Loss of Ras activity in *Saccharomyces cerevisiae* is suppressed by disruptions of a new kinase gene, *YAKI*, 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 *YAKI* 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 *tpkl*, *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 kinase protein.
established, 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-Drisen 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 cAMP.

**Results**

**Isolation of ras2**

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 [YPED] 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 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 Hpal 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 mutants were tested for phenotypes, other than loss of growth, related to the loss of cAMP. Such phenotypes include increased glycogen accumulation and Gsin-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::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
isogenic MATα strains containing either of two independent temperature-sensitive mutations, *ras2-11* (SGP11) and *ras2-34* (SGP34). Thirty revertants of a MATα 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 MATα 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* 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 (YPE13) containing *BCY1*.

Mutations in the genes *PDE1* and *PDE2* eliminate the high- and low-κ 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 |
|---------------|----------|--------|
| SGP3          | MATα leu2-3,112 trp1 ura3-52 his3 ras1 :: HIS3 RAS2 | this study |
| SGP4          | SGP3 RAS2-URA3 | from SGP3 |
| SGP11         | SGP3 ras2ts11-URA3 | from SGP3 |
| SGP19         | SGP3 ras2ts19-URA3 | from SGP3 |
| SGP23         | SGP3 ras2ts23-URA3 | from SGP3 |
| SGP31         | SGP3 ras2ts31-URA3 | from SGP3 |
| SGP34         | SGP3 ras2ts34-URA3 | from SGP3 |
| SGP10         | MATα leu2-3,112 trp1 ura3-52 his3 ras1 :: HIS3 RAS2-URA3 | this study |
| 703           | MATα leu2-3,112 trp1 ura3-52 ade1 ade2 cdc25-1 | laboratory stock |
| TS0-3A        | MATα leu2-3,112 trp1 ura3-52 his1 cyr1-2 cam | Kataoka et al. (1985) |
| S7-7A × S7-5A | MATα/MATA TPK1/tpk1 :: URA3 TPK2/tpk2 :: HIS3 YCP50 TPK1/tpk1 :: TRP1 ade8/ade8 | Toda et al. (1987b) |
| SGP9          | MATα/MATA RAS1/ras1 :: HIS3 RAS2/ras2 :: URA3 his3/his3 | laboratory stock |
| 1029          | MATα/MATA his3/his3 ura3-52 ura3-52 leu2-3,112/leu2-3,112 lys2/lys2 ade2/ade2 TRP1/tp1 | this study |
| SGP40         | MATα ras1 :: HIS3 RAS2 leu2-3,112 ura3-52 his3 ade | this study |
| SGP400        | MATα leu2-3,112 trp1 ura3-52 his3 RAS2 yak1 :: LEU2 | from SGP9 |
| SGP430        | MATα leu2-3,112 trp1 ura3-52 his3 ras2 :: URA3 YAK1 | from SGP9 |
| SGP431        | MATα leu2-3,112 trp1 ura3-52 his3 ras2 :: URA3 yak1 :: LEU2 | from SGP9 |
| SGP406        | MATα leu2-3,112 trp1 ura3-52 his3 tpk1 :: URA3 tpk2 :: HIS3 | transformed with yak1 :: LEU2 |
| SGP432        | MATα leu2-3,112 trp1 ura3-52 his3 tpk1 :: URA3 tpk2 :: HIS3 tpk3 :: TRP1 yak1 :: LEU2 | as SGP406 |
| Plasmids      |          |        |
| pSB32         | CEN4 ARS4 LEU2 | J. Truehart, unpubl. |
| pGS65         | CEN4 ARS4 LEU2 (SalI +) | this study |
| YCFS0         | CEN4 ARS4 URA3 | Rose and Fink 1987 |
| pGS21         | RAS2 in pSB32 | this study |
| pGS22         | Δras2-1 in pSB32 | this study |
| pGS100/101/102 | YAK1 in pSB32 | this study |
| pRJ520-1      | RAS2-URA3 | Deschenes and Broach (1987) |
| ppdel :: LEU2  | pde1 :: LEU2 | Nikawa et al. (1987b) |
| ppde2 :: URA3  | pde2 :: URA3 | Sass et al. (1986) |
| pbcy1 :: LEU2  | bcy1 :: LEU2 | Toda et al. (1987a) |
| YEpl3-BCY1    | BCY1 | Toda et al. (1987a) |
| pGS129        | YAK1 in CVO3 | this study |
| pGS130-2      | yak1 :: HIS3 | this study |
| pGS136-A      | yak1 :: LEU2 | this study |

transplanted into the ras1 ras2\textsuperscript{a} 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, iral/ppd1, was isolated previously as a suppressor of a defect [cyrb2] in the cAMP pathway [Matsumoto et al. 1985]. Mutations in IRA1 also might be expected to suppress the ras2\textsuperscript{a} 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 MATα ras1 :: HIS3 ras2\textsuperscript{a} – URA3 strain SGP10. None of the suppressor alleles tested exhibited linkage to LYS2 (results not shown). Therefore, iral/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
both heat shock and nitrogen starvation than the wild-type RAS2+ BCY1+ strain. Strains of the sra9 and sralO 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 bcyl mutations. In contrast, suppression of the pleiotropic ras1 ras2 phenotypes by the yakl 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 yakl 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 nonpermissive temperatures. Of ~16,000 Leu+ colonies tested (8000 from each revertant), 5 regained the temperature-sensitive growth phenotype of the ras1 ras2 YAK1 parent.

To confirm that temperature-sensitive growth was a result of the presence of the LEU2 marker, plasmid DNA from each Leu+ strain was used as a source of DNA for a second transformation into both ras1 ras2 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 sralO 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 sralO 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* |
|---------|-------|--------------|-------------------|
| Recessive | yak1   | 10           | +                 |
|         | sra9   | 20           | -                 |
|         | sralO  | 20           | -                 |
|         | bcyl   | 5            | +                 |
|         | unassigned | 35      | ND                |
| Dominant |        | 10           | ND                |

* RAS independent (+) or dependent (−) growth, as measured by germination and growth of a ras1 :: HIS3 ras2 :: URA3 spore. [ND] Not determined.
YAK1 may encode a protein kinase

To determine whether YAK1 encodes a product with homology to any proteins found in one of the available databases, 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 sequence [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 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 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 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 HI S3 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. (I) Plasmid/yeast chromosomal junction; (P) PvuII; (B) BglII, (E) EcoRV; (S) SalI; (H) HindIII; (Bg) BgIII.
Figure 4. Nucleotide and predicted amino acid sequence of YAK1. The nucleotide sequence extends 3599 bp from the YCp50-yeast 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 | Percent identity | Reference                  |
|---------------------------------------|-----------------|------------------|----------------------------|
| Schizosaccharomyces pombe cdc2        | 349             | 32               | Hindley and Phear [1984]   |
| S. cerevisiae CDC28                   | 288             | 31               | Lorinez and Reed [1984]    |
| Mouse phosphorylase kinase (γ)        | 245             | 23               | Chamberlain et al. [1987]  |
| Mouse cAPK                            | 197             | 20               | Uhler et al. [1986]        |
| STE7                                  | 156             | 24               | Teague et al. [1986]       |

* 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 transplanted 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 ras2Δ strain, 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 ras2Δ 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 transplanted 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 x 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 x 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
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; Kunitsawa et al. 1987; Toda et al. 1987a) and one may be allelic 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 | 25°C | 35°C | EtOH* |
|-------------------------|------------|---------|------|------|-------|
| ras1 :: HIS3 ras2::S3   | YAK1+      | +       | -    | -    | -     |
|                         | yak1 :: LEU2| +       | +    | +    |       |
| RAS1+ ras2 :: LEU2      | YAK1+      | +       | +    | -    |       |
|                         | yak1 :: HIS3| +       | +    | +    |       |
| cyr1-1                  | YAK1+      | +       | -    | -    |       |
|                         | yak1 :: LEU2| +       | -    | +    |       |
| cdc25-1                 | YAK1+      | +       | +    | +    |       |
|                         | yak1 :: LEU2| +       | -    | +    |       |
| ras1 :: HIS3 ras2 :: URA3| YAK1+    | -       | -    | -    |       |
|                         | yak1 :: LEU2| ±       | ±    | ±    |       |
| tpk1 tpk2 tpk3          | YAK1+      | -       | -    | -    |       |
|                         | yak1 :: LEU2| ±       | ±    | ±    |       |

* (+) Growth; (-) no growth; (±) slow growth.
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 sta9 and sta10 revert all of the pleiotropic Ras− phenotypes, albeit to degrees consistent with a hierarchy of suppressor strength. According to these criteria, the sta9 and sta10 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 STA10 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 [ΔlacX74 galU galK hisD RY38 lacY1 leu26 pyrF Δ::Tn5] and JM101 [Δlac-pro+:: thi F. traD36 proA B lacI72 lacZΔM15] 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.
DNA manipulations

Plasmid DNA was prepared from E. coli using the alkaline 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 deoxy sequencing (Sanger et al. 1977) with a-32P-labeled dATP as radioactive label. The 3599 nucleotides shown in Figure 4, from the HindIII 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 l2 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 EcoRI and HindIII and used to transform competent cells of strain SGP10. Over several days, the Ura+ transformants were replicated to 25°C and 35°C on rich medium (YEPD), as well as minimal medium. Colonies that grew at the lower temperature were selected only as potential ras2ts strains.

Selection of ras revertants

Individual colonies from two MATa ras1 :: HIS3 ras2ts strains, SGP11 and SGP34, were patched to YEPD medium agar and incubated for 48 hr at 35°C. From those patches on which colonies arose, a single colony was selected and purified at 25°C on YEPD. 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 MA-Ta ras1 :: HIS3 ras2ts 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 α counterparts by selecting for prototrophic growth. Individual α strains, along with their temperature-sensitive parents and the RAS2+ strain SGP4, were patched to YEPD agar, incubated for several days at 25°C, and then replica-plated to a lawn of the appropriate α revertant on minimal medium. After several days of prototrophic selection at 25°C, diploids were tested for growth at 35°C on YEPD 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 MA-Ta ras1 :: HIS3 RAS2+ strain SGP40. Tetrad colonies were scored in situ by inverting a plate over l2 crystals and using a sample of his strain (SGP10) to enable complementation analysis. The procedure outlined in Materials and methods. The RAS2+ colonies were then replica-plated to YEPD agar, incubated for several days at 25°C, and then replica-plated to a lawn of the appropriate α revertant on minimal medium, 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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S Garrett and J Broach

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