Loss of Ras activity in *Saccharomyces cerevisiae* is suppressed by disruptions of a new kinase gene, *YAK1*, whose product may act downstream of the cAMP-dependent protein kinase

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The yeast *Saccharomyces cerevisiae* contains two functionally redundant genes *RAS1* and *RAS2*, which are homologous to the mammalian *ras* gene family and are required for vegetative growth. We isolated and characterized five temperature-sensitive alleles of *RAS2*. In a *ras1* strain, these alleles cause growth arrest at the G₁ stage of the cell cycle. Revertants capable of growth at the nonpermissive temperature define four recessive, extragenic complementation groups. Suppressors in one complementation group (designated *yak1*) are particularly intriguing because they appear to alleviate only the growth defect of the temperature-sensitive *ras* mutants and do not show any of the phenotypes, such as heat shock sensitivity or starvation sensitivity, associated with increased production of cAMP. The *YAK1* gene has been cloned, and disruptions generated in vitro reveal that it is not essential for growth and that its loss confers growth to a strain deleted for *tpk1*, *tpk2*, and *tpk3*, the structural genes for the catalytic subunit of the cAMP-dependent protein kinase. These results place Yak1 downstream from, or on a parallel pathway to, the kinase step in the Ras/cAMP pathway. Finally, the coding region predicts a protein with significant homology to the family of protein kinases, suggesting that loss of cAMP-dependent protein kinase function can be suppressed by the loss of a second protein kinase.

[Key Words: *YAK1*; kinase; *S. cerevisiae*; cAMP; *RAS*]

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In the yeast *Saccharomyces cerevisiae*, cAMP and its attendant proteins play a crucial role in cell-cycle progression. A cell deficient in the synthesis of cAMP arrests growth as an unbudded cell early in G₁, at a stage commonly known as 'START' (Pringle and Hartwell 1981). Some of the genes necessary for cAMP synthesis and turnover have been identified and their roles in the cAMP pathway are shown in Figure 1. These include *CYR1* (Matsumoto et al. 1982; Boutelet et al. 1985; Casperson et al. 1985; Kataoka et al. 1985), the structural gene for adenylate cyclase, as well as two homologs of the mammalian *ras* oncogenes, *RAS1* and *RAS2* (Defeo-Jones et al. 1983; Kataoka et al. 1984; Powers et al. 1984). The products of the latter two genes are membrane-associated GTP-binding proteins (Fujiyama and Tamanoi 1986; Powers et al. 1986; Deschenes and Broach 1987) that stimulate adenylate cyclase (Toda et al. 1985). A fourth protein necessary for cAMP production is the product of the cell-division cycle gene *CDC25* (Camonis et al. 1986; Broek et al. 1987; Robinson et al. 1987). Although the role of Cdc25 is not understood, genetic evidence suggests that it may function to activate

the Ras proteins, which, in turn, stimulate adenylate cyclase (Camonis et al. 1986; Broek et al. 1987). Two other genes, *PDE1* and *PDE2*, encode the low- and high-affinity cAMP phosphodiesterases, respectively (Sass et al. 1986; Nikawa et al. 1987b).

The primary function of cAMP in most eukaryotes, including yeast, is activation of the cAMP-dependent protein kinase (cAPK). This activation occurs by cAMP binding to the kinase regulatory subunit and causing its dissociation from the catalytic subunit. Genetic and sequence analysis has shown that the catalytic subunits are encoded by three functionally redundant genes, *TPK1*, *TPK2*, and *TPK3* (Cannon and Tatchell 1987; Toda et al. 1987b), whereas a single gene, *BCY1*, specifies the regulatory subunit (Uno et al. 1982; Cannon and Tatchell 1987; Kunisawa et al. 1987; Toda et al. 1987a). Toda et al. (1987b) have shown that the cell-cycle defect of a cAMP-deficient strain can be suppressed by overexpression of one of the *TPK* genes or by disruption of *BCY1*. Thus, to a first approximation, the role of cAMP in cell-cycle progression can be explained entirely by the cAMP-dependent phosphorylation of one or more cellular substrates. Although components of the Ras/cAMP pathway required for activation of the cAPK are well es-

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tablished, almost nothing is known about the proteins whose cAMP-dependent phosphorylation is essential for cell-cycle progression. Several enzymes involved in carbon storage and metabolism have been shown to be regulated by their phosphorylation state (e.g., PFK2, NAD-dependent glutamate dehydrogenase, trehalase; Uno et al. 1983b, 1984a). However, no straightforward link exists between the function of these genes and progression through the cell cycle.

Several groups have isolated unlinked suppressors of *ras* and *cyr1* mutations as a means of identifying other components in the Ras/cAMP pathway. Tatchell and colleagues (Cannon et al. 1986) exploited the inability of a *RAS1 ras2* strain to grow on nonfermentable carbon sources (Tatchell et al. 1985). By selecting revertants able to grow on ethanol (EtOH) and acetate, they identified several genes, including *BCY1* and *PDE2* (Cannon and Tatchell 1987; Wilson and Tatchell 1988), whose products have been shown to function in the Ras/cAMP pathway. A different approach was taken by Fasano and colleagues (De Vendittis et al. 1986), who isolated revertants of a strain that was conditional for growth as a result of a temperature-sensitive *ras2* allele. One of the suppressors was shown to have lost a potential phosphorylation site of adenylate cyclase. An explanation for the biochemical basis for this suppression is that the mutation activates adenylate cyclase by diminishing feedback inhibition from the cAMP-dependent protein kinase (De Vendittis et al. 1986; Nikawa et al. 1987b; Resnick and Racker 1988). Finally, the *ira1* (formerly *ppd1*) mutation was isolated as a suppressor of an undefined defect in the Ras/cAMP pathway and most likely defines the gene for an inhibitor of Ras as shown in Figure 1 (Uno et al. 1984b; Matsumoto et al. 1985; Tanaka et al. 1989).

Despite the previous isolation of second-site suppressors of mutations in the Ras/cAMP pathway, we decided to undertake a similar mutant hunt by isolating temperature-sensitive *ras2* mutants and using them to isolate revertants. Our reasons for undertaking this project were as follows. First, the scheme used by Cannon and Tatchell relies on the ability of the *RAS1 ras2* revertants to grow on nonfermentable carbon sources. Although this selection identified several new genes in the Ras pathway, we reasoned that this regimen biased the types of revertants that might be obtained, particularly in light of the evidence pointing to the inter-relationship between cAMP-dependent phosphorylation and carbon metabolism (Fraenkel 1985; Breviario et al. 1986; Tatchell et al. 1986; Fasano et al. 1988; Cherry et al. 1989). Second, whereas Fasano and colleagues (De Vendittis et al. 1986) used a selection identical to ours, they concentrated on only one suppressor, and it was in a known gene, *CYR1*. Finally, some genes for proteins known to be involved in the Ras/cAMP pathway, for example, those encoding protein phosphatases (Wingender-Drissen and Becker 1983), have not been identified yet.

The following communication describes the isolation and characterization of five independent, temperature-

sensitive mutations in the *RAS2* gene, as well as the isolation of revertants of the resulting conditional-growth defect. Characterization of the revertants has shown that the suppressors fall into four major recessive complementation groups. One of the suppressors has been cloned, and its sequence reveals a large open reading frame that predicts a protein bearing significant homology to protein kinases. Disruptions of the gene are not lethal and support growth of a strain lacking the three genes for the catalytic subunits of cAPK.

Results

Isolation of *ras2^{ts}* mutations

To isolate a collection of temperature-sensitive mutations in *RAS2*, we took advantage of the gene replacement technique described previously (Winston et al. 1983). A *RAS2* fragment marked with the selectable *URA3* gene was mutagenized in vitro and used to transform a *ras1 :: HIS3 RAS2* strain to Ura⁺ at 25°C. Approximately 30,000 Ura⁺ colonies were tested on both minimal medium and rich (YEPD) medium for a growth defect at the nonpermissive temperature 35°C. Five colonies (0.017%) exhibited temperature-sensitive growth on rich medium and were candidates for *ras2^{ts}* mutants.

The five temperature-sensitive strains were shown to contain mutations in *Ras2* by several criteria, including complementation tests with a low-copy *CEN RAS2* plasmid (pGS21) and diploid analysis. The inability of diploids formed with a *ras2 :: LEU2* strain to grow on EtOH was taken as evidence that conditional growth was the result of a mutation in *RAS2*. In addition, the temperature-sensitive phenotype cosegregated with the *URA3* marker in all five mutants. Finally, the mutations were shown to reside within the 1.1-kb *HpaI RAS2* fragment (Powers et al. 1984) by marker rescue with a gapped version of a *RAS2 CEN* vector (pGS22), and subsequent transplacement of a *ras1 :: HIS3 RAS2* strain to Ura⁺ and temperature-sensitive growth with the recovered DNA (data not shown).

Because yeast Ras is essential for adenylate cyclase activity, two of the *ras2^{ts}* mutants were tested for phenotypes, other than loss of growth, related to the loss of cAMP. Such phenotypes include increased glycogen accumulation and G₁-specific cell-cycle arrest (De Vendittis et al. 1986). As expected, the temperature-sensitive mutants were shown to behave in a manner consistent with their containing conditional *ras2* mutations (data not shown). In addition, the growth defect of the temperature-sensitive strains was shown to be related to their inability to accumulate cAMP by transplacing the *ras1 :: HIS3* and *ras2^{ts}-URA3* mutations into a cAMP-permeable strain (Matsumoto et al. 1982) derived from strain 460 (Table 1) and observing growth at 35°C on medium containing exogenous cAMP (Fig. 2).

Revertant isolation

To increase the spectrum of mutations isolated as suppressors of the Ras⁻ defect, we selected revertants of

Figure 1. Model of the Ras pathway in yeast.

isogenic *MAT α* strains containing either of two independent temperature-sensitive mutations, *ras2-11* (SGP11) and *ras2-34* (SGP34). Thirty revertants of a *MAT α* strain (SGP10) carrying the *ras2-34* allele also were selected to facilitate complementation analysis. Single colonies grown at the permissive temperature were patched to glucose-rich medium and grown for several days at 35°C. The number of colonies that grew from a patch varied but rarely exceeded six. To ensure that all of the suppressors were independent only one colony from each patch was purified and retested for growth at the non-permissive temperature. Over 120 revertants from each of the *MAT α* temperature-sensitive mutants were isolated and subjected to genetic analysis.

Complementation tests were performed to assign the recessive suppressors to individual loci. Revertants of the α strain were mated to *a* strain revertants, as described in Materials and methods, and the diploids were tested for growth at the nonpermissive temperature, with either glucose, galactose or EtOH/glycerol as the carbon source. The results of the diploid analysis are summarized in Table 2. Approximately 10% of the revertants contained dominant mutations, in agreement with previous results (Cannon et al. 1986).

The remainder of the revertants fell into five major classes: four distinct complementation groups which, in sum, constituted 55% of the temperature-resistant strains and a class, comprised of 35% of the strains, that remain unassigned. Strains in this class were not assigned to complementation groups either because their

growth properties on diploid analysis were unclear or they were the lone representative of that group.

Recessive suppressors define at least four complementation groups

Because several recessive suppressors of the Ras pathway have been described previously in *S. cerevisiae*, we performed genetic tests to determine whether our suppressors defined any of the same genes. The gene *BCY1* encodes the regulatory subunit of the cAPK. Disruption of *BCY1* has been shown to alleviate the growth defect of a *ras1 ras2* strain (Matsumoto et al. 1982; Cannon and Tatchell 1987; Toda et al. 1987a). A *bcy1::LEU2* disruption was shown to suppress the growth defect of the *ras1 ras2^{ts}* strains, and subsequent diploid analysis showed that suppressors of one major complementation group were allelic with *bcy1* (Table 2). This was confirmed by complementation tests with a high-copy plasmid vector (YEpl3) containing *BCY1*.

Mutations in the genes *PDE1* and *PDE2* eliminate the high- and low- K_m cAMP phosphodiesterase, respectively (Uno et al. 1983a; Sass et al. 1986; Nikawa et al. 1987b; Wilson and Tatchell 1988). Disruptions of *PDE2* allow growth of a *RAS1 ras2* strain on EtOH, presumably by decreasing the cAMP turnover of the cell (Sass et al. 1986; Wilson and Tatchell 1988). To investigate whether one of the three remaining complementation groups defined either phosphodiesterase gene, disruptions of *PDE1* (*pde1::LEU2*) or *PDE2* (*pde2::URA3*) were

Table 1. Strain and plasmid list

Strain/plasmid	Genotype	Source
Strains		
SGP3	MAT α <i>leu2-3,112 trp1 ura3-52 his3 ras1 :: HIS3 RAS2</i>	this study
SGP4	SGP3 <i>RAS2-URA3</i>	from SGP3
SGP11	SGP3 <i>ras2ts11-URA3</i>	from SGP3
SGP19	SGP3 <i>ras2ts19-URA3</i>	from SGP3
SGP23	SGP3 <i>ras2ts23-URA3</i>	from SGP3
SGP31	SGP3 <i>ras2ts31-URA3</i>	from SGP3
SGP34	SGP3 <i>ras2ts34-URA3</i>	from SGP3
SGP10	MAT α <i>leu2-3,112 trp1 ura3-52 his3 ras1 :: HIS3 ras2ts34-URA3</i>	this study
703	MAT α <i>leu2-3,112 trp1 ura3-52 ade1 ade2 cdc25-1</i>	laboratory stock
T50-3A	MAT α <i>leu2-3,112 trp1 ura3-52 his3 cyr1-2 cam</i>	Kataoka et al. (1985)
S7-7A $\times$ S7-5A	MAT α /MAT α <i>TPK1/tpk1 :: URA3 TPK2/tpk2 :: HIS3</i> <i>TPK3/tpk3 :: TRP1 ura3/ura3 his3/his3 trp1/trp1 leu2/leu2</i> <i>ade8/ade8</i>	Toda et al. (1987b)
SGP9	MAT α /MAT α <i>RAS1/ras1 :: HIS3 RAS2/ras2 :: URA3 his3/his3</i> <i>ura3-52/ura3-52 leu2-3,112/leu2-3,112</i>	this study
1029	MAT α /MAT α <i>his3/his3 ura3-52/ura3-52 leu2-3,112/leu2-3,112</i> <i>lys2/lys2 ADE2/ade2 TRP1/trp1</i>	laboratory stock
SGP40	MAT α <i>ras1 :: HIS3 RAS2 leu2-3,112 ura3-52 his3 ade</i>	this study
SGP400	MAT α <i>leu2-3,112 trp1 ura3-52 his3 RAS2 yak1 :: LEU2</i>	from SGP9
SGP430	MAT α <i>leu2-3,112 trp1 ura3-52 his3 ras2 :: URA3 YAK1</i>	from SGP9
SGP431	MAT α <i>leu2-3,112 trp1 ura3-52 his3 ras2 :: URA3 yak1 :: LEU2</i>	from SGP9
SGP406	MAT α <i>leu2-3,112 trp1 ura3-52 his3 tpk1 :: URA3</i> <i>tpk2 :: HIS3 tpk3 :: TRP1 yak1 :: LEU2</i>	from S7-7A $\times$ S7-5A transformed with <i>yak1 :: LEU2</i> as SGP406
SGP432	MAT α <i>leu2-3,112 trp1 ura3-52 his3 tpk1 :: URA3 tpk2 :: HIS3</i> <i>tpk3 :: TRP1 yak1 :: LEU2</i>	
Plasmids		
pSB32	<i>CEN4 ARS4 LEU2</i>	J. Truehart, unpubl.
pGS65	<i>CEN4 ARS4 LEU2 (SalI⁺)</i>	this study
YCP50	<i>CEN4 ARS4 URA3</i>	Rose and Fink 1987
pGS21	<i>RAS2</i> in pSB32	this study
pGS22	Δ <i>ras2-1</i> in pSB32	this study
pGS100/101/102	<i>YAK1</i> in pSB32	this study
pRJ520-1	<i>RAS2-URA3</i>	Deschenes and Broach (1987)
<i>ppde1 :: LEU2</i>	<i>pde1 :: LEU2</i>	Nikawa et al. (1987b)
<i>ppde2 :: URA3</i>	<i>pde2 :: URA3</i>	Sass et al. (1986)
<i>pbcy1 :: LEU2</i>	<i>bcy1 :: LEU2</i>	Toda et al. (1987a)
YEpl3-BCY1	<i>BCY1</i>	Toda et al. (1987a)
pGS129	<i>YAK1</i> in CVO3	this study
pGS130-2	<i>yak1 :: HIS3</i>	this study
pGS136-A	<i>yak1 :: LEU2</i>	this study

transplanted into the *ras1 ras2^{ts}* strains SGP34 or SGP42, respectively. The *pde2* mutation, but not the *pde1* mutation, appeared to suppress the growth defect of these strains. Thus, none of the complementation groups is likely to be allelic to *PDE1*. Complementation tests between the *pde2* transformant and the revertants gave equivocal results for two of the complementation groups (referred to as *sra9* and *sra10*, for Suppressor of RAs, Cannon et al. 1986). However, the *pde2* mutant clearly complemented the *yak1* revertants shown in Table 2; accordingly, *YAK1* is a distinct gene from *PDE2*.

A single recessive mutation, *ira1/ppd1*, was isolated previously as a suppressor of a defect (*cyr2*) in the cAMP pathway (Matsumoto et al. 1985). Mutations in *IRA1* also might be expected to suppress the *ras2^{ts}* defect of the strains used in this selection, although they do not alleviate the growth defect of a strain carrying a dominant *RAS2(Ts)* mutation (Tanaka et al. 1989). Because

IRA1 has been mapped to the right arm of chromosome II, 7.7 cM from *LYS2*, several suppressors from the complementation groups *yak1*, *sra9*, and *sra10* were mapped relative to the *lys2* allele of MAT α *ras1 :: HIS3 ras2^{ts}-URA3* strain SGP10. None of the suppressor alleles tested exhibited linkage to *LYS2* (results not shown). Therefore, *ira1/ppd1* is not allelic with *yak1*, *sra9*, or *sra10*.

The pattern of suppression exhibited by the revertants of any one complementation group often can be used in subsequent characterization of the suppressors. Thus, the recessive revertants were tested for glycogen accumulation, growth on various carbon sources, heat shock sensitivity, and survival of nitrogen starvation, according to the procedures outlined in Materials and methods. The *ras2 bcy1* revertants exhibited the widest phenotypic variation and included strains that appeared to accumulate less glycogen and were more sensitive to

Figure 2. Suppression of *ras2^{ts}* by cAMP. Growth was tested by replicating patches to agar at 35°C, with or without exogenous cAMP (100 mM). [CAM] cAMP impermeable; [cam] cAMP permeable. Strains are *RAS2* (*ras1 :: HIS3 RAS2*), 2-11 (*ras1 :: HIS3 ras2^{ts11}*), 2-34 (*ras1 :: HIS3 ras2^{ts34}*).

both heat shock and nitrogen starvation than the wild-type *RAS2⁺ BCY1⁺* strain. Strains of the *sra9* and *sra10* complementation groups also exhibited varying degrees of suppression for temperature-sensitive growth, glycogen accumulation, and sensitivity to stress; however, none of the suppressors conferred phenotypes as extreme as the *bcy1* mutations. In contrast, suppression of the pleiotropic *ras1 ras2* phenotypes by the *yak1* alleles seemed confined to the growth defect because the revertants of that complementation group exhibited healthy growth on several different carbon sources, yet still hyperaccumulated glycogen.

Cloning YAK1

Because the *yak1* suppressors were recessive, the wild-type allele was isolated by complementation from a *S. cerevisiae* library in a low-copy *LEU2 CEN4* vector, pSB32 (see Materials and methods). Two revertants containing alleles *yak1-39* and *yak1-82*, respectively, were transformed to *Leu⁺* with the plasmid library at the permissive temperature 25°C. After several days of growth, the transformants were replicated to minimal medium and YEPD and incubated at the permissive and nonper-

missive temperatures. Of ~16,000 *Leu⁺* colonies tested (8000 from each revertant), 5 regained the temperature-sensitive growth phenotype of the *ras1 ras2^{ts} YAK1* parent.

To confirm that temperature-sensitive growth was a result of the presence of the *LEU2* marker, plasmid DNA from each *Leu⁺*, temperature-sensitive colony was used as a source of DNA for a second transformation into both *ras1 ras2^{ts} yak1* revertants. Only three of the five potential clones appeared to confer temperature sensitivity on retransformation (see Fig. 3A). One plasmid (pGS100, Fig. 3B) was isolated as complementing the *yak1-39* allele, whereas two others (pGS101 and pGS102) were isolated in the *yak1-82* strain. Each plasmid was tested for its ability to complement several different *yak1* alleles, as well as an allele each of the *sra9* and *sra10* complementation groups. All three plasmids complemented each of four *yak1* alleles (18, 39, 79, and 82) but failed to complement the suppressors in the *sra9* or *sra10* revertants.

To identify the *YAK1*-coding region, we constructed a physical map of plasmids pGS100, pGS101, and pGS102. All three plasmids contained the large region of homology shown in Figure 3B. To delineate the regions necessary for *yak1* complementation, several smaller fragments were subcloned into the original *LEU2* cloning vector pSB32, as well as a variant of that vector, pGS65 (see Table 1), and tested for their ability to complement the *yak1* mutations. The fragments tested, along with the complementation pattern, are shown in Figure 3B. These results suggest that a region between 3300 and 3800 bp appears necessary for full complementation.

To prove that the DNA fragment represented in Figure 3B contains *YAK1* sequences, we showed that it directed an appropriate yeast marker into a region of the chromosome genetically linked to the *yak1* suppressors. Plasmid pGS129 (*LEU2 YAK1*) was integrated into the

Table 2. Spontaneous suppressors

Class	Locus	Frequency (%)	Ras independence ^a
Recessive	<i>yak1</i>	10	+
	<i>sra9</i>	20	–
	<i>sra10</i>	20	–
	<i>bcy1</i>	5	+
	unassigned	35	ND
Dominant		10	ND

^a RAS independent (+) or dependent (–) growth, as measured by germination and growth of a *ras1 :: HIS3 ras2 :: URA3* spore. [ND] Not determined.

Figure 3. Cloning and restriction map of *YAK1*. (A) Strains were patched onto YEPD medium and incubated for 3 or 2 days at 25°C and 35°C, respectively. All strains contain the *ras1::HIS3* mutation and are *RAS2* (SGP4), *ras2* (SGP34), *ras2 yak1* (SGP34 *yak1*-39), *ras2 yak1* (pYAK1) [SGP34 *yak1*-39 (pGS100)], and *ras2 yak1* (pSB32) [SGP34 *yak1*-39 (pSB32)]. (B) Solid lines represent different yeast chromosomal fragments (top line represents pGS100) used to test complementation of several *yak1* alleles. (+) Complementation; (±), weak complementation; (-), no complementation. (J) Plasmid/yeast chromosomal junction; (P) *PvuII*; (B) *BamHI*; (V) *EcoRV*; (S) *Sall*; (H) *HindIII*; (Bg) *BglII*.

chromosome of strain SGP10 (MATa *ras1::HIS3* *ras2^{ts34}-URA3 YAK1*) through homology with the cloned fragment, and a *Leu⁺* transformant was crossed with two appropriately marked *Yak1* strains (MA-Ta *ras1::HIS3* *ras2^{ts}-URA3 yak 1-39* and (MA-Ta *ras1::HIS3* *ras2^{ts}-URA3 yak 1-82*). Of 50 tetrads containing two *Leu⁺* spores, there were 49 parental ditypes, 0 non-parental ditypes, and 1 tetratype. This segregation pattern suggests that the cloned fragments contain the wild-type *YAK1* gene rather than DNA that encodes a similar function.

YAK1 may encode a protein kinase

To determine whether *YAK1* encodes a product with homology to any proteins found in one of the available data bases, we sequenced the smallest region capable of fully complementing alleles *yak1*-39 and *yak1*-82. One long open reading frame (ORF) is found in the 3599 bp sequenced (Fig. 4). The *YAK1* gene would encode a protein of 807 amino acids if the first methionine in the ORF is used. An in-frame stop codon is found just 9 nucleotides upstream from that ATG.

The coding region of *YAK1* predicts a protein bearing striking homology with known protein kinases (Table 3). Several amino acid sequences diagnostic for protein kinases are emphasized in Figure 4 (Hanks et al. 1988). The ATP-binding site is thought to include the sequence Gly X Gly X X Gly X Val (X is any amino acid), followed by an invariant Lys 7–16 amino acids later. This sequence is denoted by stars in Figure 4. Three other sites common to protein kinases are underlined and appear 116 residues downstream from the ATP-binding site: Glu Leu, Asp Phe Gly, and Ala Pro Glu. A computer search of the translated form of GenBank reveals significant homology between the product of *YAK1* and the entire protein kinase family (Table 3). These results suggest that *YAK1* encodes a protein kinase. Although the predicted kinase domain of *YAK1* exhibits greater identity to some proteins than to others (it appears more related to the products of *cdc2* and *CDC28* than *STE7*),

none of these homologies would appear sufficient to allow the assignment of the *YAK1* product to a particular class of kinases (Hanks et al. 1988). Most significant is the relatively low identity between the catalytic domain of the cAMP-dependent protein kinases and the kinase domain predicted for the *YAK1* product. This contrasts with the very high degree of identity between the cAPK proteins and the predicted amino acid sequence of a gene (*SCH9*) isolated as a high-copy suppressor of a defect in *CDC25* (Toda et al. 1988).

One intriguing feature of the predicted amino acid sequence of *Yak1* is the presence of an extended amino-terminal domain that is distinct from the conserved kinase domain of the protein. This organization is similar to a variety of other protein kinases, such as *src*, protein kinase C, and *Sch9*, although the actual sequence within this domain is unrelated to any protein in the GenBank data base. This amino-terminal domain encompasses several noteworthy features. A glutamine-rich region is present from amino acid 56 to 85, including a stretch of 12 consecutive glutamine residues. Similar glutamine-rich regions have been observed in *SSN6* and *GAL11*, although a role for this feature has not been identified in either case (Shultz and Carlson 1987; M. Ptashne, pers. comm.). Also found within the amino-terminal domain are a series of four consensus sites for cAPK phosphorylation, Arg Arg X Ser (Edelman et al. 1987). Although the function of these sites is unknown, their potential role in regulation of *Yak1* activity is discussed below.

YAK1 is not essential

Because *yak1* mutations were recessive and relatively common, it seemed likely that suppression of the temperature-sensitive *ras* defect was a result of the loss of *Yak1* function. To test this assumption, it was first necessary to determine whether *YAK1* was essential. Two different disruptions were constructed on the basis of our knowledge of the *YAK1* complementing region (Fig. 3B) and predicted coding sequence (Fig. 4). The first disruption placed the *HIS3* gene into the single *BamHI*

Figure 4. Nucleotide and predicted amino acid sequence of *YAK1*. The nucleotide sequence extends 3599 bp from the YCp50–yeast DNA junction to the *Hind*III site in Fig. 3B. Sequences conserved in protein kinases are indicated by stars (ATP binding) or by underlining (catalysis). The termination codon is represented by hyphens. Putative sites of cAPK phosphorylation are denoted by overlines and underlines.

Table 3. Protein kinase homologies

Protein kinase	Optimized score ^a	Percent identity ^b	Reference
<i>Schizosaccharomyces pombe cdc2</i>	349	32	Hindley and Phear (1984)
<i>S. cerevisiae CDC28</i>	288	31	Lorincz and Reed (1984)
Mouse phosphorylase kinase (γ)	245	23	Chamberlain et al. (1987)
Mouse cAPK	197	20	Uhler et al. (1986)
<i>STE7</i>	156	24	Teague et al. (1986)

^a Optimized score, as determined by the FASTP program (Lipman and Pearson 1985).

^b Percent amino acid identity over a stretch of 250–300 residues encompassing the putative kinase domain.

site of the coding region, so that only the first 189 amino acids would be expressed from the endogenous promoter. A second disruption removed all of the kinase domain between *EcoRV* sites at nucleotides 842 and 1702 and replaced it with the *LEU2* marker. Both disruptions were shown to inactivate Yak1 function in a complementation test similar to the one used in the subcloning experiments (Fig. 3A; data not shown). Each disruption was transplaced into diploid strain 1029, and two independent heterozygous *YAK1/yak1* diploids from each transformation were sporulated and scored. The disruptions segregated 2 : 2, as judged by the His and Leu phenotypes of the resulting colonies. Two tetrads from each diploid were checked by hybridization analysis, and in each case, the presence of the selectable auxotrophic marker correlated with the shift in fragment size expected for the disrupted allele (data not shown). Because the *HIS3* insertion was within the amino terminus of the protein and the *LEU2* marker displaced all of the putative kinase domain, we assume that at least one of these disruptions represents a complete loss of function. Thus, the *Yak1* gene is not essential for growth or germination.

Physiology of the *yak1* strains

Because none of the spontaneous *yak1* mutations suppressed the glycogen hyperaccumulation by the *ras2^{ts}* strains, we tested several wild-type strains containing either of two *yak1* disruptions for phenotypes related to those exhibited by other mutants of the Ras/cAMP pathway. Several *yak1* colonies were examined for heat shock sensitivity, glycogen accumulation, sensitivity to nitrogen starvation, and sporulation defects. All of the *yak1* strains appeared wild type for nitrogen starvation and heat shock sensitivity, as well as glycogen accumulation (see Fig. 5B) and sporulation competence. To make certain the phenotype conferred by the disruptions was the same as that conferred by the original suppressors, several spontaneous alleles (*yak1-39*, *yak1-79*, and *yak1-82*) were moved into a wild-type *RAS2* background by the crosses outlined in Materials and methods. Once again, the *RAS2 yak1* strains appeared as wild type, although a slight alteration might not be detected by these assays.

Loss of the *Yak1* kinase suppresses *ras2^{ts}* and *tpk* mutations

To determine whether suppression of the temperature-sensitive *ras* defect was a result of the loss of Yak1 function, the *yak1 :: LEU2* disruption (Materials and methods) was transplaced into strains SGP11 and SGP34 (*ras1 :: HIS3 ras2-11-URA3* and *ras1 :: HIS3 ras2-34-URA3*, respectively) by selecting for growth on minimal medium at 25°C. Four Leu⁺ colonies from each transformation were tested for growth at the nonpermissive temperature. In all cases, transplacement conferred temperature resistance. Thus, suppression of the *ras* defect appeared to be a result of the loss of Yak1 function. To determine the point of Yak1 action in the Ras/cAMP pathway, tests of epistasis were performed on the mutant strains listed in Table 4. All of the strains exhibiting conditional growth, including those carrying temperature-sensitive mutations in *ras2*, *cdc25*, and *cyr1*, reverted to nonconditional growth on transplacement with the *yak1* disruptions.

Although the growth defect of strain 460 (*cyr1-2*) was suppressed by both *yak1* disruptions (Table 4), it was possible that suppression was dependent on residual cyclase activity or functioned immediately downstream from Cyr1. Therefore, we determined whether either disruption could suppress the complete loss of Ras or cAPK activity. Heterozygous *ras* and *tpk* diploids (SGP9 and S7-7A × S7-5A) were transformed to Leu⁺ with the *yak1 :: LEU2* disruption, sporulated, and picked onto synthetic complete-minimal glucose agar. Haploid spores containing *ras1 ras2* or *tpk1 tpk2 tpk3* disruptions failed to germinate (as judged by segregation of the auxotrophic markers) unless they contained the mutant *yak1* allele. Figure 5A shows the growth of spores from several tetrads of a *yak1 :: LEU2* transformant of diploid strain S7-7A × S7-5A. The large colonies contained at least one wild-type *TPK* gene, and the tiny colonies bore disruptions of all three *TPK* genes and the *LEU2* marker indicative of the *yak1* disruption. For each tetrad resulting in growth of only two or three colonies, segregation of the auxotrophic markers predicted that the spores that failed to germinate contained all three *tpk* disruptions and the wild-type *YAK1* allele.

It is apparent from Figure 5B that although the *tpk yak1* strains are viable, they are compromised for

Figure 5. Suppression of *ras* and *tpk* mutations by *yak1*. (A) Diploid strain S7-7A × S7-5A (*MATa/MATα TPK1/tpk1 :: URA3 TPK2/tpk2 :: HIS3 TPK3/tpk3 :: TRP1 leu2/leu2*) was transformed to *Leu*⁺ with the *yak1 :: LEU2* disruption and tetrads were dissected and scored on the appropriate minimal medium agar to determine the segregation of the *tpk* and *yak1* disruptions. All of the small colonies contained disruptions of the three *tpk* genes, as well as the *yak1 :: LEU2* mutation. (B) Strains indicated at top left are WT (SGP4), *yak1* (SGP400), *ras2* (SGP430), *ras2 yak1* (SGP431), *tpk1,2,3 yak1* (SGP406), *tpk1,2,3 yak1* (SGP432). Patches of these strains were replicated to rich medium containing EtOH/glycerol (top right) or glucose (bottom right and bottom left) as the carbon source. (Bottom right) patches were scored for glycogen accumulation by inverting them over *I*₂ vapors, as outlined in the text.

growth and hyperaccumulate glycogen. This latter phenotype is not simply the result of the slow growth of these strains, because a *Ras1 ras2* strain exhibits wild-type growth on glucose medium but hyperaccumulates glycogen regardless of the *YAK1* allele (*YAK1* or *yak1 :: LEU2*) (Fig. 5B).

Discussion

By isolating revertants of a *S. cerevisiae* strain deficient in the Ras/cAMP pathway, we identified at least one new locus (*YAK1*), whose product may be involved in cell-cycle regulation. This locus was defined by a recessive complementation group isolated as a suppressor of the conditional-growth defect of several temperature-sensitive *ras* mutants. Three other genes also were identified in the screen. One corresponds to the previously isolated *BCY1* gene (Cannon and Tatchell 1987; Kuni-sawa et al. 1987; Toda et al. 1987a) and one may be al-

lelic to *PDE2* (Sass et al. 1986; Wilson and Tatchell 1988). Thus, at least one, and possibly two, additional novel genes involved in the Ras/cAMP pathway have emerged from this analysis.

The most interesting result from these studies is that disruptions of *YAK1* suppress the cell-cycle defect of a strain deleted for all three *TPK* genes. This makes *YAK1* the only gene whose loss of function has been shown to suppress the growth defect of a *tpk* deletion strain. This result is somewhat paradoxical because the *YAK1*-coding region predicts a protein kinase of 807 amino acids. Thus, the loss of function of three kinase proteins is alleviated by the loss of yet another kinase. This situation contrasts with that for suppression of *tpk* by *SCH9*. *SCH9* also encodes a protein kinase, exhibiting significant homology with the cAPK catalytic subunits (Toda et al. 1988). However, overexpression of *SCH9*, not its elimination, suppresses *tpk*. Given the sequence similarities of *Sch9* and the cAPK subunits, suppression of a

Table 4. Epistasis studies with several *yak1* disruptions

Strain	Suppressor	Glucose		EtOH ^a
		25°C	35°C	
<i>ras1 :: HIS3 ras2^{ts34}</i>	<i>YAK1</i> ⁺	+	—	—
	<i>yak1 :: LEU2</i>	+	+	+
<i>RAS1⁺ ras2 :: LEU2</i>	<i>YAK1</i> ⁺	+	+	—
	<i>yak1 :: HIS3</i>	+	+	+
<i>cyr1-1</i>	<i>YAK1</i> ⁺	+	—	—
	<i>yak1 :: LEU2</i>	+	+	+
<i>cdc25-1</i>	<i>YAK1</i> ⁺	+	—	—
	<i>yak1 :: LEU2</i>	+	+	+
<i>ras1 :: HIS3 ras2 :: URA3</i>	<i>YAK1</i> ⁺	—	—	—
	<i>yak1 :: LEU2</i>	±	±	±
<i>tpk1 tpk2 tpk3</i>	<i>YAK1</i> ⁺	—	—	—
	<i>yak1 :: LEU2</i>	±	±	±

^a (+) Growth; (—) no growth; (±) slow growth.

tpk strain by overexpression of *SCH9* most likely occurs as a result of overlapping specificity of the two kinases. Such an explanation clearly does not account for *yak1* suppression of *tpk*.

The roles of Sch9 and Yak1 in the Ras/cAMP pathway are still not understood. Available evidence would suggest that Sch9 might be on a parallel pathway (Toda et al. 1988). Although we have not eliminated the possibility that Yak1 is also part of a parallel pathway, our results are consistent with a model in which Yak1 is a G₁-specific inhibitor of the cell cycle, and it, in turn, is regulated negatively by cAPK activity. According to this model, strains exhibiting decreased cAPK activity, such as a *tpk* deletion mutant, would fail to grow because Yak1 would actively block cell-cycle progression. Growth would be restored to these strains by mutations in *YAK1* that result in its loss of function. Although Yak1 could be regulated by cAPK activity in an indirect manner, it is tempting to speculate that one or more of the cAPK consensus sites within the amino terminus of Yak1 is phosphorylated in vivo. In this scenario, phosphorylation of Yak1 would lead to its inactivation and alleviation of its inhibition of cell-cycle progression.

An intriguing feature of the *yak1* mutations, including the disruptions, is that they do not revert all of the phenotypes associated with diminished cAPK activity. For example, loss of Yak1 function restores growth to strains with defects at different points in the Ras/cAMP pathway yet does not alter their propensity to accumulate glycogen. Moreover, a strain deficient only in Yak1 activity exhibits wild-type glycogen stores and is resistant to heat shock and nitrogen starvation. The simultaneous reversion of several phenotypes conferred by a primary defect is often used as indirect proof that the suppressor mutation defines a function integral to the pathway under study. Although this reasoning often proves correct, it is probably wrong to assume that all suppressors within a pathway must revert all of the phenotypes associated with the original defect. This is particularly true for genes whose products act beyond a point of divergence in the pathway. Because the initial selection was for reversion of the Ras⁻ growth defect, we reasoned that suppressors of the temperature-sensitive growth arrest would be interesting even if other phenotypes associated with the loss of RAS function were not alleviated similarly. Thus, the failure of mutations in *yak1* to revert all of the Ras⁻ associated phenotypes can be reconciled with a role for Yak1 in the Ras/cAMP pathway if Yak1 acts beyond the phosphorylation/dephosphorylation branch point. Our epistasis results support this conclusion. In addition, the assignment of Yak1 to only one branch in the pathway might account for the rather slow growth of *tpk* deletion strains containing the *yak1* suppressors. These strains would remain compromised for all other metabolic functions regulated by cAPK phosphorylation, including carbohydrate storage and utilization (Uno et al. 1983b).

Our model for the role of Yak1 is tentative; however, it makes several predictions that can be tested. First, as a negative regulator of cell growth, the Yak1 protein

might be expected to be dispensable for viability. Because disruptions of both the amino- and carboxy-terminal (kinase) domains of Yak1 result in cell viability, it seems likely that *YAK1* is a nonessential gene. Second, enhanced Yak1 activity, perhaps by overproduction, should result in the same cell-cycle arrest observed in strains deficient in cAPK activity. Although strains containing *YAK1* on a high-copy plasmid do not grow appreciably slower than strains carrying the vector alone (S. Garrett and J. Broach, unpubl.), a conclusion about this prediction must await its placement under control of an inducible promoter. Finally, the model suggests that Yak1 is regulated negatively by cAPK activity and that Yak1 activity is determined by its state of phosphorylation. Further characterization of *YAK1* and its product should be helpful in determining the answers to these questions.

In contrast to the limited suppression pattern conferred by the *yak1* mutations, suppressors from the complementation group *sra9* and *sra10* revert all of the pleiotropic Ras⁻ phenotypes, albeit to degrees consistent with a hierarchy of suppressor strength. According to these criteria, the *sra9* and *sra10* suppressors most resemble mutations in *bcy1*, which are also pleiotropic. The pattern of suppression by the *bcy1* alleles is consistent with a role for its product upstream of the branch point in the Ras/cAMP pathway (defined by the state of phosphorylation of a variety of cellular substrates). Molecular characterization of BCY1 has shown this to be true (Cannon and Tatchell 1987; Toda et al. 1987a). Although we have no molecular evidence for the product of *SRA9* or *sra10* affecting the phosphorylation state of the cellular substrates, the pattern of suppression is consistent with this assumption. Such roles might include a protein that functioned as an intermediate in the feedback mechanism attenuating cAMP production (Nikawa et al. 1987b; Resnick and Racker 1988) or a protein phosphatase activity that counteracts cAPK activity (Wingender-Drissen and Becker 1983; Matsumoto et al. 1985). Further evaluation should allow us to pinpoint the role of these genes in the pathway.

Materials and methods

Strains and plasmids

All yeast strains are listed in Table 1. Bacterial strains MC1066 [$\Delta(lac)X74$ *galU galK strA^r hsdR trpC9830 leuB6 pyrF::Tn5*] and JM101 [$\Delta(lac-pro)supE thi/F'$ *traD36 proAB lacI_q ZAM15*] were used for construction and growth of all plasmids, and phage and have been described (Messing et al. 1981; Casadaban et al. 1983). Phage mp11 and mp18 (Messing 1983) were used for the generation of single-stranded DNA for sequencing. The *LEU2*-based *CEN4* vector pSB32 was constructed by J. Truehart by deleting the *URA3* gene from YCp50 and replacing it with *LEU2*. The *S. cerevisiae* library constructed with this vector was the gift of P. Hieter (The Johns Hopkins University). Plasmid pRJ520-1 (Deschenes and Broach 1987) consists of the 3-kb *EcoRI*–*HindIII* *Ras2* fragment into which the 1.1-kb *URA3* fragment has been inserted 150 bp past the *RAS2* termination codon. Plasmids *Yep13-BCY1*, *ppde1::LEU2*, and *ppde2::URA3* are from Mike Wigler and have been described

(Sass et al. 1986; Nikawa et al. 1987b; Toda et al. 1987a). The YAK1-disruption plasmid pGS130-2 was constructed by inserting the 1.7-kb *Bam*HI fragment of *HIS3* into the single *Bam*HI site of *Yak1* on pGS100. Plasmid pGS136-A was constructed by inserting the 2.5-kb *Bam*HI–*Sal*I *YAK1* fragment into the corresponding sites of pUC8, dropping out the 600-bp *Eco*RV fragment within *YAK1* (Fig. 3B), attaching *Xho*I linkers, and inserting the 2.2-kb *Sal*I–*Xho*I fragment carrying *LEU2*. Mapping plasmid pGS129 was constructed by inserting the 6-kb *Pvu*II fragment carrying *YAK1* into the integrating *LEU2* vector CV03 (Broach and Hicks 1980).

DNA manipulations

Plasmid DNA was prepared from *E. coli* using the alkali lysis method (Maniatis et al. 1982). Yeast DNA, plasmid and chromosomal, was prepared according to the method of Nasmyth (Nasmyth and Reed 1980). All enzymes were used according to the specifications of their suppliers (New England Biolabs), and cloning techniques were as described (Maniatis et al. 1982). Hybridizations of yeast DNA were performed as described (Maniatis et al. 1982), except for the substitution of Church buffer (Church and Gilbert 1984).

DNA sequencing

Overlapping fragments were cloned into the M13 derivatives mp11 and mp18 and used as substrates for single-strand di-deoxy sequencing (Sanger et al. 1977) with α -³⁵S-labeled dATP as radioactive label. The 3599 nucleotides shown in Figure 4, from the *Hind*III site to the junction between yeast DNA and YCp50, were determined by sequencing both strands.

Genetic techniques and media

Transformation of yeast was by the lithium acetate method (Ito et al. 1983), and other yeast genetic manipulations were performed as described (Sherman et al. 1986). Glycogen staining was scored in situ by inverting a plate over *I*₂ crystals (Deschenes and Broach 1987). Nitrogen-poor medium contained 0.17% yeast nitrogen base without ammonium sulfate or amino acids (Difco) and 2% glucose. Determination of survival of the revertants to nitrogen starvation and heat treatment was performed as described (Cannon et al. 1986; Sass et al. 1986). Sporulation efficiency was scored essentially as outlined (Toda et al. 1985). All media, including yeast-rich and minimal media, as well as bacterial media, were prepared as described previously (Deschenes and Broach 1987).

Isolation of *ras2* temperature-sensitive alleles

Hydroxylamine mutagenesis of the *RAS2* plasmid pRJ520-1 was carried out for 30 hr at 37°C, following published procedures (Rose and Fink 1987). The mutagenized plasmid DNA was digested to completion with *Eco*RI and *Hind*III and used to transform competent cells of strain SGP3 to Ura⁺ at 25°C. After several days, the Ura⁺ transformants were replicated to 25°C and 35°C on rich medium (YEPA), as well as minimal medium. Colonies that grew at the lower temperature were selected only as potential *ras2^{ts}* strains.

Selection of *ras* revertants

Individual colonies from two MAT α *ras1* :: *HIS3 ras2^{ts}* strains, SGP11 and SGP34, were patched to YEPA medium agar and incubated for 48 hr at 35°C. From those patches on which colo-

nies arose, a single colony was selected and purified at 25°C on YEPA. Potential revertants were then retested for growth at 25°C and 35°C on rich medium agar, using 2% glucose, 2% galactose, or 2% EtOH/2% glycerol as carbon source. Over 120 revertants were isolated from each strain. In addition, 30 temperature-resistant revertants were isolated from a MAT α *ras1* :: *HIS3 ras2^{ts}* strain (SGP10) to enable complementation tests of the revertants.

Complementation analysis of the revertants

The temperature-resistant revertants of the α strains SGP11 and SGP34 were mated to their counterparts by selecting for prototrophic growth. Individual α strains, along with their temperature-sensitive parents and the *RAS2⁺* strain SGP4, were patched to YEPA agar, incubated for several days at 25°C, and then replica-plated to a lawn of the appropriate revertant on minimal medium. After several days of prototrophic selection at 25°C, diploids were tested for growth at 35°C on YEPA medium. Complementation between recessive alleles was indicated by lack of growth at the nonpermissive temperature.

Crossing the *yak1* mutations into a *RAS2* strain

Several *yak1* alleles were moved into a *RAS2* background by crossing the respective revertants with the MAT α *ras1* :: *HIS3 RAS2⁺* strain SGP40. Tetrads that exhibited two temperature-sensitive colonies coincident with the presence of the *URA3* marker (*ras2^{ts34}–URA3*) were assumed to be nonparental ditype, and the Ura[–] (*RAS2⁺*) colonies were examined for such phenotypes as heat shock sensitivity, glycogen accumulation, sensitivity to nitrogen starvation, and sporulation defects by the procedures outlined in Materials and methods. The *RAS2⁺* colonies were tested for the presence of suppressor alleles by backcrossing them to a *ras1* :: *HIS3 ras2^{ts34}* :: *LEU2* strain, sporulating the diploids, and testing the tetrads for growth at the nonpermissive temperature. Whereas a wild-type *ras1* :: *HIS3 RAS2⁺ YAK1⁺* strain generated tetrads containing only two temperature-resistant colonies, the *ras1* :: *HIS3 RAS2⁺ yak1* strains led to the appearance of two, three, and four temperature-resistant colonies within a tetrad.

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