade8 tpk2::HIS3 tpk3::TRP1 bcy1::LEU2*
S15-1D[f]	*MATa his3 leu2 ura3 trp1 ade8 tpk1::URA3 tpk2^{w1} tpk3::TRP1 bcy1::LEU2*
S18-1D[g]	*MATα his3 leu2 ura3 trp1 ade8 tpk1^{w1} tpk2::HIS3 tpk3::TRP1*
S18-4A[g]	*his3 leu2 ura3 trp1 ade8 tpk1^{w1} tpk2::HIS3 bcy1::LEU2*
TF1.5[b]	*MATα his3 leu2 ura3 trp1 ade8 tpk2::HIS3 tpk3::TRP1 bcy1::URA3*
TF2.1[b]	*MATα his3 leu2 ura3 trp1 ade8 tpk1::URA3 tpk3::TRP1 bcy1::LEU2*
TF3.1[b]	*MATa his3 leu2 ura3 trp1 ade8 tpk1::URA3 tpk2::HIS3 bcy1::LEU2*
RS13-58A-1[h]	*MATa his3 leu2 ura3 trp1 ade8 tpk1^{w1} tpk2::HIS3 tpk3::TRP1 bcy1::LEU2*
RS13-58A-11[h]	*MATa his3 leu2 ura3 trp1 ade8 tpk1^{w11} tpk2::HIS3 tpk3::TRP1 bcy1::LEU2*
RS13-7C-1[h]	*MATa his3 leu2 ura3 trp1 ade8 tpk1::URA3 tpk2^{w1} tpk3::TRP1 bcy1::LEU2*
RS13-29D-12[h]	*MATα his3 leu2 ura3 trp1 ade8 tpk1::URA3 tpk2::HIS3 tpk3^{w12} bcy1::LEU2*
RTF1.5-2[h]	*MATα his3 leu2 ura3 trp1 ade8 tpk1^{w2} tpk2::HIS3 tpk3::TRP1 bcy1::URA3*
RTF1.5-6[h]	*MATα his3 leu2 ura3 trp1 ade8 tpk1^{w6} tpk2::HIS3 tpk3::TRP1 bcy1::URA3*
RTF1.5-7[h]	*MATα his3 leu2 ura3 trp1 ade8 tpk1^{w7} tpk2::HIS3 tpk3::TRP1 bcy1::URA3*
RTF1.5-8[h]	*MATα his3 leu2 ura3 trp1 ade8 tpk1^{w8} tpk2::HIS3 tpk3::TRP1 bcy1::URA3*
RTF1.5-9[h]	*MATα his3 leu2 ura3 trp1 ade8 tpk1^{w9} tpk2::HIS3 tpk3::TRP1 bcy1::URA3*
RTF1.5-10[h]	*MATα his3 leu2 ura3 trp1 ade8 tpk1^{w10} tpk2::HIS3 tpk3::TRP1 bcy1::URA3*
RTF1.5-12[h]	*MATα his3 leu2 ura3 trp1 ade8 tpk1^{w12} tpk2::HIS3 tpk3::TRP1 bcy1::URA3*
RTF1.5-13[h]	*MATα his3 leu2 ura3 trp1 ade8 tpk1^{w13} tpk2::HIS3 tpk3::TRP1 bcy1::URA3*
RTF3.1-1[h]	*MATa his3 leu2 ura3 trp1 ade8 tpk1::URA3 tpk2::HIS3 tpk3^{w1} bcy1::LEU2*
TF14.1[i]	*MATa his3 leu2 ura3 trp1 ade8 tpk1^{w1} tpk2::HIS3 tpk3::TRP1 bcy1::LEU2 cyr1::URA3*
TF16.1[i]	*MATa his3 leu2 ura3 trp1 ade8 tpk1^{w1} tpk2::HIS3 tpk3::TRP1 bcy1::LEU2 cdc25::URA3*
TF17.2[i]	*MATa his3 leu2 ura3 trp1 ade8 tpk1^{w1} tpk2::HIS3 tpk3::TRP1 bcy1::LEU2 ras2::ADE8*
TF18.2[j]	*MATa his3 leu2 ura3 trp1 ade8 tpk1^{w1} tpk2::HIS3 tpk3::TRP1 bcy1::LEU2 ras1::URA3 ras2::ADE8*
TF19.1[k]	*MATa his3 leu2 ura3 trp1 ade8 tpk1^{w1} tpk2::HIS3 tpk3::TRP1 bcy1::LEU2 ade8::pADE8*
TF20.1[l]	*MATa his3 leu2 ura3 trp1 ade8 tpk1^{w1} tpk2::HIS3 tpk3::TRP1 bcy1::LEU2 cyr1::URA3 ade8::pADE8*
TF22.1[m]	*MATa his3 leu2 ura3 trp1 ade8 tpk1^{w1} tpk2::HIS3 tpk3::TRP1 bcy1::LEU2 cdc25::URA3 ade8::pADE8*
TF23.1[l]	*MATa his3 leu2 ura3 trp1 ade8 tpk1^{w1} tpk2::HIS3 tpk3::TRP1 bcy1::LEU2 ade8::pADE8*
TF26.1[n]	*MATa his3 leu2 ura3 trp1 ade8 tpk2::HIS3 tpk3::TRP1 ade8::pADE8*

Strains beginning with S are tetrad segregants from a diploid strain; those beginning with TF were derived by transformation with DNA; those beginning with R are phenotypic revertants derived by selection.

[a] These strains are from the Cold Spring Harbor Laboratory collection, and are the original parents of all strains used in this work. SP1 was derived from a cross between DC124 and DC5, another strain from the collection.

[b] These strains have been described by Toda et al. (1987c).

[c] TF5.4 was derived by transformation of S7 with DNA.

[d] The S7 series are tetrad segregants from the strain S7.

[e] The S13 series are tetrad segregants from the strain TF5.4.

[f] S15-1D is a tetrad segregant from a diploid strain formed by mating RS13-7C-1 and S7-1A.

[g] S18-1D and S18-4A are tetrad segregants derived from a diploid strain formed by mating RS13-58A-1 and S7-21B.

[h] These strains are independent phenotypic revertants derived by selection.

[i] These strains were derived by transformation of RS13-58A-1 with DNA.

[j] TF18.2 was derived by transformation of TF17.2 with DNA.

[k] TF19.1 was derived by transformation of S13-58A with DNA.

[l] TF20.1 was derived by transformation of TF14.1 with DNA.

[m] TF22.1 was derived by transformation of TF16.1 with DNA.

[n] TF26.1 was derived by transformation of S7-1A with DNA.

TPK3, and *BCY1* genes (see Table 1 for a description of strains used in this work). After sporulation and the dissection of complete asci, the genotypes of segregants from TF5.4 were deduced by replica plating to synthetic medium to determine the auxotrophic requirements of the strains.

Segregants from TF5.4 were analyzed for their ability to grow on various carbon sources and for their ability to survive heat shock or starvation for nitrogen. As expected, only strains with at least one functional cAPK catalytic subunit gene were viable and rapid growing (Toda et al., 1987b). All strains with a wild-type *BCY1* gene grow effi-
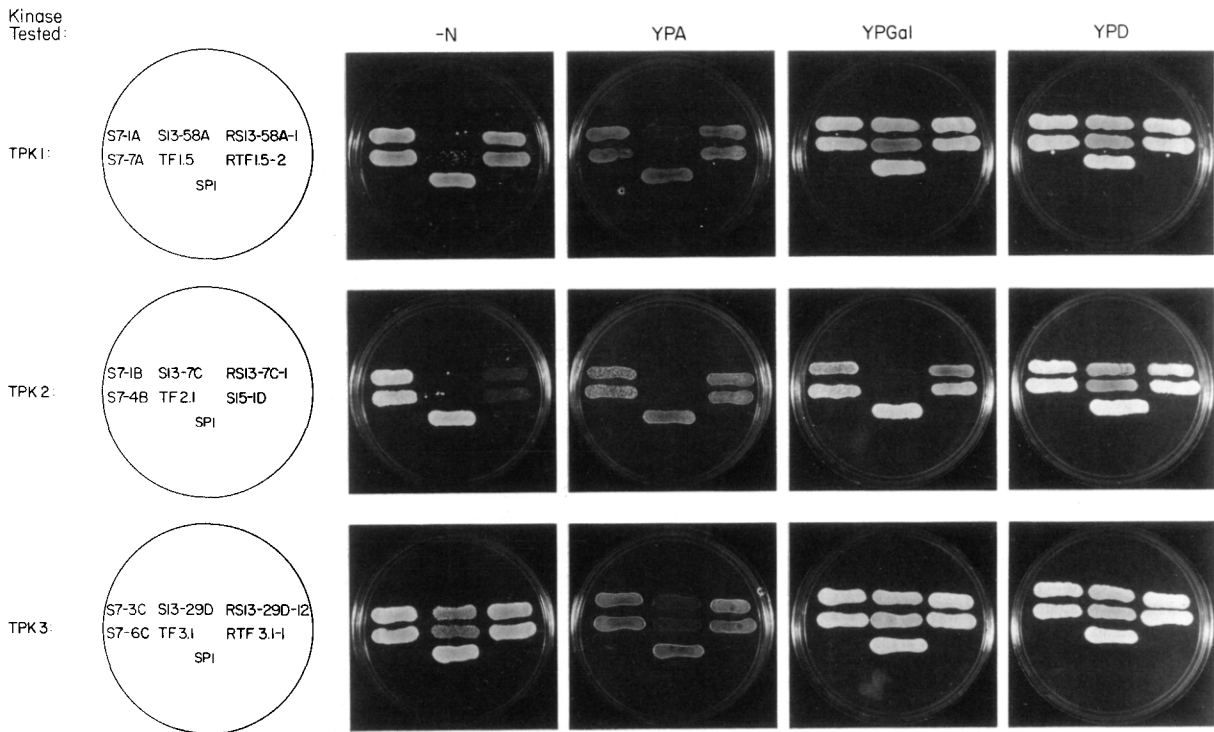
Figure 1. Characterization of Yeast Strains Containing Single *TPK* Genes

Yeast strains were tested to examine the effect of cAPK catalytic activity on the abilities to survive starvation for nitrogen and to utilize carbon sources other than glucose. Yeast strains were inoculated into YPD and grown at 30°C with shaking, collected by centrifugation, washed, and resuspended at known cell densities in either water or nitrogen starvation medium. The samples diluted in water were inoculated with a sterile loop onto plates containing the carbon sources acetate (YPA), galactose (YPGal), or glucose (YPD). Strains inoculated into nitrogen starvation medium were incubated for 1 week, and were then transferred with an inoculating loop onto YPD plates. All plates were grown at 30°C for 3 days before being photographed. The line drawings provide a key for identifying the yeast strains tested (see Table 1). Each plate has two representatives of each of three different genotypes. On the left side of each plate are two *BCY1* strains containing a single wild-type *TPK* gene. In the center of each plate are two *bcy1⁻* strains containing a single wild-type *TPK* gene. On the right side of each plate are two *bcy1⁻* strains containing a *tpkʷ* allele. SP1 is a wild-type strain. The top row of four plates presents the phenotypes of yeast strains containing only *TPK1*, the center row those containing only *TPK2*, and the bottom row those containing only *TPK3*. The phenotypes induced by a particular cAPK catalytic subunit can be assessed by examining the four plates of one row. To compare the effects of the different catalytic subunits on a single phenotype, a column can be examined. Note that the two *bcy1⁻* strains containing the mutant allele of *TPK2* form thin patches after starvation for nitrogen. The thin patch formed by these strains is not due to sensitivity to starvation for nitrogen, but rather to the slow recovery after starvation of strains containing the mutant allele of *TPK2*. The resistance of all strains to nitrogen starvation was quantitated by plating dilutions before and after the starvation treatment. All the *bcy1⁻* revertants are as resistant to nitrogen starvation as the *BCY1* controls. In this experiment, approximately 1% of the *bcy1⁻* strains containing *TPK3* as the only functional *TPK* gene survived the nitrogen starvation. Approximately 0.1% of *bcy1⁻* strains containing *TPK1* or *TPK2* survived the starvation. Nitrogen starvation medium, YPA, YPGal, and YPD have been described (Toda et al., 1985).

ciently on acetate and galactose, and are resistant to the lethal effects of heat shock or nitrogen starvation. All *bcy1⁻* segregants are very sensitive to heat shock (data not presented), but the severity of the other *bcy1⁻* defects observed depends upon which cAPK catalytic subunits are present (see Figure 1). *bcy1⁻* strains containing only *TPK3* grow well on glucose and galactose, grow poorly on acetate as the primary carbon source, and are only somewhat sensitive to nitrogen starvation. *bcy1⁻* strains containing only *TPK1* grow on glucose or galactose, grow very poorly on acetate, and are very sensitive to nitrogen deprivation. *bcy1⁻* strains containing only *TPK2* demonstrate the most severe defects of growth. They form a thin patch even on rich, glucose-containing plates, are entirely unable to grow on galactose- or acetate-based media, and are very sensitive to nitrogen starvation. The characteristic defects induced by the presence of a given cAPK cata-

lytic subunit are dominant. For example, *bcy1⁻* strains containing a functional *TPK2* gene do not grow on galactose, regardless of the presence or absence of *TPK1* or *TPK3* (data not presented). The phenotype of a *bcy1⁻* strain is therefore at least partially a consequence of the cumulative actions of the products of the *TPK1*, *TPK2*, and *TPK3* genes, with *TPK2* causing the most severe defects and *TPK3* the least.

Mutant *TPK* Genes Can Suppress *bcy1⁻*

When *bcy1⁻* strains containing only a single functional cAPK catalytic subunit gene are incubated for a prolonged period of time as a patch on a plate, papillations appear on the lawn of cells. It seemed likely that the papillations might contain mutations that improve the ability of *bcy1⁻* strains to survive sub-optimal nutrient conditions. Indeed, we found that the papillations contain cells that, unlike the

Table 2. Genetic Linkage of bcy1⁻ Suppressor Phenotype to the TPK1 Gene

Diploid Genotype			
TPK1	TPK2	TPK3	BCY1
−/∗	−/−	+/−	+/−

Segregant Genotype				Fraction Heat
TPK1	TPK2	TPK3	BCY1	Shock Resistant
∗	−	+	+	22/22
	−	+	+	15/15
∗	−	−	+	15/15
∗	−	+	−	0/11
∗	−	−	−	19/19
−	−	+	−	0/18

This tetrad data is designed to test whether a phenotypic revertant from a bcy1⁻ strain contains a suppressor linked to the TPK1 gene. An asterisk identifies the TPK1 gene that was originally present in the phenotypically reverted bcy1⁻ strain. The diploid strain was constructed to be heterozygous at BCY1, heterozygous for a disruption of TPK1, heterozygous for a disruption of TPK3, and homozygous for disruption of TPK2. Heterozygosity at BCY1 is necessary to avoid the sporulation defect characteristic of bcy1⁻ strains. Heterozygosity at the TPK1 locus allows identification of the cAPK catalytic subunit gene originally present in the phenotypic revertant. The strain RS13-58A-1 (bcy1⁻ tpk1ʷ¹ tpk2⁻ tpk3⁻) was crossed to the strain S7-21B (BCY1 tpk1⁻ tpk2⁻ TPK3), and diploids were selected by amino acid prototrophy. Following sporulation and tetrad analysis, segregants were scored for resistance to heat shock treatment. Genotypes were determined from the auxotrophic requirements of strains.

parental bcy1⁻ strains, can efficiently utilize acetate as the carbon source for growth. As will be described below, these phenotypic revertants do not display any of the defects characteristic of strains lacking the regulatory subunit of the cAPK. Such complete phenotypic revertants could not be obtained when starting with a bcy1⁻ strain containing multiple functional TPK genes.

Mutation of the sole gene encoding a functional cAPK catalytic subunit might be expected to suppress the defects that result from the loss of the regulatory subunit, and to be genetically recessive. All of 40 independent phenotypic revertants isolated from bcy1⁻ strains having a single functional cAPK catalytic subunit gene contained recessive mutations (see Experimental Procedures and Table 2 for a description of the genetic analysis). Ten revertants were analyzed from bcy1⁻ strains containing only TPK1, one from a bcy1⁻ strain containing only TPK2, and twenty-nine from bcy1⁻ strains containing only TPK3. Complementation analysis of the revertants revealed that all ten isolates from the bcy1⁻ TPK1 tpk2⁻ tpk3⁻ strains formed a single complementation class, as did all twenty-nine isolates from the bcy1⁻ tpk1⁻ tpk2⁻ TPK3 strains.

We used a genetic approach to test whether the phenotypic revertants from bcy1⁻ strains contain mutant cAPK catalytic subunit genes. A revertant from a bcy1⁻ strain containing TPK1 as the sole gene encoding a functional cAPK catalytic subunit was crossed to a strain containing a TPK1 gene that was disrupted by insertion of the URA3 marker. The diploid was heterozygous at BCY1 to avoid the sporulation defect characteristic of bcy1⁻ mutants, homozygous for disruption of the TPK2 genes, and hetero-

zygous for a disruption of the TPK3 gene (see Table 2 for details). Analysis of tetrad segregants following sporulation and dissection of complete asci indicates that the suppression of the bcy1⁻ phenotype is due to a single mutation linked to the TPK1 gene originally found in the revertant (Table 2). Four observations from the tetrad data support this conclusion. First, all of the bcy1⁻ strains are resistant to heat shock whenever they contain the allele of TPK1 originally present in the revertant as their sole functional TPK gene. Second, none of the bcy1⁻ strains containing a wild-type allele of TPK3 are resistant to heat shock. Third, spores that contain the allele of TPK1 from the revertant as the sole gene encoding a cAPK catalytic subunit germinate and form colonies at close to the statistically expected frequency. Fourth, no viable and rapidly growing tpk spores appear. We can thus rule out the possibility of two mutations in the revertant: one that abolishes cAPK catalytic activity, thus suppressing the bcy1⁻ phenotype, and a second mutation that renders these strains viable in the absence of cAPK activity. If such a tpk⁻ suppressor were present, half of the tpk1⁻ tpk2⁻ tpk3⁻ spores would have been viable and rapid growing, and half of the spores that contain null alleles at TPK2 and TPK3, and the TPK1 allele from the revertant, would have been inviable or extremely slow growing. After similar tetrad analysis of revertants from bcy1⁻ strains containing either TPK2 or TPK3 as the sole functional TPK gene, we obtained analogous results (data not presented). In each case, suppression of the bcy1⁻ phenotype is due to a single mutation genetically linked to the cAPK catalytic subunit gene that was originally present in the revertant. Since the three TPK genes are not genetically linked, we conclude that the phenotypic revertants from bcy1⁻ strains contain mutant TPK genes. We designate these mutant alleles tpkʷ.

The tpk1ʷ¹ Allele Shows Reduced Kinase Activity

To demonstrate more concretely that revertants of the bcy1⁻ phenotypes actually contain mutant cAPK catalytic subunit genes, we recovered from the chromosome and sequenced the TPK1 gene from one revertant strain, RS13-58A-1 (see Experimental Procedures for details). The isolated gene was completely sequenced from approximately 300 nucleotides upstream of the initiating ATG through the termination codon. A single point mutation of TTG to TCG in the codon for the 217th amino acid was found. This mutation changes a leucine to a serine in a highly conserved region of the cAPKs (Figure 2), and presumably accounts for the bcy1⁻ suppressing phenotype of this cAPK catalytic subunit gene. We designate this mutant allele of the TPK1 gene tpk1ʷ¹.

We tested the product of the tpk1ʷ¹ allele for catalytic activity. Extracts were prepared from yeast strains containing either a wild-type TPK1 gene or the tpk1ʷ¹ allele encoding the sole cAPK catalytic subunit. These strains were constructed to contain a functional BCY1 gene, which is required for a cAMP-dependent protein kinase activity (Matsumoto et al., 1982; Toda et al., 1987b; Johnson et al., 1987). Extracts from both strains were fractionated by ion-exchange chromatography and assayed for
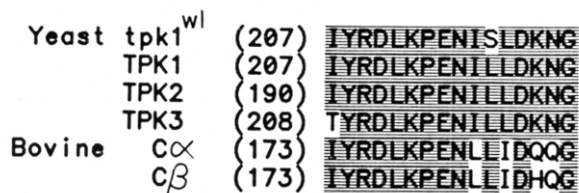
Yeast tpk1^{wl} (207) IYRDLKPENISLDKNG
TPK1 (207) IYRDLKPENILLDKNG
TPK2 (190) IYRDLKPENILLDKNG
TPK3 (208) TYRDLKPENILLDKNG
Bovine Cα (173) IYRDLKPENLLIDQQG
Cβ (173) IYRDLKPENLLIDHQG

Figure 2. Amino Acid Sequence of the Mutant Region of the *tpk1^{wl}* Allele

A portion of the amino acid sequence of the *tpk1^{wl}* allele is aligned with corresponding sequences of the three yeast (Toda et al., 1987c) and two bovine (Shoji et al., 1981; Showers and Mauver, 1986) cAPK catalytic subunits. The conserved leucine at amino acid position 217 of *TPK1* is changed to a serine in the mutant. The *tpk1^{wl}* allele of *TPK1* was isolated from the strain RS13-58A-1 (see Experimental Procedures for a description of the isolation and sequencing strategies).

cAPK activity as described previously (Toda et al., 1987b). A cAMP-dependent phosphorylating activity is present in the extract from the strain containing a wild-type *TPK1* gene encoding the cAPK catalytic subunit (see Figure 3A). No activity is detectable in the extract from the strain containing the mutant allele of *TPK1* (Figure 3B). A trivial explanation for the failure to detect kinase activity could be that the fractions assayed from the yeast strain having the mutant cAPK catalytic subunit gene do not contain intact protein. Therefore, we determined the amount of immunoreactive *TPK1*-encoded protein in the column fractions. Comparable amounts of *TPK1* protein are present in the extracts from yeast strains containing either a wild-type or a mutant *TPK1* gene (Figures 3A and 3B). Thus it is likely that the *tpk1^{wl}* allele isolated as a suppressor of the *bcy1$^-$* mutation encodes a catalytic subunit of the cAPK with a reduced capacity for phosphorylating relevant substrates. We expect that most, if not all, of the products of the various mutant *TPK* genes are attenuated. Justification for this expectation is provided in the Discussion.

All the *bcy1$^-$* Phenotypes Are Suppressed by Mutant *TPK* Genes

Unlike the parental strains, the revertant *bcy1$^-$* strains that contain the mutant *TPK* genes grow well on acetate- or galactose-based media, and are fully resistant to nitrogen starvation (Figure 1). They are reverted for other *bcy1$^-$* phenotypes as well. Strains lacking the regulatory subunit of the cAPK do not accumulate the storage carbohydrates glycogen or trehalose (Uno et al., 1983; Cannon et al., 1986); however, all of nine independent revertants isolated from *bcy1$^-$* strains containing only *TPK1*, *TPK2*, or *TPK3* stored more carbohydrate than wild-type strains (data not presented). *bcy1$^-$/bcy1$^-$* homozygous diploid strains do not sporulate in response to nutrient limitation (Matsumoto et al., 1983b; Cannon et al., 1986; Toda et al., 1987b); however, *bcy1$^-$/bcy1$^-$* homozygous diploid strains containing only mutant *TPK* genes are competent for sporulation (see below). In crosses, suppression of all the *bcy1$^-$* phenotypes cosegregated with the mutant *tpk^w* alleles, and all tetrad segregants with similar genotypes displayed similar phenotypes (data not presented and Table 2).
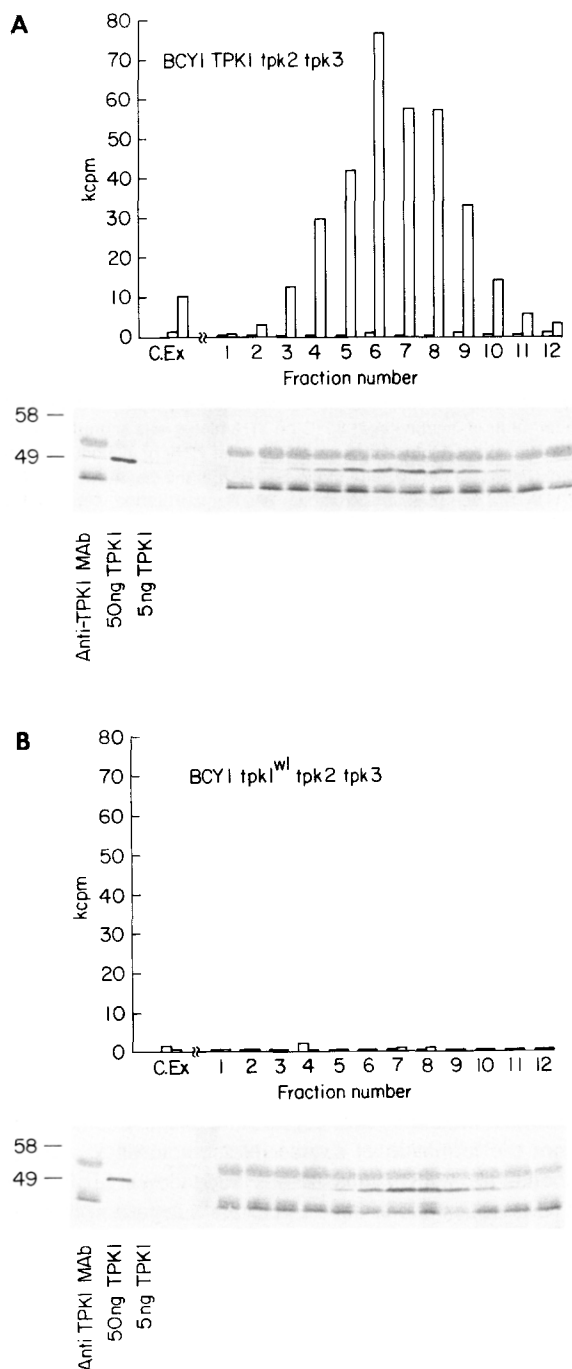

Figure 3. Reduced Kinase Activity of the *tpk1^{wl}* Allele

Yeast cell lysates were prepared from strains containing either a wild-type or an attenuated allele of *TPK1* encoding the sole cAPK catalytic subunit. After chromatography on a DEAE-Sephacel column, fractions were assayed for protein kinase activity in the absence (lefthand bar of each pair) or presence (righthand bar of each pair) of 10 μM cAMP by using the synthetic peptide Kemptide (Kemp et al., 1977) as substrate. Cell lysates were prepared and assayed basically as described by Toda et al. (1987b). (A) Strain S7-1A, containing a wild-type *TPK1* gene. (B) Strain S18-1D, containing the attenuated allele *tpk1^{wl}*. kcpm, kilocounts per minute. An immunoblot of immune-precipitated column fractions for *TPK1*-encoded protein is aligned below a bar graph of cAPK activity in that fraction (see Experimental Procedures for details).

Table 3. Sporulation Efficiency of bcy1 tpk1ʷ Homozygous Diploids

Cross	Diploid Genotype				Medium	
	BCY1	TPK1	TPK2	TPK3	YPA[a]	SPO[b]
RS13-58A-11 × RTF1.5-2	−/−	w11/w2	−/−	−/−	2%	69%
RS13-58A-11 × RTF1.5-6	−/−	w11/w6	−/−	−/−	0%	3%
RS13-58A-11 × RTF1.5-7	−/−	w11/w7	−/−	−/−	0%	0%
RS13-58A-11 × RTF1.5-8	−/−	w11/w8	−/−	−/−	0%	46%
RS13-58A-11 × RTF1.5-9	−/−	w11/w9	−/−	−/−	0%	2%
RS13-58A-11 × RTF1.5-10	−/−	w11/w10	−/−	−/−	0%	5%
RS13-58A-11 × RTF1.5-12	−/−	w11/w12	−/−	−/−	0%	46%
RS13-58A-11 × RTF1.5-13	−/−	w11/w13	−/−	−/−	0%	1%
S13-58A × RTF1.5-8	−/−	+/w8	−/−	−/−	0%	0%

This table presents an examination of the sporulation properties of a series of bcy1⁻ tpk1ʷ tpk2⁻ tpk3⁻ homozygous diploid strains. The bcy1⁻ tpk1ʷ¹¹ tpk2⁻ tpk3⁻ strain RS13-58A-11 was crossed to eight phenotypic revertants isolated from the strain TF1.5. Each RTF1.5-n has the genotype bcy1⁻ tpk1ʷⁿ tpk2⁻ tpk3⁻. Diploid cells were selected by amino acid prototrophy and transferred to YPA medium. The percent of asci formed after 48 hr of incubation at 30°C on YPA plates was scored by microscopic examination of 200 cells. The cells were then transferred from YPA medium to sporulation medium (SPO). After 72 hr of incubation at 30°C on plates containing sporulation medium, the percent of asci formed was determined as before. The strain S13-58A is the parent of RS13-58A-11; it contains an unregulated wild-type cAPK catalytic subunit.
[a] YPA is a rich medium containing 2% Bacto-Peptone, 1% yeast extract, and 2% potassium acetate.
[b] Sporulation medium contains 0.25% yeast extract, 1.5% potassium acetate, 0.1% glucose, and supplements of adenine (2 μg/ml), uracil (2 μg/ml), histidine (10 μg/ml), leucine (10 μg/ml) and tryptophan (10 μg/ml).

Diploid bcy1⁻ tpkʷ Strains Can Sporulate Appropriately

Glycogen accumulation, heat shock resistance, and sporulation are regulated responses to nutritional stress in wild-type cells. bcy1⁻ tpkʷ cells should lack a cAMP-responsive cAPK. It was therefore of great interest to determine whether these responses were regulated in such cells. Sporulation normally occurs when diploid cells adapted for growth on a nonfermentable carbon source such as acetate are starved for nitrogen (Esposito and Klapholz, 1981). The process of spore formation in yeast can be disrupted by genetic manipulation of the cAMP pathway. Diploid yeast containing temperature-sensitive components of the cAMP pathway sporulate inappropriately, forming spores on nitrogen-rich, acetate-based medium (Shilo et al., 1978; Matsumoto et al., 1983b). In contrast, mutations that activate the cAMP pathway prevent the formation of spores (Matsumoto et al., 1983b; Toda et al., 1985; Tatchell et al., 1985; Toda et al., 1987b). These observations have led to the suggestion that the cAMP pathway controls the transition from mitotic growth to meiosis and sporulation (Shilo et al., 1978; Matsumoto et al., 1983b). To test this, we examined the ability of bcy1⁻ tpkʷ strains to sporulate.

We constructed a series of diploid yeast strains that were homozygous for a disruption of the BCY1 gene. In all cases, the genes encoding TPK2 and TPK3 were disrupted. Some of the mutant strains were designed to contain two attenuated alleles of TPK1 as the only cAPK catalytic subunit genes. As a control, some strains contained one wild-type allele of TPK1 and one attenuated allele of TPK1. The diploids were patched onto rich, acetate-based medium and incubated for 48 hr, at which time they were replica plated onto medium lacking nitrogen ("sporulation medium"; see Table 3 for a detailed description). The sporulation efficiency of each diploid strain was determined at various stages by microscopic examination (Table 3). As expected, the bcy1⁻ strains that contained even

a single wild-type TPK gene did not sporulate under any conditions. After incubation on sporulation medium, however, three of the bcy1⁻ diploid strains containing only tpk1ʷ alleles sporulated efficiently. None of the strains having only tpkʷ alleles sporulated prematurely, that is, on nitrogen-rich, acetate-based medium. A similar experiment conducted with bcy1⁻ diploid strains containing attenuated alleles of TPK3 produced similar results (data not presented). The results suggest that bcy1⁻ diploid strains containing only mutant TPK genes are capable of appropriately regulating the sporulation response.

bcy1⁻ tpkʷ Strains Can Regulate Glycogen Content

When nutrient limited, normal cells accumulate the storage carbohydrates glycogen and trehalose (Lillie and Pringle, 1980). Storage carbohydrate accumulation is enhanced in strains with decreased activity of the cAMP pathway (Tatchell et al., 1985), and is prevented in strains with increased activity of the pathway (Uno et al., 1983; Toda et al., 1985; Cannon et al., 1986). We have examined the regulation of glycogen accumulation in bcy1⁻ strains containing an attenuated cAPK catalytic subunit gene. A series of five strains were inoculated at low cell density into liquid YPD medium and grown at 30°C with shaking. Aliquots of the cultures were collected at various times during the growth curve, and the glycogen content per cell was quantitated (Gunja-Smith et al., 1977; see Figure 4).

The wild-type strain SP1 and the strain S7-1A, which lacks TPK2 and TPK3 but is otherwise wild type, both have a cAPK that is responsive to changing cAMP concentrations. Both strains store little glycogen during the logarithmic phase of growth. As the culture approaches saturation, the cellular glycogen content of both strains increases. This phenomenon has been previously observed in wild-type yeast (Lillie and Pringle, 1980). When diluted into fresh medium, the strains SP1 and S7-1A transiently accumulate a large quantity of glycogen which then rapidly decreases as the cells re-enter the logarithmic phase of
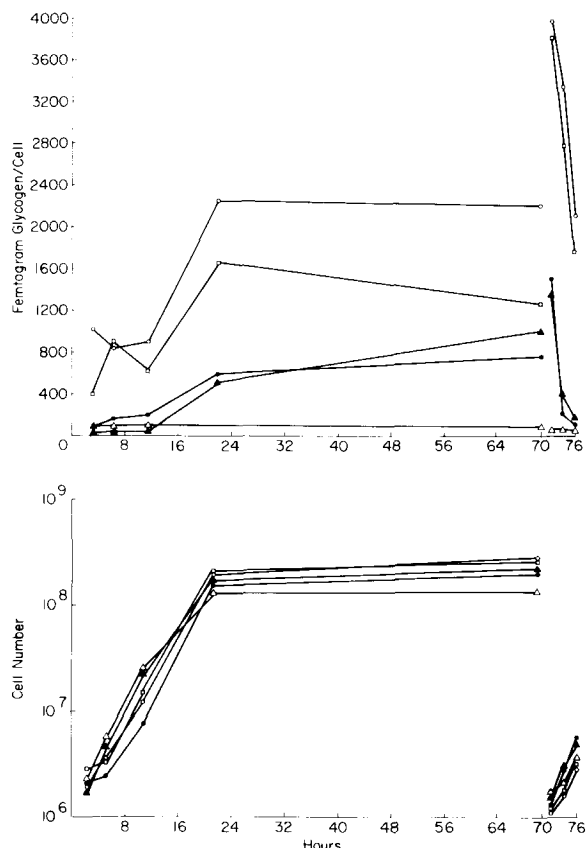
Figure 4. Regulated Glycogen Content of Yeast Cells

Yeast strains were inoculated into liquid YPD medium and grown at 30°C with shaking. Cells were collected at the indicated times and the glycogen content per cell was determined (Gunja-Smith et al., 1977). After 70 hr of growth, cultures were diluted back to 1 × 10⁶ cells per ml in YPD, and the glycogen content per cell was determined at timepoints as before. The lower graph indicates the number of cells per ml of culture at timepoints. The strains are as follows: (□) RS13-58A-1, a *bcy1⁻ tpk1ʷ¹ tpk2⁻ tpk3⁻* strain, (○) TF14.1, a *bcy1⁻ tpk1ʷ¹ tpk2⁻ tpk3⁻ cyr1⁻* strain, (△) S13-58A, a *bcy1⁻ TPK1 tpk2⁻ tpk3⁻* strain, (▲) S7-1A, a *BCY1 TPK1 tpk2⁻ tpk3⁻* strain, and (●) SP1, a wild-type strain.

growth. As expected, the *bcy1⁻* strain S13-58A, which contains an unregulated wild-type cAPK catalytic subunit, fails to store glycogen under any conditions. RS13-58A-1, a *bcy1⁻* strain containing the *tpk1ʷ¹* allele encoding the only cAPK catalytic subunit, stores a high basal level of glycogen relative to the wild-type strains, but regulates this store in parallel with wild type. This strain stores its lowest level of glycogen while actively growing, and accumulates more as the culture approaches saturation. Upon dilution into fresh medium, RS13-58A-1 transiently increases the pool of stored glycogen. As in the wild-type strains, this pool is then rapidly diminished. As is described in detail below, adenylyl cyclase is not required for the viability of *bcy1⁻ tpk1ʷ¹* strains. Strain TF14.1 is a *bcy1⁻ tpk1ʷ¹* strain that lacks the *CYR1* gene. Significantly, despite the lack of adenylyl cyclase and of a cAMP-responsive protein kinase in strain TF14.1, the levels of glycogen accumulated in this strain are regulated in parallel with those in a wild-type strain.

bcy1⁻ tpkʷ Strains Can Dynamically Control Heat Shock Resistance

When actively growing, yeast do not survive heat shock treatment (Schenberg-Frascino and Moustacchi, 1972; Iida and Yahara, 1984; Powers et al., 1986). When nutrient limited, wild-type yeast enter a physiologic state that confers resistance to heat shock (Schenberg-Frascino and Moustacchi, 1972; Iida and Yahara, 1984). Some mutant strains of yeast with reduced activity of the cAMP pathway show enhanced resistance to heat treatment (Iida and Yahara, 1984; Shin et al., 1987). Mutational activation of the cAMP pathway—for example, by disruption of the *BCY1* gene—prevents the acquisition of thermotolerance (Toda et al., 1987b; Shin et al., 1987). We have examined in detail the thermotolerance of *bcy1⁻* strains containing an attenuated cAPK catalytic subunit gene. Two representatives of the *bcy1⁻ tpk1ʷ¹* strain RS13-58A-1, and the necessary controls, were inoculated into liquid YPD medium and grown at 30°C with shaking. Aliquots of the cultures were withdrawn at various times during growth and subjected to a heat shock of 50°C for 30 min. Appropriate dilutions of the heat-shocked cells were plated to determine the fraction of surviving cells relative to cells that were not heat shocked (see Figure 5).

The wild-type strain SP1 and the strain S7-1A are both very sensitive to heat shock during the logarithmic phase of growth. At this stage of growth, approximately 0.01% of the cells in these strains survive the heat shock treatment. After 95 hr of growth the cultures are at high density, and essentially all of the SP1 and S7-1A cells have become resistant to the heat treatment. This demonstrates the regulated thermotolerance of yeast strains containing a functional cAMP effector pathway. The *bcy1⁻* strain S13-58A, which contains an unregulated wild-type cAPK catalytic subunit, is sensitive to heat shock while growing and fails to become thermotolerant, even in dense culture. RS13-58A-1, which is a *bcy1⁻* strain containing an attenuated cAPK catalytic subunit, is as sensitive as wild-type strains to heat shock during the logarithmic phase of growth. Unlike the parental *bcy1⁻* strain S13-58A, however, RS13-58A-1 becomes resistant to the heat shock treatment as the culture approaches saturation. Curiously, RS13-58A-1 acquires a thermotolerant state at a somewhat earlier stage of growth than do wild-type strains. The results of this experiment suggest that in yeast, the acquisition of thermotolerance in response to changing nutrient conditions does not require a regulated cAPK system.

Regulation in *bcy1⁻ tpkʷ* Strains Is Independent of *CYR1*

The results of the previous sections establish that *BCY1*-mediated control of cAPK catalytic activity is not required for the regulation of sporulation, glycogen metabolism, or thermotolerance. Nevertheless, it is possible that these processes are regulated by cAMP, but that the *BCY1* and *TPK1*, *TPK2*, and *TPK3* genes do not encode the relevant cAMP-responsive regulatory elements. Mutant strains of yeast having a deletion of the *CYR1* gene, which encodes adenylyl cyclase, do not contain detectable cAMP (Toda et
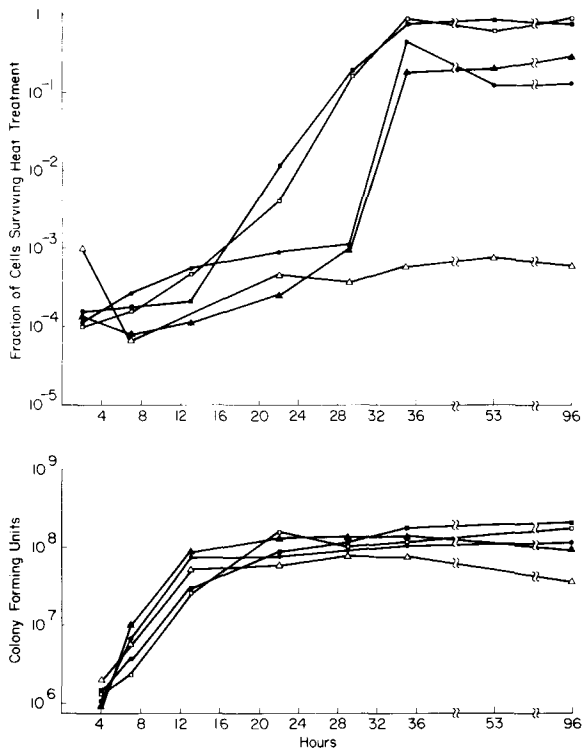
Figure 5. Regulated Thermotolerance of Yeast Strains

Yeast strains were inoculated into liquid YPD medium (at an initial density of 1 × 10⁶ cells per ml) and grown at 30°C with shaking. At the indicated times, aliquots were withdrawn and subjected to a heat shock of 50°C for 30 min. The upper graph presents the fraction of cells at different stages of growth surviving this heat shock relative to a control culture that was not heat shocked. The lower graph presents the number of colony-forming units at the timepoints. The strains are (□, ■), two subclones of RS13-58A-1, a *bcy1⁻ tpk1ʷ¹ tpk2⁻ tpk3⁻* strain, (△) S13-58A, a *bcy1⁻ TPK1 tpk2⁻ tpk3⁻* strain, (▲) S7-1A, a *BCY1 TPK1 tpk2⁻ tpk3⁻* strain, and (●) SP1, a wild-type strain.
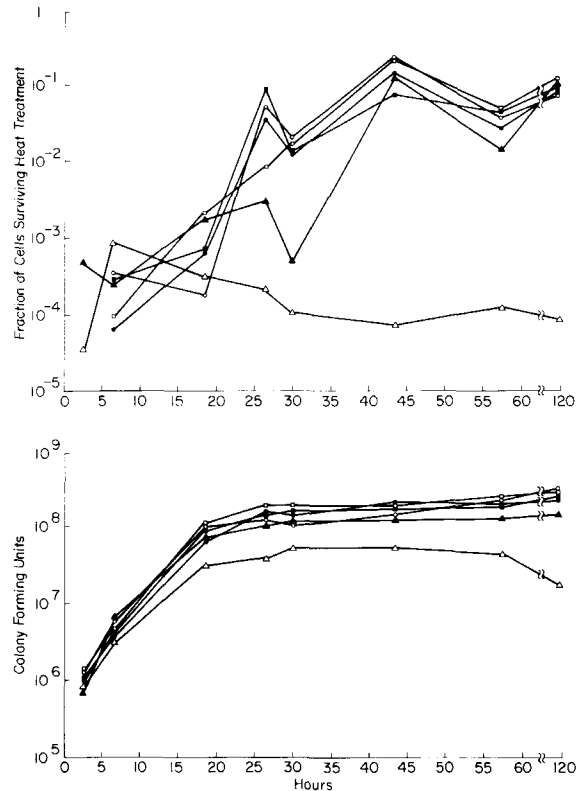

Figure 6. Regulated Thermotolerance of Yeast Strains Lacking *CYR1*, *RAS*, or *CDC25*

Yeast strains were inoculated into liquid YPD medium (at an initial density of 1 × 10⁶ cells per ml) and grown at 30°C with shaking. At the indicated times, aliquots were withdrawn and subjected to a heat shock of 50°C for 30 min. The upper graph presents the fraction of cells at different stages of growth surviving this heat treatment relative to a control culture that was not heat shocked. The lower graph presents the number of colony-forming units at the timepoints. The strains are (□) TF23.1, a *bcy1⁻ tpk1ʷ¹ tpk2⁻ tpk3⁻* strain, (●) TF20.1, a *bcy1⁻ tpk1ʷ¹ tpk2⁻ tpk3⁻ cyr1⁻* strain, (■) TF18.2, a *bcy1⁻ tpk1ʷ¹ tpk2⁻ tpk3⁻ ras1⁻ ras2⁻* strain, (○) TF22.1, a *bcy1⁻ tpk1ʷ¹ tpk2⁻ tpk3⁻ cdc25⁻* strain, (△) TF19.1, a *bcy1⁻ TPK1 tpk2⁻ tpk3⁻* strain, and (▲) TF26.1, a *BCY1 TPK1 tpk2⁻ tpk3⁻* strain.

al., 1987a). We have tested whether cAMP is required for the viability or nutrient-regulated phenotype of a *bcy1⁻ tpkʷ* strain. The *bcy1⁻ tpk1ʷ¹* strain RS13-58A-1 was transformed by a DNA fragment constructed to replace the entire coding region of the *CYR1* gene with the *URA3* gene. Normal numbers of Ura⁺ transformants were obtained, and deletion of the gene for adenylyl cyclase was confirmed by blot hybridization (data not shown). This result indicates that cAMP is not required for the viability of *bcy1⁻ tpkʷ* strains. When the adenylyl cyclase–containing strain RS13-58A-1 (*bcy1⁻ tpk1ʷ¹*) and the adenylyl cyclase–lacking transformants TF14.1, TF14.2, and TF14.3 (*bcy1⁻ tpk1ʷ¹ cyr1⁻*) were compared for resistance to heat shock or nitrogen starvation, accumulation of storage carbohydrates, or ability to utilize nonglucose carbon sources, no significant differences were observed (see Figures 4 and 6, and data not presented). Modulation of cAMP levels is thus not likely to be responsible for the regulation of cellular processes observed in *bcy1⁻ tpkʷ* strains.

Regulation in *bcy1⁻ tpkʷ* Strains Is Independent of *RAS* and *CDC25*

The results of the previous section suggest that there may be a cellular mechanism for responding to changing nutrient conditions that does not require mediation by cAMP or adenylyl cyclase. The products of the *RAS* genes, *RAS1* and *RAS2*, are established as modulators of adenylyl cyclase activity (Toda et al., 1985; Broek et al., 1985). The product of the *CDC25* gene is likely to control the activity of the *RAS* proteins (Robinson et al., 1987; Broek et al., 1987). In addition to their roles as controlling elements of adenylyl cyclase, the *RAS* and *CDC25* proteins may have other regulatory functions in mediating the response to nutrients. We have therefore examined whether the products of the *RAS* and *CDC25* genes are required for the proper regulation of the nutrient response in a *bcy1⁻ tpkʷ* strain. RS13-58A-1 (*bcy1⁻ tpk1ʷ¹*) was transformed by

DNA fragments to disrupt *RAS2* by the *ADE8* gene. A strain that resulted from this transformation was then transformed by a DNA fragment to disrupt *RAS1* by the *URA3* gene. A normal number of Ura⁺ transformants was obtained, and disruption of both *RAS* genes in several transformants was confirmed by blot hybridization (data not presented). In addition, a *bcy1⁻ tpk1ʷ¹* strain having a deletion of the *CDC25* gene replaced by the *URA3* gene was constructed by transformation. As before, the genotype of the Ura⁺ transformants was confirmed by blot hybridization (data not presented). The successful construction of these strains indicates that neither the *CDC25* nor the *RAS* proteins are required for viability in a strain lacking the regulatory subunit of the cAPK and containing an attenuated cAPK catalytic subunit gene. We then examined the acquisition of thermotolerance in these strains as before (Figure 6). Loss of the *CDC25* gene, both the *RAS* genes, or adenylyl cyclase does not affect regulation of the heat shock resistance of a *bcy1⁻ tpk1ʷ¹* strain. This result suggests that the products of the *CDC25* and *RAS* genes are not required to control the response to nutrients in a *bcy1⁻ tpkʷ* strain of yeast.

Discussion

Cells lacking the regulatory subunit of the cAPK are defective in responding to nutrient limitation. We have shown here that the phenotype of a *bcy1⁻* strain is affected by manipulation of the genes for the cAPK catalytic subunits, and is completely dependent upon the presence of a wild-type cAPK catalytic subunit gene. These observations provide convincing evidence that it is the unbridled activity of the cAPK catalytic subunits in a *bcy1⁻* strain, and not the loss of the product of the *BCY1* gene per se, that is responsible for the *bcy1⁻* phenotypes.

The mutant alleles of the cAPK catalytic subunit genes (*tpkʷ* alleles) may encode functionally attenuated proteins. We recovered one mutant allele of the *TPK1* gene from the chromosome and determined its DNA sequence. This mutant allele contains a single missense mutation in the codon for an amino acid very conserved among cAPK catalytic subunits. The protein produced by this mutant cAPK catalytic subunit gene has a greatly reduced ability to phosphorylate a peptide substrate in vitro. It seems likely that this observation reflects a loss of catalytic activity in vivo as well, and that the other *tpkʷ* alleles encode proteins that are similarly defective. In support of this idea, most of the mutant yeast strains containing *tpkʷ* alleles show a modest decrease in growth rate (data not presented), consistent with cAPK catalytic activity being required for optimum growth (Matsumoto et al., 1982, 1983a, 1983b; Toda et al., 1987c). We have also demonstrated that yeast strains having a *tpkʷ* allele encoding the sole cAPK catalytic subunit have extremely high cAMP levels, probably as the consequence of a powerful feedback mechanism responding to the attenuated cAPK catalytic activity found in such strains (Nikawa et al., 1987a).

The biochemical basis for the attenuated catalytic activity of *tpkʷ*-encoded proteins is unknown. We have been unable to detect protein kinase activity in extracts from yeast strains containing the *tpk1ʷ¹* allele of *TPK1*, but it is unlikely that the mutant proteins encoded by the *tpkʷ* alleles are entirely inactive. Although yeast strains lacking any functional cAPK catalytic subunit genes are either inviable or extremely slow growing (Toda et al., 1987c), most strains containing a *tpkʷ* allele encoding the sole cAPK catalytic subunit show only a modest decrease in growth rate. This indicates that the mutant catalytic subunits retain some biological activity required for cell viability. Analysis of the biochemical properties of mutant catalytic subunits may identify amino acid residues required for proper catalytic function of the cAMP-dependent protein kinase.

We demonstrate in the work reported here that yeast cells can dynamically regulate an appropriate response to nutrient conditions in the absence of a cAMP-responsive cAPK. We have examined the regulation of three processes in response to changing nutrient conditions: sporulation, glycogen accumulation, and the acquisition of thermotolerance. In each case, wild-type strains, and strains lacking the regulatory subunit of the cAPK but containing an attenuated allele of the sole functional catalytic subunit gene (*bcy1⁻ tpkʷ* strains), respond similarly to nutrient conditions. *bcy1⁻ tpkʷ* homozygous diploid strains form spores only when transferred to medium lacking both nitrogen and a fermentable carbon source. *bcy1⁻ tpkʷ* strains change their glycogen content to reflect growth conditions. *bcy1⁻ tpkʷ* strains change their resistance to heat shock treatment in response to nutrient limitation. Yeast strains containing attenuated cAPK catalytic subunits do show some differences from wild-type yeast. Relative to wild-type strains, strains with attenuated cAPK catalytic subunit genes store larger amounts of glycogen. Similarly, *bcy1⁻ tpkʷ* strains acquire resistance to heat shock earlier in the growth curve than wild-type strains (Figure 6), and are slow to leave a heat shock–resistant state after dilution into fresh medium (data not presented). These phenomena may reflect the attenuated nature of the cAPK catalytic subunit in *bcy1⁻ tpkʷ* strains. Alternatively, a regulatable cAPK system may be required for the optimal timing of cellular responses to changes in the environment.

If the cAPK regulatory subunit is the sole essential receptor for cAMP in yeast, we would predict that adenylyl cyclase should be dispensable in a *bcy1⁻ tpkʷ* strain. We confirmed this by deleting the *CYR1* gene, which encodes adenylyl cyclase, from a *bcy1⁻ tpkʷ* strain. *cyr1⁻ bcy1⁻ tpkʷ* strains are viable. Significantly, these strains remain responsive to nutrient conditions. This argues convincingly that the cAMP pathway does not have an absolutely required role in mediating a response to nutrients. It follows that at least one regulatory process other than modulation of cAMP levels is capable of controlling the response to nutrients in yeast. The *RAS* and *CDC25* proteins, which modulate the activity of adenylyl cyclase (Toda et al., 1985; Broek et al., 1985; Robinson et al., 1987; Broek et al., 1987), could have additional functions in mediating the response to nutrients in yeast. These func-

tions, while cryptic in wild-type yeast, could in theory be essential for the nutrient responsiveness of a $bcy1^-$ tpk^w strain. We therefore deleted the RAS and CDC25 genes from a $bcy1^-$ tpk^w strain, and found the strains able to respond to nutrients. The results suggest regulatory mechanisms in $bcy1^-$ tpk^w strains that can function independently of the known components of the cAMP pathway. It is also possible that cAMP-independent regulatory mechanisms act by affecting the activity of the cAPK catalytic subunits. $bcy1^-$ tpk^w strains may be useful in identifying these mechanisms.

The yeast S. cerevisiae undergoes marked changes in response to nutrient limitation. Diploid cells will sporulate under appropriate starvation conditions. Haploid cells respond by becoming heat shock resistant and by accumulating storage carbohydrates. These events are frequently likened to the entry of yeast cells into a state in the cell cycle that in mammalian cells is called G_0, since these events do not occur in the normal course of the cell cycle. Since entry into this state can be mimicked by mutations that lower the activity of the cAPK and can be blocked by mutations that raise the activity of the cAPK, it is natural to assume that physiologic modulation of cAPK activity by cAMP regulates the entry of yeast cells into this state. There is no direct evidence in support of this idea, however. While our work does not conflict with this idea, we can conclude that there are other cAMP-independent mechanisms that can regulate events associated with the G_0 state. We do not wish to imply that a single concerted mechanism controls sporulation, glycogen accumulation, and the acquisition of thermotolerance; rather, it seems likely to us that these events are under multiple regulatory controls.

Experimental Procedures

DNA

DNA restriction endonucleases, polymerases, and ligase were used as recommended by suppliers (New England BioLabs, Inc.; Boehringer Mannheim Biochemicals; or Bethesda Research Laboratories, Inc.). Standard molecular cloning techniques and nitrocellulose filter hybridizations were performed as described by Maniatis et al. (1982). DNA sequence analysis was done using the dideoxy chain–termination method of Sanger et al. (1977).

Strains, Disruption Fragments, Media, and Nomenclature

The yeast strains used in this work are listed in Table 1. The BCY1 locus was disrupted by using the BamHI fragments of pbcy1::LEU2 or pbcy1::URA3 (Toda et al., 1987b). RAS1 was disrupted by using the BamHI–EcoRI fragment of pras1::URA3 (Kataoka et al., 1984). RAS2 was disrupted by using the HindIII–NcoI fragment of pras2::ADE8. pras2::ADE8 was constructed by cleaving pRAS2 with HpaI and inserting the BamHI–BglII fragment of the ADE8 gene (White et al., 1985). CDC25 was disrupted by using the SalI fragment of pcdc25::URA3 (Broek et al., 1987). CYR1 was disrupted by using the BglII fragment of pcyr1::URA3. This plasmid was constructed by digesting pCYR1-2 (Kataoka et al., 1985) with PvuII and BalI. These enzymes cleave in the 5'- and 3'-flanking regions of CYR1, respectively. The PvuII to BalI region was replaced with the HindIII–SmaI fragment of URA3. The E. coli strains HB101 and MC1061 (Casadaban and Cohen, 1980) were used for the isolation of plasmid DNA. The E. coli strain BSJ 72 (gift of S. Henikoff) was used to prepare single-stranded DNA for sequence analysis. All media have been described (Toda et al., 1985). In the text, wild-type alleles or dominant mutant alleles are represented by capital letters. Recessive mutant alleles are represented by lowercase letters.

abc::XYZ indicates that XYZ is integrated at and disrupts the ABC locus. This latter case is abbreviated abc⁻

Genetic Methods and Physiologic Assays

Standard yeast genetic procedures for diploid construction, sporulation, and tetrad analysis were used (Sherman et al., 1986). For complementation analysis, revertants from $bcy1^-$ strains containing a single functional TPK gene were mated with each of two $bcy1^-$ strains: a $bcy1^-$ strain containing a known wild-type TPK gene, and a $bcy1^-$ strain containing a known mutant TPK allele. Prototrophy for amino acid markers was used to select diploid products from a cross. This required that revertants be raised from $bcy1^-$ strains of opposite mating types and with suitable auxotrophic markers for selection. For example, all of the revertants from $bcy1^-$ strains containing TPK1 as the sole functional TPK gene were selected from the strains TF1.5 (MATα his3 leu2 ura3 trp1 ade8 tpk2::HIS3 tpk3::TRP1 bcy1::URA3) and S13-58A (MATα his3 leu2 ura3 trp1 ade8 tpk2::HIS3 tpk3::TRP1 bcy1::LEU2). Diploid strains formed by crosses between these parental strains, and revertants derived from them, were selected on synthetic medium supplemented only with adenine. For the purposes of complementation analysis, suppression of the $bcy1^-$ phenotype was scored by assessing the heat shock sensitivity of $bcy1^-$ diploid strains. Diploid strains selected from a cross were patched onto a YPD plate, grown at 30°C for two days, and then replica plated to a YPD plate that had been preheated to 55°C. After incubation at 55°C for 30 min, the plate was transferred to 30°C and allowed to recover overnight before being scored. When revertants from the strain TF1.5 were crossed to the strain S13-58A, the resulting diploids were sensitive to heat shock, indicating that the suppressor mutations were recessive. The same result was obtained when revertants from RS13-58A were crossed to TF1.5. Crosses among revertants from the TF1.5 and S13-58A strains produced only heat shock–resistant diploid strains, indicating a single complementation group. Similar methods were used to place revertants from $bcy1^-$ strains containing TPK3 into complementation groups.

Isolation and Sequence Analysis of $tpk1^{w1}$

The $tpk1^{w1}$ allele of TPK1 was retrieved from the chromosome by mitotic gap repair (Orr-Weaver et al., 1983). The plasmid designed for this purpose, pKRP-1, was constructed as follows. A 2.6 kb SphI–EcoRI fragment containing TPK1 was inserted into the corresponding sites of a pUC118 (Vieira and Messing, 1987) derivative that had been made by destroying the HindIII site. This plasmid was cleaved at a HindIII site in the TPK1 gene found approximately 200 nucleotides 3' of the termination codon of the gene, and the ends were filled with the large fragment of DNA polymerase I. This DNA was then cleaved at an EcoRV site approximately 300 nucleotides 5' of the initiating ATG of the TPK1 gene. The vector sequences were isolated, and an XhoI linker was inserted by ligation to create a new plasmid, pTPK1.Xho. To provide both a selectable marker for transformation and an origin for replication in yeast, the 1.4 kb EcoRI fragment containing the TRP1 gene was inserted at the unique EcoRI site in pTPK1.Xho. The resulting plasmid is called pKRP-1.

The $tpk1^{w1}$-containing yeast strain S18-4A was transformed with XhoI-linearized pKRP-1. The Trp⁺ transformants were pooled, and DNA was prepared and transformed into the E. coli strain MC1061 (Casadaban and Cohen, 1980). To identify recombinant pKRP-1 plasmids containing the mutant tpk1 gene, colony-filter hybridization (Maniatis et al., 1982) using the 1.0 kb XbaI fragment of TPK1 as probe was used (Toda et al., 1987c). A recombinant plasmid carrying the mutant $tpk1^{w1}$ allele was isolated and transferred to the E. coli strain BSJ 72 (gift of S. Henikoff). Single-stranded DNA was prepared after transfection with the helper phage M13-KO7 (Vieira and Messing, 1987). Six oligonucleotides were designed to prime sequencing reactions for the TPK1 gene starting from the EcoRV site 300 nucleotides 5' of the initiating ATG, through the complete coding sequence, and to the HindIII site 200 nucleotides 3' of the termination codon. One strand of the DNA was completely sequenced alongside the wild-type sequence.

Kinase Assay and Immunoblot Analysis

Preparation of cell extracts, column chromatography, and assays for cAMP-dependent kinase activity were performed as described previ-

ously (Toda et al., 1987b), but the kinase assay reaction conditions were changed to increase sensitivity. The modified reaction mix contained the following in a volume of 50 μl: 50 mM MOPS (3-[N-morpholino]propanesulfonic acid; pH 7.0), 10 mM $MgCl_2$, 250 μg of bovine serum albumin per ml, 100 μM [γ-^{32}P]ATP at 750 cpm/pmol, 500 μM Kemptide (Kemp et al., 1977), 5 μl of extract, and, where indicated, 10 μM cAMP. Reactions were terminated after 8 min at 30°C by spotting 15 μl of the reaction mix onto phosphocellulose paper (1 × 2 cm; P-81; Whatmann Inc.) and washing as described by Toda et al. (1987b).

To detect *TPK1*-encoded protein in column fractions, 1 ml of each column fraction was treated with 20 μl of hybridoma supernatant containing a monoclonal antibody (MAbZ48) directed against the *TPK1*-encoded protein (Zoller et al., 1988). Immune complexes were precipitated by incubation with 100 μl of Protein-A agarose (Bio-Rad) and solubilized in boiling sample buffer, and one-quarter of each precipitate was loaded and run on a 12.5% polyacrylamide gel in the presence of sodium dodecyl sulfate. The proteins were then transferred to nitrocellulose filters, blocked overnight in phosphate-buffered saline containing 3% bovine serum albumin and 1% gelatin (PBG), and incubated with 50-fold diluted MAbZ48 in PBG for 2 hr. *TPK1*-encoded protein was visualized by treatment with gold-conjugated anti-mouse IgG (Bio-Rad) and subsequent silver enhancement according to the manufacturer's instructions.

Determination of Glycogen Content

Yeast strains were inoculated at a density of 1 × 10^6 cells per ml in 2 liter flasks containing 1 liter of YPD. Cultures were grown at 30°C with shaking. At the indicated times, cells were collected on a nitrocellulose filter (0.60 μm pores; Schleicher and Schuell, Inc.), rinsed with ice-cold water, resuspended in 25 ml of ice-cold water, counted by using a hemocytometer, and pelleted by centrifugation. The cell pellets were stored at −20°C before the cellular glycogen content was determined (Gunja-Smith et al., 1977).

Determination of Heat Shock Resistance

Yeast strains were inoculated at a density of 1 × 10^6 cells per ml in 250 ml flasks containing 100 ml of YPD. Cultures were grown at 30°C with shaking. At the indicated times, 1 ml aliquots were withdrawn into 12 × 75 mm borosilicate glass tubes and heat shocked in an oil bath at 50°C for 30 min. After the heat treatment, dilutions were prepared and plated on YPD plates to determine the fraction of surviving cells relative to non-heat shocked control platings.

Acknowledgments

Received February 5, 1988; revised March 11, 1988.

References

Beebe, S. J., and Corbin, J. D. (1986). Cyclic nucleotide-dependent protein kinases. In The Enzymes, Vol. 17, P. D. Boyer, ed. (New York: Academic Press), pp. 43–111.

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.

Cannon, J., and Tatchell, K. (1987). Characterization of *Saccharomyces cerevisiae* genes encoding subunits of cyclic AMP-dependent protein kinase. Mol. Cell. Biol. *7*, 2653–2663.

Cannon, J. F., Gibbs, J. B., and Tatchell, K. (1986). Suppressors of the *ras2* mutation of Saccharomyces cerevisiae. Genetics *113*, 247–264.

Casadaban, M. J., and Cohen, S. N. (1980). Analysis of gene control signals by DNA fusion and cloning in Escherichia coli. J. Mol. Biol. *138*, 179–207.

Edelman, A. M., Blumenthal, D. K., and Krebs, E. G. (1987). Protein serine/threonine kinases. Ann. Rev. Biochem. *56*, 567–613.

Esposito, R. E., and Klapholz, S. (1981). Meiosis and ascospore development. In The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance, J. N. Strathern, E. W. Jones, and J. R. Broach, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory), pp. 211–287.

Gunja-Smith, Z., Patil, N. B., and Smith, E. E. (1977). Two pools of glycogen in Saccharomyces. J. Bacteriol. *130*, 818–825.

Iida, H., and Yahara, I. (1984). A heat shock-resistant mutant of *Saccharomyces cerevisiae* shows constitutive synthesis of two heat shock proteins and altered growth. J. Cell Biol. *99*, 1441–1450.

Johnson, K. E., Cameron, S., Toda, T., Wigler, M., and Zoller, M. (1987). Expression in *Escherichia coli* of *BCY1*, the regulatory subunit of the cAMP-dependent protein kinase from *Saccharomyces cerevisiae*. J. Biol. Chem. *262*, 8636–8642.

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., Powers, S., Cameron, S., Fasano, O., Goldfarb, M., Broach, J., and Wigler, M. (1985). Functional homology of mammalian and yeast *RAS* genes. Cell *40*, 19–26.

Kemp, B. E., Graves, D. J., Benjamini, E., and Krebs, E. G. (1977). Role of multiple basic residues in determining the substrate specificity of cAMP-dependent protein kinase. J. Biol. Chem. *252*, 4888–4898.

Kunisawa, R., Davis, T. N., Urdea, M. S., and Thorner, J. (1987). Complete nucleotide sequence of the gene encoding the regulatory subunit of the 3',5'-cyclic AMP-dependent protein kinase from the yeast *Saccharomyces cerevisiae*. Nucl. Acids Res. *15*, 368–369.

Lillie, S. H., and Pringle, J. R. (1980). Reserve carbohydrate metabolism in Saccharomyces cerevisiae: responses to nutrient limitation. J. Bacteriol. *143*, 1384–1394.

Lisziewicz, J., Godany, A., Förster, H., and Küntzel, H. (1987). Isolation and nucleotide sequence of a *Saccharomyces cerevisiae* protein kinase gene suppressing the cell cycle start mutation *cdc25*. J. Biol. Chem. *262*, 2549–2553.

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

Marshall, M. S., Gibbs, J. B., Scolnick, E. M., and Sigal, I. S. (1987). Regulatory function of the *Saccharomyces cerevisiae RAS* C-terminus. Mol. Cell. Biol. *7*, 2309–2315.

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. (1983a). Control of cell division in *Saccharomyces cerevisiae* mutants defective in adenylate cyclase and cAMP-dependent protein kinase. Exp. Cell Res. *146*, 151–161.

Matsumoto, K., Uno, I., and Ishikawa, T. (1983b). Initiation of meiosis in yeast mutants defective in adenylate cyclase and cyclic AMP-dependent protein kinase. Cell *32*, 417–423.

Nikawa, J., Cameron, S., Toda, T., Ferguson, K. M., and Wigler, M. (1987a). Rigorous feedback control of cAMP levels in Saccharomyces cerevisiae. Genes Dev. *1*, 931–937.

Nikawa, J., Sass, P., and Wigler, M. (1987b). Cloning and characterization of the low-affinity cyclic AMP phosphodiesterase gene of *Saccharomyces cerevisiae*. Mol. Cell. Biol. *7*, 3629–3636.

Orr-Weaver, T. L., Szostak, J. W., and Rothstein, R. J. (1983). Genetic

applications of yeast transformation with linear and gapped plasmids. Meth. Enzymol. *101*, 228–245.

Powers, S., Michaelis, S., Broek, D., Santa Anna-A., 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.

Pringle, J. R., and Hartwell, L. H. (1981). The *Saccharomyces cerevisiae* cell cycle. In The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance, J. N. Strathern, E. W. Jones, and J. R. Broach, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory), pp. 97–142.

Robinson, L. C., Gibbs, J. B., Marshall, M. S., Sigal, I. S., and Tatchell, K. (1987). CDC25: a component of the RAS-adenylate cyclase pathway in *Saccharomyces cerevisiae*. Science *235*, 1218–1221.

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

Schenberg-Frascino, A., and Moustacchi, E. (1972). Lethal and mutagenic effect of elevated temperature on haploid yeast. 1. Variations in sensitivity during cell cycle. Mol. Gen. Genet. *115*, 243–257.

Sherman, F., Fink, G. R., and Hicks, J. B. (1986). Laboratory Course Manual for Methods in Yeast Genetics, F. Sherman, G. R. Fink, and J. B. Hicks, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

Shilo, V., Simchen, G., and Shilo, B. (1978). Initiation of meiosis in cell cycle initiation mutants of *Saccharomyces cerevisiae*. Exp. Cell Res. *112*, 241–248.

Shin, D.-Y., Matsumoto, K., Iida, H., Uno, I., and Ishikawa, T. (1987). Heat shock response of *Saccharomyces cerevisiae* altered in cyclic AMP-dependent protein phosphorylation. Mol. Cell. Biol. *7*, 244–250.

Shoji, S., Parmelee, D., Wade, R., Kumar, S., Ericsson, L., Walsh, K., Neurath, H., Long, G., Demaille, J., Fischer, E., and Titani, K. (1981). Complete amino acid sequence of the catalytic subunit of bovine cardiac muscle cyclic AMP-dependent protein kinase. Proc. Natl. Acad. Sci. USA *78*, 848–851.

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.

Sigal, I. S., Gibbs, J. B., Dalonzo, J. S., Temeles, G. L., Walonski, B. S., Socher, S. H., and Scolnick, E. M. (1986). Mutant *RAS*-encoded proteins with altered nucleotide-binding exert dominant biological effects. Proc. Natl. Acad. Sci. USA *83*, 952–956.

Tatchell, K., Robinson, L. C., and Breitenbach, M. (1985). *RAS2* of *Saccharomyces cerevisiae* is required for gluconeogenic growth and proper response to nutrient limitation. Proc. Natl. Acad. Sci. USA *82*, 3785–3789.

Toda, T., 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., Broek, D., Field, J., Michaeli, T., Cameron, S., Nikawa, J., Sass, P., Birchmeier, C., Powers, S., and Wigler, M. (1987a). Exploring the function of *RAS* oncogenes by studying the yeast *Saccharomyces cerevisiae*. In Oncogenes and Cancer, S. A. Aaronson, ed. (Tokyo, Japan: Japan Sci. Soc. Press), pp. 253–260.

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

Toda, T., Cameron, S., Sass, P., Zoller, M., and Wigler, M. (1987c). Three different genes in S. cerevisiae encode the catalytic subunits of the cAMP-dependent protein kinase. Cell *50*, 277–287.

Tschumper, G., and Carbon, J. (1980). Sequence of a yeast DNA frag-

ment containing a chromosomal replicator and the TRP1 gene. Gene *10*, 157–166.

Uno, I., Matsumoto, K., Aduchi, K., and Ishikawa, T. (1983). Genetic and biochemical evidence that trehalase is a substrate of cAMP-dependent protein kinase in yeast. J. Biol. Chem. *258*, 10867–10872.

Vieira, J., and Messing, J. (1987). Production of single-stranded plasmid DNA. Meth. Enzymol. *153*, 3–11.

White, J. H., Lusnak, K., and Fogel, S. (1985). Mismatch-specific postmeiotic segregation frequency in yeast suggests a heteroduplex recombination intermediate. Nature *315*, 350–352.

Zoller, M. J., Kuret, J., Cameron, S., Levin, L., and Johnson, K. (1988). Purification of C1, the catalytic subunit of Saccharomyces cerevisiae cAMP-dependent protein kinase encoded by *TPK1*. J. Biol. Chem., in press.