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Suppression of a Yeast Cyclic AMP-Dependent Protein Kinase Defect by Overexpression of *SOK1*, a Yeast Gene Exhibiting Sequence Similarity to a Developmentally Regulated Mouse Gene

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***Saccharomyces cerevisiae* cyclic AMP-dependent protein kinase (A kinase) activity is essential for growth and cell cycle progression. Dependence on A kinase function can be partially relieved by the inactivation of a second kinase encoded by the gene *YAK1*. We have isolated two new genes, *SOK1* and *SOK2* (suppressor of kinase), as gene dosage suppressors of the conditional growth defect of several temperature-sensitive A kinase mutants. Overexpression of *SOK1*, like lesions in *YAK1*, also restores growth to a strain (*tpk1 tpk2 tpk3*) lacking all A kinase activity. The *SOK1* gene is not essential, but a *sok1::HIS3* disruption abrogates suppression of an A kinase defect by *yak1*. These results suggest that Yak1 and Sok1 define a linear pathway that is partially redundant with that of the A kinase. Activation of Sok1, by *SOK1* overexpression or by inactivation of the negative regulator Yak1, renders a cell independent of A kinase function. The implications of such a model are particularly intriguing in light of the nuclear localization pattern of the overexpressed Sok1 protein and the primary sequence homology between *SOK1* and a recently described, developmentally regulated mouse gene.**

In the yeast *Saccharomyces cerevisiae*, cyclic AMP (cAMP)-dependent protein kinase (A kinase) activity is essential for growth and cell cycle progression. Cells deficient in this activity stop growing and arrest in G_1 in a manner similar to that observed for wild-type cells deprived of nutrients (16, 20, 21). In contrast, mutations yielding elevated A kinase activity prevent cells from arresting in G_1 following nutrient starvation or heat shock (4). Such mutations also cause sporulation deficiency, loss of carbohydrate reserves, and sensitivity to various forms of stress, including heat shock and nutrient starvation. Together, these phenotypes have been taken as evidence that the yeast A kinase pathway plays a central role in mediating the growth and cell cycle arrest of starved cells (2). Low-level activity promotes exit from the mitotic cycle and entry into G_0, whereas high-level activity precludes access to G_0.

Yeast A kinase activity is regulated by a complex signal transduction pathway that includes the yeast homologs of the mammalian *ras* products. *S. cerevisiae* contains two *RAS* genes (*RAS1* and *RAS2*) which encode functionally redundant, membrane-associated proteins that bind and hydrolyze GTP (32). In their active, GTP-bound state, yeast Ras proteins activate adenylate cyclase, which is encoded by a single gene, *CYR1* (18). Like its mammalian counterpart, yeast A kinase is a heterotetrameric protein consisting of two catalytic subunits and two regulatory subunits. The yeast catalytic subunits are encoded by three functionally redundant genes, *TPK1*, *TPK2*, and *TPK3* (31), whereas the regulatory subunit is specified by a single gene, *BCY1* (4). Binding of cAMP to Bcy1 results in its dissociation from the catalytic subunits and in stimulation of A kinase activity.

The growth arrest and cell cycle arrest of conditional Ras and A kinase mutants are consistent with the notion that A kinase phosphorylation regulates many cellular processes. Targets of the yeast A kinase have been described and include proteins involved in processes such as carbohydrate storage and metabolism, phospholipid metabolism, and transcriptional regulation, as well as functions involved in the synthesis and breakdown of cAMP (for reviews, see references 1 and 2). Nevertheless, it remains unclear whether A kinase phosphorylation of these known targets can influence whether the cell exits the cell cycle or continues proliferation (3).

Previous attempts to identify downstream effectors of the Ras/A kinase pathway have exploited classical and gene dosage (high-copy-number) suppressor analyses (4, 9, 13, 24, 30). Two genes exhibiting significant homology to known protein kinase genes were identified in separate selections for suppressors of conditional defects in the A kinase pathway. Null mutations of one gene, *YAK1*, allowed strains completely deficient in A kinase activity to grow; *tpk1 tpk2 tpk3 YAK1*$^+$ strains are inviable, but *tpk1 tpk2 tpk3 yak1* strains grow (13). These and other results led us to propose that Yak1 served as a negative regulator of cell growth, in a pathway parallel to that of the A kinase, with overlapping but antagonistic effects (14). The second gene exhibiting protein kinase gene homology, *SCH9*, was isolated as a high-copy-number suppressor of a temperature-sensitive *cdc25*(Ts) mutation. Although the mechanism by which Sch9 overproduction alleviates the A kinase defect is not known, the reciprocal suppression of null mutations in both pathways (a *tpk* strain grows in the presence of a *SCH9* high-copy-number plasmid, and the slow-growth defect caused by an *sch9* disruption is alleviated by high levels of A kinase activity) is consistent with a model in which Sch9 and the A kinase have partially overlapping functions. An alternate model, based in part on the significant sequence similarity between the two kinases, suggests that hyperactivation of either kinase results in the abnormal phosphorylation of essential kinase substrates of the opposite pathway (15, 30).

Since previous suppressor selections were conducted with mutations in *RAS* (5, 8, 13) and *CDC25* (30), we elected to isolate temperature-sensitive *tpk2* mutants [*tpk1 tpk2*(Ts) *tpk3* mutants] and use them to isolate second-site suppressors. We

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TABLE 1. Yeast strains used in this study

Strain	Genotype	Source or reference
S7-7A × S7-5A	*MAT***a**/*MATα tpk1*::*URA3*/*TPK1 tpk2*::*HIS3*/*TPK2 tpk3*::*TRP1*/*TPK3 ura3-52*/*ura3-52 leu2-3,112*/*leu2-3,112 his3*/*his3 trp1*/*trp1 ade8*/*ade8*	31
SGY356	*MATα tpk1*::*URA3 TPK2 tpk3*::*TRP1 bcy1*::*LEU2 ura3-52 his3 leu2-3,112 trp1 ade8*	This study
SGP3	*MATα ras1*::*HIS3 RAS2 his3 ura3-52 leu2-3,112 trp1 ade8*	13
SGP34	*MATα ras1*::*HIS3 ras2-34-URA3*(Ts) *his3 ura3-52 leu2-3,112 trp1 ade8*	13
MWY63	SGY356 *tpk2-63*(Ts)	This study
MWY65	SGY356 *tpk2-65*(Ts)	This study
MWY123	MWY63 *yak1*::*HIS3*	This study
MWY131	MWY65 *yak1*::*HIS3*	This study
SGY398	MWY63 *tpk1*::*ADE8*	This study
1029	*MAT***a**/*MATα ura3*/*ura3 leu2-3,112*/*leu2-3,112 his3*/*his3 lys2*/*lys2 trp1*/*TRP1 ade2*/*ADE2*	13
MWY264	1029 *sok1*::*HIS3*/*SOK1*	This study
MWY285	MWY63 *sok1*::*HIS3*	This study
MWY273	MWY63 *yak1*::*ADE8*	This study
MWY313	MWY63 *sok1*::*HIS3 yak1*::*ADE8*	This study
SGP406	*MAT***a** *leu2-3,112 trp1 ura3-52 his3 tpk1*::*URA3 tpk2*::*HIS3 tpk3*::*TRP1 yak1*::*LEU2*	13
RS13.58A-1	*MAT***a** *tpk1*$^{WI a}$ *tpk2*::*HIS3 tpk3*::*TRP1 bcy1*::*LEU2 ura3-52 his3 leu2-3,112 trp1 ade8*	3

[a] WI is the notation used by Cameron et al. (3) for the wimp alleles of *TPK1*, *TPK2*, and *TPK3*.

reasoned that by doing so, and thereby eliminating the majority of suppressors already isolated (*CDC25*, *RAS1* and *RAS2*, and the three *TPK* genes, etc.), we would identify rare or weak suppressors that might originally have been overlooked. This communication describes the isolation and characterization of several independent, temperature-sensitive mutations in the *TPK2* gene, as well as the isolation of two gene dosage suppressors of the resulting conditional growth defect. Characterization of one of the high-copy-number suppressors has identified a novel gene, *SOK1*, that exhibits sequence similarity to a developmentally regulated mouse gene. Tests of epistasis are consistent with a model in which activation of Sok1, by *SOK1* overexpression or by inactivation of Yak1, results in suppression of the A kinase defect.

MATERIALS AND METHODS

Media and growth conditions. Media used, including yeast rich and minimal media as well as bacterial media, were prepared as described previously (7, 13). Yeast cells were heat shocked by replicating patches to prewarmed agar and placing the agar plates in a shallow water bath set to 55°C. After 10 min at the elevated temperature, the plates were incubated at 23 or 30°C for several days. Physiological characterization of the temperature-sensitive A kinase mutants for glycogen accumulation and growth arrest was carried out by essentially the same methods described previously (13). Gene dosage suppressors of the A kinase conditional mutants were isolated by transforming two temperature-sensitive *tpk2*(Ts) strains, MWY63 and MWY65, to adenine prototrophy and temperature resistance (34.5 and 36°C, respectively) with a high-copy-number plasmid library based on the *ADE8* 2μm vector YEp*ADE8* (14).

Strains and plasmids. Yeast strains are listed, with references where appropriate, in Table 1. Bacterial strains MC1066 [Δ(*lac*)*X74 galU galK strA hsdR trpC9830 leuB6 pyrF*::Tn*5*] and DH5 [F′/*endA hsdR17*(r_K m_K) *supE44 thi-1 recA1 gyrA relA1*Δ(*lacZYA-argF*)*U169* (Δ80*dlac* (*lacZ*)*M15*)] have been described previously (6, 35). The high-copy-number yeast vector YEp*ADE8* was constructed by Toda and Cameron and has been described previously (14). To circumvent the repeated isolation of plasmids containing *TPK1*, *TPK2*, and *TPK3*, a high-copy-number library was constructed by digesting chromosomal DNA of a *tpk*-deficient strain (*tpk1*::*URA3 tpk2*::*HIS3 tpk3*::*TRP1 yak1*::*LEU2*) (SGP406) to partial completion with *Sau*3A and ligating size-selected fragments into the single *Bam*HI site of YEp*ADE8*. Eight separate pools of transformants (>8,000 colonies per pool) were collected and tested for the fraction of plasmids with inserts as well as for insert size. Physical analysis of plasmids isolated from random bacterial colonies showed that >80% (10 of 12) contained inserts, with an average insert size of 14 kb. The bacterial vector pBSK$^+$ has been described previously (Stratagene Product Catalogue). Plasmids pMW25 and pMW42 are class I suppressors, whereas plasmid pMW43 is a class II suppressor.

DNA manipulations. Plasmid DNA was prepared from *Escherichia coli* by the alkali lysis method (19). All enzymes were used according to the specifications of their suppliers (New England Biolabs or Bethesda Research Laboratories), and cloning techniques were as described previously (19).

The *HIS3* disruption of *SOK1* was constructed by digesting plasmid pMW22 (YEp*ADE8* containing a 6-kb *Bgl*II *SOK1* fragment at the *Bam*HI site; see Fig. 3, plasmid C) with *Nco*I, filling in the ends with Klenow fragment, ligating on *Bam*HI linkers, and then inserting the 1.7-kb *Bam*HI fragment of *HIS3*. The resulting *sok1*::*HIS3* plasmid was designated pMW26. The *yak1*::*HIS3* and *yak1*::*ADE8* disruptions have been described previously (13).

The sequence of *SOK1* was determined by a modification of the method of Sanger et al. (28), using double-stranded plasmid DNA containing random deletions of *SOK1*. Random deletions of *SOK1* in pBSK$^+$ were constructed by digesting pMP11 and pMP13 with *Kpn*I and *Xho*I and then sequentially digesting with exonuclease III and S1 nuclease according to the instructions of the supplier (Promega).

The *SOK1* gene was epitope tagged by converting the single *Hin*dIII site (bp 312 in Fig. 4) within the 5′ end of the coding region to a *Not*I site and inserting a 112-bp *Not*I DNA fragment (GTEP) containing three repeats of a 27-codon sequence specifying the hemagglutinin (HA) peptide, YPYDVPDYA (11, 26a). The *Not*I site was created by cutting *SOK1* with *Hin*dIII, filling in the 5′ overhang with Klenow fragment, and inserting *Not*I linkers (8-mers from New England Biolabs). One of the clones containing the correct linkers was digested with *Not*I, dephosphorylated with calf intestinal phosphatase, and then ligated with the GTEP fragment. Plasmids containing a single insert were tested by digestion with several restriction

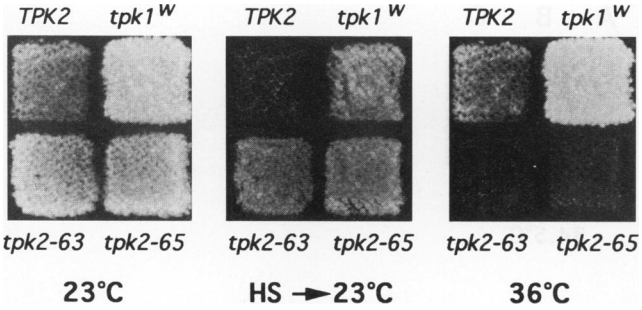

FIG. 1. Characterization of conditional A kinase mutants. Growth was tested by replicating patches to agar that was either incubated at 23°C, heat shocked (HS) for 10 min and returned to 23°C, or incubated at 36°C. Strains: *TPK2*, SGY356 (*TPK2 bcy1*::*LEU2*); *tpk1*^{w}, RS13.58A-1 (*tpk1*^{WI} *bcy1*::*LEU2*); *tpk2-63*, MWY63 [*tpk2-63*(Ts) *bcy1*::*LEU2*]; *tpk2-65*, MWY65 [*tpk2-65*(Ts) *bcy1*::*LEU2*].

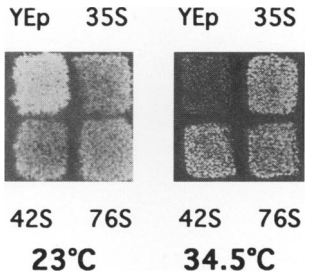

FIG. 2. High-copy-number suppressors of conditional A kinase mutants. Patches of strain MWY63 [*tpk2-63*(Ts) *ade8*] containing the indicated high-copy-number plasmids were replicated to minimal medium (lacking adenine) and incubated at 23 and 34.5°C for several days. Plasmids were YEp (YEp*ADE8*), 35S (YEp*ADE8-SOK1*) (pMW25), 42S (YEp*ADE8-SOK1*) (pMW42), and 76S (YEp*ADE8-SOK2*) (pMW43).

enzymes to monitor the orientation of the insert (there is a single *Bam*HI site positioned asymmetrically within the GTEP fragment). Finally, the single *Sst*I fragment from one of the derivatives containing the GTEP insert in the correct orientation (as well as a clone containing the same fragment in the opposite orientation) was inserted into the unique *Sst*I site of the original vector (YEp*ADE8*).

Immunofluorescence of Sok1-HA. Strains were stained for indirect immunofluorescence essentially as described previously (27). The primary antibody was anti-HA monoclonal antibody 12CA5 (the kind gift of Ken Ferguson and Mike Wigler) and was diluted 1:1,000 for staining. The secondary antibody was fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G from Boehringer Mannheim.

RESULTS

Isolation and characterization of A kinase temperature-sensitive mutations. To isolate temperature-sensitive A kinase mutations, we took advantage of the stress-sensitive phenotype of strains with elevated A kinase activity (4). Strains lacking the regulatory subunit of the A kinase and containing one or more of the three, redundant catalytic subunit genes fail to survive regimens such as brief exposure to high temperatures (10 min, 55°C) and nutrient deprivation. This stress sensitivity was previously exploited to isolate partial-loss-of-function mutations in the A kinase catalytic subunit genes (3). Although the mutations isolated by Cameron et al. (3) were not analyzed for conditional activity, we reasoned that a subset of them might result in the complete loss of A kinase function at an elevated temperature. Strains containing such mutations would be resistant to heat shock and viable at the permissive temperature (i.e., 23°C) and would arrest in G_1 on a shift to the nonpermissive temperature. A similar scheme was used by others to identify conditional alleles of *RAS2* (26).

Stress-resistant revertants of a *tpk1 TPK2 tpk3 bcy1* strain were isolated by exposing cells to 55°C for 10 min and then incubating the cells at 23°C for 3 days. To ensure that all of the revertants were independent, only one heat shock-resistant colony from each patch was saved (see Materials and Methods). Stress-resistant revertants were then retested for their ability to survive exposure to extreme heat as well as for their viability at 36°C (Fig. 1). Of a total of 150 heat shock-resistant survivors, 30 (20%) exhibited a temperature-sensitive growth defect.

At least three of the stress-resistant revertants contain temperature-sensitive mutations in *TPK2* as judged by several criteria (Table 2). First, the temperature-sensitive growth defect of each strain was recessive and was complemented by a low-copy-number plasmid carrying *TPK1* or *TPK3*. Second, the temperature-sensitive mutations were shown to be tightly linked to *TPK2* by mating each of the revertants to a *TPK1 tpk2*::*HIS3 tpk3*::*TRP1 BCY1* strain and scoring tetrads. Only His^- Ura^+ strains were temperature sensitive for growth (of 50 tetrads), suggesting that the conditional mutation was tightly linked to *TPK2* and was masked by the presence of a wild-type *TPK1* allele. Third, the temperature-sensitive growth defect was suppressed by the disruption of a gene, *YAK1*, identified previously as a recessive suppressor of conditional A kinase activity (13). Finally, all of the mutants accumulated at least moderate levels of glycogen, in contrast to the stress-sensitive *TPK2 bcy1* parent, which was unable to accumulate measurable glycogen under any condition. In *S. cerevisiae*, glycogen synthesis and degradation have been shown to be

TABLE 2. Characterization of *tpk2*(Ts) mutants[a]

Strain	Relevant genotype	Growth at:			HS[b]	Iodine[c]	Complementation		*TPK2* linkage
		23°C	34.5°C	36°C			TPK1	TPK2	
SGY356	*TPK2*	+	+	+/−	−	Y	ND	ND	ND
MWY63	*tpk2-63*(Ts)	+	−	−	+	B	+	+	Yes
MWY65	*tpk2-65*(Ts)	+	+/−	−	+	B	+	+	Yes
MWY123	*tpk2-63*(Ts) *yak1*::*HIS3*	+	+	+/−	ND	ND	ND	ND	ND
MWY131	*tpk2-65*(Ts) *yak1*::*HIS3*	+	+	+/−	ND	ND	ND	ND	ND

[a] +, growth; +/−, slow growth; −, no growth; ND, not done; Y, yellow (no glycogen accumulation); B, brown (glycogen accumulation).
[b] Growth at 23°C after exposure to 55°C for 10 min.
[c] Color of colonies after exposure to iodine vapors.

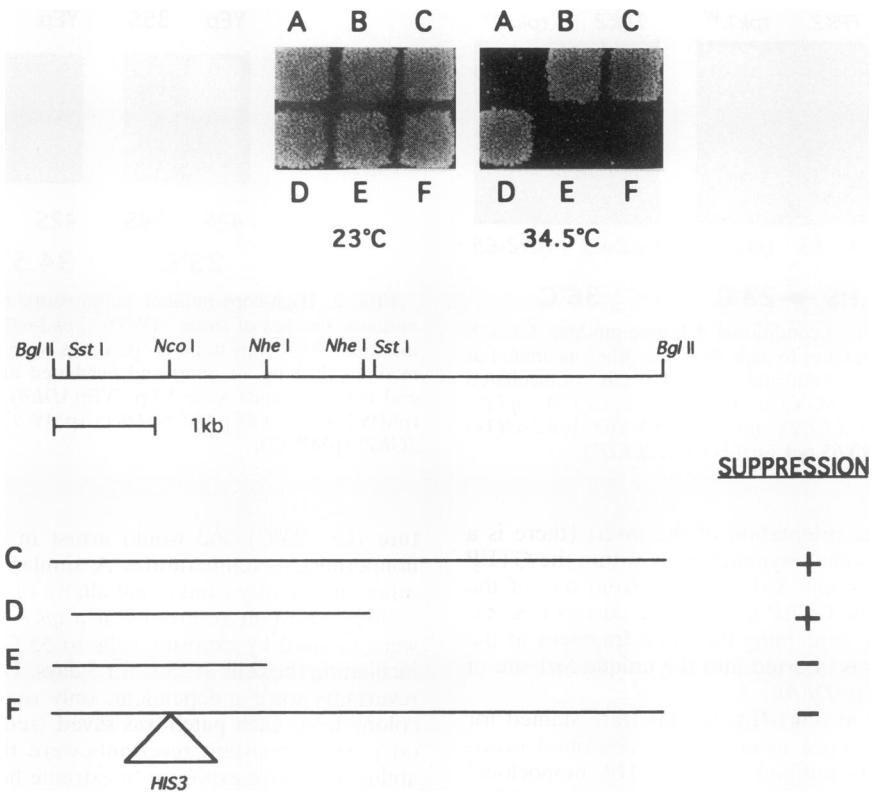

FIG. 3. Cloning and restriction map of *SOK1*. Patches of strain MWY63 (*tpk2-63 ade8*) containing the indicated plasmids were replicated to minimal medium (lacking adenine) and incubated for several days at 23 and 34.5°C. The yeast DNA fragments of some of the plasmids are shown at the bottom along with the results of the test of suppression. Plasmids not shown are A (YEp*ADE8*) and B (pMW25, the original *SOK1* clone).

regulated by A kinase phosphorylation, with glycogen levels exhibiting an inverse relation to cellular A kinase activity (4, 12). By these criteria, the conditional growth defects of at least three of the heat shock-resistant revertants were due to a temperature-sensitive mutation in *TPK2* (Table 2). Eight other conditional mutants contained recessive, temperature-sensitive mutations in *TPK2* as judged by their inability to complement one of the *tpk2*(Ts) mutants (*tpk2-63* mutant) of the opposite mating type.

Isolation of gene dosage suppressors of the conditional A kinase mutations. The technical difficulties of characterizing and cloning dominant suppressors have been partially circumvented by the recent exploitation of gene dosage, or high-copy-number, suppressors. Gene dosage suppressors of the conditional A kinase mutations were isolated by transforming two of the temperature-sensitive A kinase mutants (*tpk2-63* and *tpk2-65* mutants) with a high-copy-number plasmid library and incubating the transformants at the nonpermissive temperature for several days. Since A kinase phosphorylation may affect many essential processes, we determined the lowest temperature at which each of the strains could be incubated without exhibiting any growth. By doing so, we hoped to make the phosphorylation of a single essential substrate limiting. In addition, the plasmid library from which the high-copy-number suppressors were isolated was generated from a *tpk1 tpk2 tpk3 yak1* strain (see Materials and Methods). The use of this library eliminated the reisolation of the three *TPK* genes, any one of which was able to complement the conditional A kinase mutation and alleviate the growth defect.

To confirm that the temperature-resistant growth of the transformants was plasmid dependent, colonies that grew at the nonpermissive temperature were allowed to lose the plasmid (by growth in nonselective medium at 23°C for several days) and then retested for conditional growth. By this criterion, 30 colonies (of a total of 55,000 Ade^+ transformants) exhibited plasmid-dependent, temperature-resistant growth. Plasmid DNA from each of the 30 colonies was rescued in *E. coli* and used to retransform the original temperature-sensitive yeast strain (MWY63 or MWY65) from which each was derived. In this second screen, plasmid DNA from 8 of the 30 temperature-resistant colonies was able to retransform the conditional A kinase mutants to temperature resistance (Fig. 2). At least 6 of the remaining 22 colonies may have contained a mixture of high-copy-number plasmids, since temperature resistance was restored to greater than one-eighth of the yeast transformants when plasmid DNA from a pooled bacterial transformation, rather than a single colony, was used.

The gene dosage suppressors define two new genes, *SOK1* and *SOK2*. Since the plasmid library was constructed from a strain lacking the three A kinase catalytic genes, we could rule out the possibility that any of the eight gene dosage suppressors contained a functional *TPK* gene. To determine the identities of the inserts contained within the eight high-copy-number plasmids, each plasmid was subjected to restriction fragment analysis. Plasmids with nonidentical but overlapping inserts were grouped into two classes: class I and class II were defined by five and three plasmids, respectively. Consistent with this physical assignment, class II plasmids suppressed the temperature-sensitive growth defect more weakly than the five class I suppressors (see below). Despite this quantitative

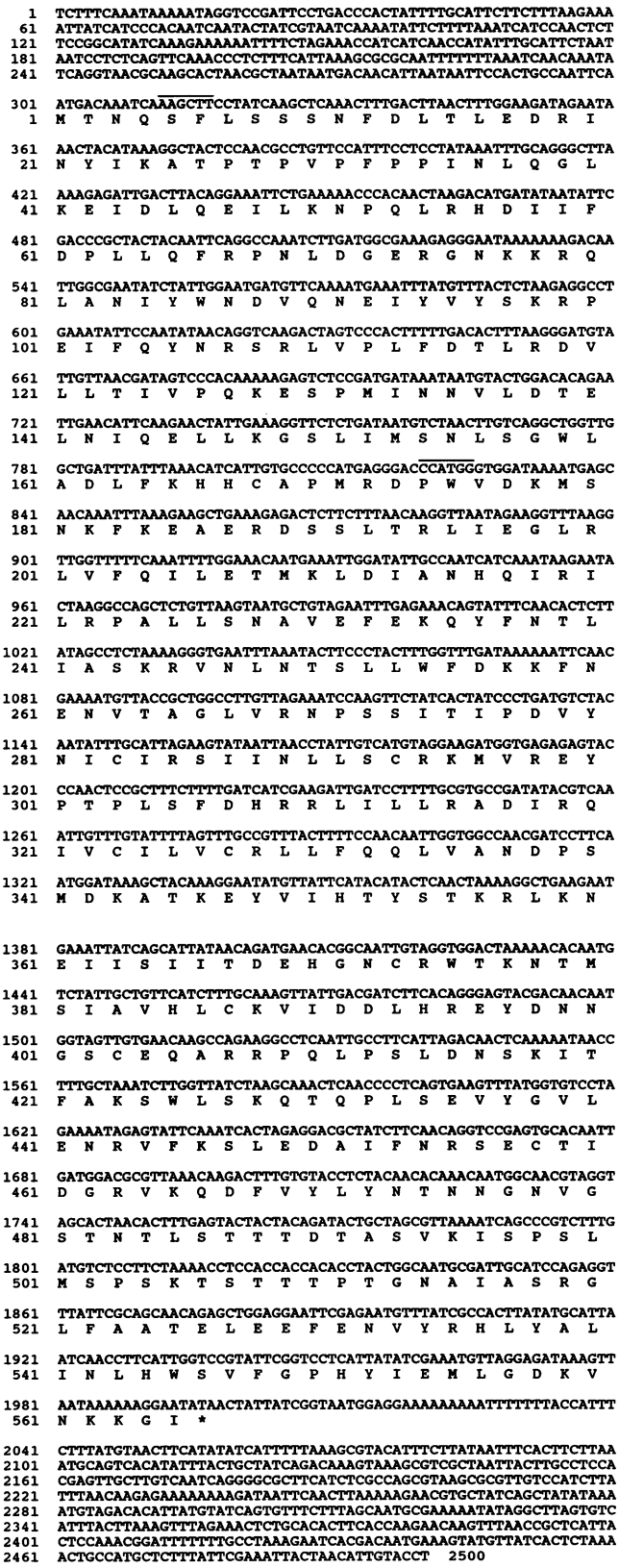

FIG. 4. Nucleotide and predicted amino acid sequences of *SOK1*. The nucleotide sequence extends 2,500 bp from the leftmost *Sst*I site (the sequence does not include the *Sst*I site) to about halfway between the two *Nhe*I sites shown in Fig. 3. The *Nco*I and *Hin*dIII restriction sites (the sites of insertion of the *HIS3* marker [Fig. 3] and the HA epitope tag, respectively) are indicated by lines above the DNA sequence, and the chain-terminating codon is denoted by an asterisk.

difference, plasmids from each class were isolated as suppressors of both conditional A kinase strains. Thus, by both physical and phenotypic analyses, the eight plasmids defined two genes whose presence in elevated copy number suppressed the growth defect of two independent A kinase conditional mutants.

The *SCH9* gene was previously identified as a dosage suppressor of a conditional defect [*cdc25*(Ts)] in the A kinase pathway (30). To confirm that none of the suppressing plasmids contained incomplete clones of *SCH9*, a labelled probe of *SCH9* DNA was hybridized to class I and class II plasmid DNAs isolated from *E. coli*. As predicted by the restriction maps, the *SCH9* probe failed to hybridize to the DNA of either suppressor class but elicited a strong signal from a control lane containing an identical amount of *SCH9* DNA (data not shown). We have confirmed that the temperature-sensitive A kinase defect can be suppressed by an *SCH9* high-copy-number plasmid but that suppression is weak relative to *SOK1* overexpression (data not shown).

Fragments from one representative plasmid of the class I suppressors were subcloned back into the YEp*ADE8* vector and screened for their ability to suppress the conditional A kinase defect. As shown in Fig. 3, the class I suppressor was defined by a DNA insert of 3 kb, and insertion of *HIS3* into the single *Nco*I site of the same fragment completely destroyed suppressor activity. The locus defined by the *HIS3* insertion was designated *SOK1*, for suppressor of A kinase. The class II suppressors have been designated *SOK2*.

The *SOK1* gene encodes a polypeptide exhibiting sequence similarity to a mouse testis-specific protein. The nucleotide sequence of the *SOK1* gene and flanking DNA was determined. The sequence revealed one long open reading frame disrupted by the *Nco*I site (Fig. 4). The open reading frame corresponding to the *SOK1* gene would encode a predicted protein of 565 amino acids if the first ATG encoded the initiating methionine. A second reading frame 2 kb downstream from and transcribed in the orientation opposite to that of *SOK1* was identical to *RAD57* (17). The physical proximity of these two genes was in agreement with genetic mapping data that revealed tight linkage between both genes and the *CEN*-linked *TRP1* gene of chromosome IV (data not shown).

The predicted *SOK1* gene product was used in a homology search with previously identified proteins. The only protein to exhibit significant homology with *SOK1* was a developmentally regulated mouse protein (the predicted product of a transcript designated pBs13) of unknown function (21). Overall, the two proteins have 25% identity and 49% similarity. The sequence identity increases in several stretches in the middle of the two proteins and includes a stretch of 146 residues exhibiting 33% identity and 59% similarity (Fig. 5). Although our analysis of the Sok1 sequence has not been extensive, the sequence does not exhibit similarity to any of the canonical transcription factors, in agreement with conclusions reached for the testis-specific mouse gene (21).

Since the *SOK1* gene was isolated as a high-copy-number suppressor of a conditional A kinase mutant, the predicted polypeptide was scanned for potential A kinase phosphorylation sites as well as for other consensus motifs with a possible role in the function of Sok1. While several putative protein kinase C phosphorylation sites were found, A kinase recogni-

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111 VPLFDTLRDVLLTIVPQKESPMINNV...LDTELNIQELLKGSLIMSNLS 157
    :.|:..::::||.:: .::|.: |::   || |:  |:  :|.| :| ||
175 LELLKEIKEILLSLLLPRQSRLKNEIEEALDMEFLQQQADRGDLNVSYLS 224
158 GWLADLFKHHCAPMRDPWVDKMSNKFKEAERDSSLTRLIEGLRLVFQILE 207
     :: :::   |||:||. |:::.|          :.  :  || :||:|:
225 KYILNMMVLLCAPIRDEAVQRLEN.....ISDPVRLLRGIFQVLG 264
208 TMKLDIANHQIRILRPALLSNAVEFEKQYF 237
    ||:|:.|. |. |.|.| .:.|:||:. |
265 QMKMDMVNYTIQSLQPQLQEHSVQFERAQF 294
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FIG. 5. Amino acid sequence homology between the *SOK1* product and a testis-specific mouse protein. Sequence similarity between Sok1 (residues 111 to 237) and the protein encoded by pBs13 is displayed. Amino acid identity is denoted by bars, and conservative changes are denoted by colons.

tion sites were conspicuously missing from the predicted protein.

An epitope-tagged Sok1 protein is localized to the nucleus. Since the primary sequence of the Sok1 protein did not suggest an obvious function or activity, we determined its cellular localization by indirect immunofluorescence (27). The Sok1 protein was epitope tagged by inserting a 115-bp oligonucleotide specifying three repeats of the 9-amino-acid HA epitope into the single *Hin*dIII site within the 5′ end of the *SOK1* coding region (Fig. 4; see Materials and Methods). Insertion of the oligonucleotide in frame and in the correct orientation did not perturb the suppressor activity of the altered *SOK1* gene (data not shown), so we reasoned that the tagged polypeptide would be properly localized. The epitope-tagged Sok1 protein was localized to the nuclei of cells containing the high-copy-number YEp*ADE8-SOK1*-GTEP plasmid by using the anti-HA monoclonal antibody 12CA5 and a fluorescein isothiocyanate-conjugated goat antimouse antibody (Fig. 6). In contrast, no nuclear staining was observed with strains bearing various control plasmids, including the YEp*ADE8* vector and a

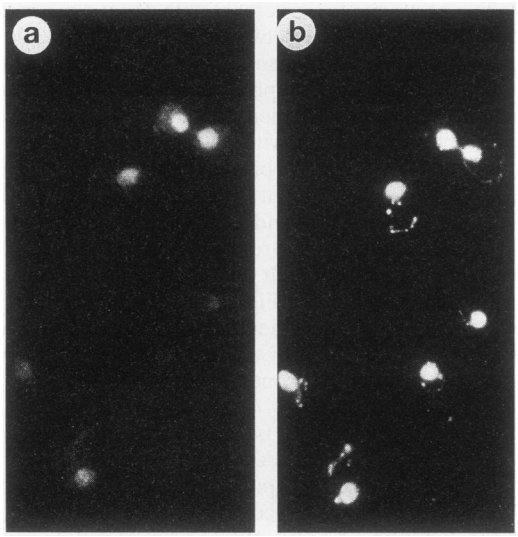

FIG. 6. Cellular localization of the Sok1 protein. The localization of an epitope-tagged derivative of the Sok1 protein was visualized by indirect immunofluorescence with fluorescein isothiocyanate-conjugated secondary antibody (a) and contrasted with the localization of cellular DNA as judged by DAPI (4′,6-diamidino-2-phenylindole) staining (b). Cells contained an epitope-tagged derivative of the original high-copy-number *SOK1* suppressor (see Materials and Methods).

TABLE 3. Suppression of A kinase defects by overexpression of *SOK1* or *SOK2*

Mutant genotype	Growth[a] with the following suppressor:		
	yak1	HC[b]-*SOK1*	HC-*SOK2*
ras1 ras2-34(Ts)	+	+	−
cdc25-5(Ts)	+	+	−
tpk1 tpk2-63(Ts) *tpk3*	+	+	+/−
tpk1 tpk2-65(Ts) *tpk3*	+	+	+/−
tpk1 tpk2 tpk3	+/−	+/−	−

[a] See Table 2, footnote *a*.
[b] HC, high copy number.

SOK1 derivative containing the epitope in the inverse orientation (data not shown). Consistent with this observation, it is possible to identify at least one potential nuclear localization signal within the Sok1 coding region (Fig. 4). The mouse testis-specific protein also contains a sequence that might serve as a nuclear localization signal, although neither sequence falls within the regions shared by the two proteins. Finally, we have confirmed by Western blot (immunoblot) analysis (data not shown) that strains bearing the *SOK1*-GTEP plasmid, but not those bearing the *SOK1*-PETG construct, specify a protein that, at 65 kDa, is consistent with the size predicted for the epitope-tagged *SOK1* gene product (565 plus 27 amino acids).

***SOK1* overexpression suppresses total loss of A kinase function.** To determine the relation of the *SOK1* and *SOK2* products to the Ras/A kinase pathway, we tested the abilities of the corresponding plasmids to suppress other mutations of the pathway. A representative high-copy-number plasmid from each class was transformed into temperature-sensitive *cdc25-5*(Ts) and *ras1 ras2*(Ts) strains, and Ade$^+$ transformants were tested for growth at several temperatures. As shown in Table 3, the *SOK1* plasmid was able to partially suppress the growth defects caused by both mutations. By contrast, the high-copy-number *SOK2* plasmid suppressed neither mutation, consistent with the observation that *SOK2* was a relatively weak suppressor of the *tpk2*(Ts) defect.

Disruption of *yak1* or overexpression of *SCH9* restored growth to a strain lacking all three A kinase catalytic genes, as well as to strains lacking *cdc35* and both *ras* genes (13, 30). Accordingly, we tested whether either high-copy-number suppressor isolated in these studies could do the same. The heterozygous *tpk* diploid S7-7A × S7-5A was transformed to Ade$^+$ with a plasmid bearing either *SOK1* or *SOK2*, sporulated, and picked to rich medium. Haploid spores containing disruptions of *TPK1*, *TPK2*, and *TPK3* germinated only when they contained the high-copy-number plasmid containing *SOK1* (Table 3). Interestingly, the pattern of suppression by *SOK1* overexpression was similar to that conferred by inactivation of *YAK1*, as judged by the slow growth (Table 3) (13) and hyperaccumulation of glycogen. Thus, neither *SOK1* overexpression nor Yak1 inactivation is capable of completely relieving the A kinase growth dependence of a cell. In contrast, colonies lacking all three catalytic subunit genes were never recovered from the diploid containing the *SOK2* plasmid, despite the high transmission frequency of the *SOK2* plasmid during sporulation.

Overexpression of *SOK1* has no effect on glycogen accumulation or sensitivity to stress. Several distinct phenotypes have been associated with strains containing diminished or elevated levels of A kinase. For example, activation of the A kinase pathway results in cells that are exquisitely sensitive to various forms of stress as well as in a failure to accumulate storage

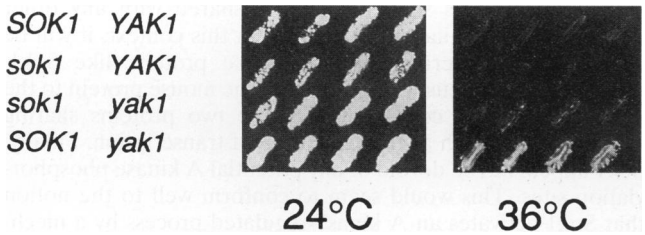

FIG. 7. Epistatic relation between Sok1 and Yak1. Four patches of the indicated strains were replicated to rich medium (yeast extract-peptone-dextrose) agar and incubated at 24 and 36°C for several days. Strains: *SOK1 YAK1*, MWY63 [*tpk2-63*(Ts) *SOK1 YAK1*]; *sok1 YAK1*, MWY285 [*tpk2-63*(Ts) *sok1*::*HIS3 YAK1*]; *sok1 yak1*, MWY313 [*tpk2-63*(Ts) *sok1*::*HIS3 yak1*::*ADE8*]; *SOK1 yak1*, MWY273 [*tpk2-63*(Ts) *SOK1 yak1*::*ADE8*].

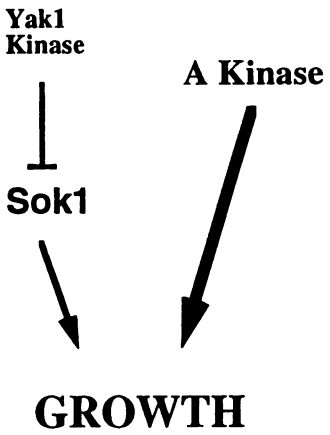

FIG. 8. Model of the A kinase-Yak1-Sok1 pathway.

carbohydrates such as glycogen. Strains that are compromised for A kinase activity are abnormally resistant to stress and accumulate elevated levels of glycogen. To examine the effect of *SOK1* overexpression on these phenotypes, we determined the heat shock and starvation sensitivities of a wild-type strain containing the high-copy-number *SOK1* plasmid or the YEp*ADE8* vector. The same plasmid-containing strains were inverted over iodine to gauge the effect of *SOK1* overexpression on glycogen accumulation. By these criteria the *SOK1*-overexpressing strain was identical to its isogenic YEp*ADE8* control and was in stark contrast to a congenic *bcy1* strain, which was exquisitely sensitive to all forms of stress and failed to accumulate glycogen even on prolonged incubation (data not shown). Disruption of the *SOK1* gene also had no appreciable effect on stress sensitivity or the capacity to accumulate glycogen.

Disruption of *SOK1* prevents suppression of the A kinase defect by loss of Yak1 function. To determine the role of the *SOK1* gene product in cell growth and division, a *sok1*::*HIS3* disruption was placed in the chromosome. The 4.7-kb fragment of plasmid pMW26 (YEp*ADE8-sok1*::*HIS3*) was used to transform diploid strain 1029 (*SOK1/SOK1 his3/his3*) to His^+, and two transformants were subjected to tetrad analysis. Both transformants contained a single disrupted copy and a wild-type copy of *SOK1* as determined by DNA-DNA hybridization (data not shown). All tetrads of both strains (17 of 17) had four equal-sized colonies, with the His^+ marker segregating 2:2 (data not shown). Thus, the *SOK1* gene is not essential for growth. Although we cannot rule out the possibility that *SOK1* is a member of a family of genes with related functions, we have been unable to detect another yeast gene exhibiting significant structural similarity as judged by low-stringency hybridization (data not shown).

Tests of epistasis placed the *SOK1* suppressor downstream from, or on a pathway parallel with that of, the A kinase gene (Table 3). Two other genes thought to encode growth regulators related to the A kinase pathway include *SCH9* and *YAK1*, identified as gene dosage and loss-of-function suppressors of mutations in the A kinase, respectively. We have previously shown that suppression by Sok1 is independent of the Sch9 kinase (15). To determine the relation between Sok1 and Yak1, the *sok1*::*HIS3* disruption was introduced into isogenic *tpk2*(Ts) *YAK1* and *tpk2*(Ts) *yak1* strains, which were then tested for growth at the permissive and nonpermissive temperatures. While the loss of Sok1 function had no apparent effect on the growth of the Yak1-proficient strain [Fig. 7; compare the growth of the *tpk2*(Ts) *YAK1 SOK1* strain with that of the *tpk2*(Ts) *YAK1 sok1* mutant], its inactivation totally blocked growth of the *tpk2*(Ts) *yak1* strain at elevated temperatures. Thus, suppression of the A kinase defect by the inactivation of Yak1 required a functional *SOK1* gene product.

DISCUSSION

We have identified a new gene, *SOK1*, whose overexpression alleviates the growth defect of yeast strains lacking A kinase activity. Although the mechanism of this suppression is not known, our results are consistent with a model in which Sok1 identifies a downstream component of the Yak1 kinase pathway (Fig. 8). In that scenario, the Yak1-Sok1 pathway would stimulate a set of essential cellular processes under A kinase control. Activation of Sok1, by *SOK1* overexpression or by inactivation of the negative regulator Yak1, would render each of the processes independent of A kinase activity. Given the nuclear localization pattern of Sok1 on overexpression, it is tempting to speculate that at least one process made independent of A kinase activity might include the general or specific activation of transcription. These results are particularly intriguing in light of the structural similarity between the predicted product of *SOK1* and the product of a recently described, developmentally regulated mouse gene (21).

The most important result of these studies is that overexpression of *SOK1* can suppress the growth defect of a strain lacking all three *TPK* genes. This marks *SOK1* as only the second gene whose overexpression is capable of bypassing the need for A kinase for growth. The first such gene, *SCH9*, encodes a protein kinase exhibiting significant homology to the yeast A kinase catalytic subunits (30). The structural similarity between Sch9 and the A kinase, along with physiological and genetic studies (15), is at least consistent with the notion that suppression of a *tpk* strain by *SCH9* overexpression occurs as a result of the overlapping specificities of the two kinases. The *SOK1* gene, in contrast, corresponds to a protein that bears no relation to known protein kinases (Fig. 4). Thus, the *SOK1* high-copy-number suppressor appears to act by a mechanism that is different from that of *SCH9*.

One intriguing possibility is that *SOK1* encodes an A kinase substrate involved in cell growth and division. Its dependence on A kinase phosphorylation might, therefore, be abrogated by an increase in *SOK1* abundance. Arguing against this proposal, however, is the fact that deletion of the *SOK1* gene does not result in a noticeable growth defect, contrary to the expectation for an effector protein in an essential pathway. This result could be reconciled if *SOK1* was a member of a duplicated

gene family. However, *SOK1* and *SOK2* have each been isolated multiple times, and genetic and physical analyses suggest that the two genes are not functionally related (for example, a *sok1 sok2 TPK* strain exhibits no obvious growth defects [33a]).

An alternate hypothesis is that the Sok1 protein participates in a pathway that is partially redundant with that of the A kinase (Fig. 8). A kinase-dependent processes would be regulated independently by Sok1, such that a decrease in A kinase activity (with a concurrent diminution in some metabolic process) could be compensated for by an increase in stimulation by Sok1. In such a model, the phenotypic effect of losing either pathway would depend on the relative contribution of each to the overall function of the process. Judging by the apparent wild-type growth of the *sok1* null mutant (Fig. 7), it seems likely that under normal conditions, the contribution by the Sok1 pathway is small. On activation, the contribution by Sok1 presumably increases to levels sufficient to relieve the A kinase requirement.

Our model also posits that Sok1 activity is normally repressed by the Yak1 kinase. Such a model explains our earlier observation (13) that Yak1 activity is antagonistic to growth of an A kinase-deficient mutant, and it predicts that the growth of a *tpk*(Ts) *yak1* strain would be abrogated by the loss of Sok1 function. In other words, relief from Yak1 repression would result in A kinase independence only if a functional Sok1 protein was present. As predicted, the growth of a *tpk*(Ts) *yak1* strain is made conditional by the disruption of *SOK1*. Thus, our results are consistent with a model in which the defect in yeast A kinase can be alleviated by activation of Sok1 function, either by an increase in *SOK1* expression or by a decrease in Yak1 kinase activity. The model shown in Fig. 8 is consistent with the possibility, but does not require, that the interaction between Yak1 and Sok1 is direct, such that Sok1 is inactivated by a Yak1-specific phosphorylation event.

The lack of identity between Sok1 and other protein kinases does not eliminate the possibility that *SOK1* overexpression activates a kinase that shares overlapping specificity with the A kinase. For example, if Sok1 stimulated *SCH9* transcription, overexpression of either *SOK1* or *SCH9* might result in suppression of the A kinase defect. However, several results argue against Sok1 regulation of the Sch9 kinase. First, the synthetic lethality of a *tpk*(Ts) *sch9* strain can be overcome by *SOK1* overexpression or disruption of *yak1* (15). Thus, the *SOK1* and *yak1* suppressors must alleviate the A kinase defect by an *SCH9*-independent mechanism, placing Sok1 function distal to Sch9 function. Second, cells lacking Sch9 activity grow extremely slowly (15, 30), whereas *sok1* deletion mutants are unaltered in growth. Thus, the two functions appear unrelated. It remains possible that Sok1 might alleviate the A kinase defect through the activation of another A kinase homolog; however, such a kinase has been notably absent from the suppressors identified to date (5, 9, 13, 30, 33a).

The structural similarity between Sok1 and a testis-specific mouse transcript thought to play a role in sperm development implicates a shared determinant or functional domain of these two proteins in the regulation of a variety of important growth and developmental processes. Unfortunately, the primary structures of the two proteins provide few clues to their specific biochemical functions or the nature of the processes they regulate. Potential sites of N glycosylation and membrane attachment (21) are not conserved between the two proteins, and several putative sites of protein kinase C phosphorylation that are present in the mouse pBs13 product are displaced in Sok1. Moreover, the region most conserved between the two proteins, a central core of 143 amino acids, does not appear to reveal a consensus sequence that is shared with any other proteins in the available data banks. In this context, it will be interesting to determine if the mouse protein, like Sok1, resides in the nucleus. Localization of the mouse protein to the nucleus would be consistent with the two proteins sharing some function, such as the regulation of transcription. Finally, Sok1 appears to be devoid of any potential A kinase phosphorylation sites. This would seem to conform well to the notion that Sok1 activates an A kinase-regulated process by a mechanism that is independent of A kinase function. Since A kinase plays a critical role in complex developmental pathways of other organisms (10, 23, 29), it is reasonable to imagine that A kinase-regulated circuits in *S. cerevisiae* might be equally complex.

In contrast with the broad pattern of suppression exhibited by overexpression of *SOK1*, the *SOK2* high-copy-number suppressors were able to alleviate the growth defects caused by only a restricted set of conditional defects in the A kinase pathway. While *SOK2* reversed the temperature-sensitive defect caused by mutations in the A kinase catalytic subunit gene *TPK2*, several conditional lesions in upstream elements of the Ras/A kinase pathway [*ras2*(Ts) and *cdc25*(Ts)] were unaffected. Although the number of alleles tested is too few to make a strong conclusion, it is tempting to speculate that this result may point to the mechanism by which *SOK2* overexpression alleviates the conditional growth of the *tpk2*(Ts) strains. One can imagine, for example, that overproduction of Sok2 might alleviate a conformational defect of the free, but altered, *tpk2*(Ts) product but have a negligible effect on the activity of a wild-type A kinase catalytic subunit sequestered by Bcy1 [as would be the case in a *ras2*(Ts) or *cdc25*(Ts) background]. Since preliminary sequence and genetic analyses have determined that *SOK1* and *SOK2* do not have overlapping functions, it will be interesting to determine the role of *SOK2* in A kinase-dependent growth control and division.

Finally, it seems likely that the temperature-sensitive A kinase mutants isolated in this study will continue to contribute to our understanding of the growth and cell cycle control processes regulated by the yeast A kinase. The two high-copy-number suppressors described here may reflect only a subset of the genes that can be altered to alleviate the A kinase defect. For example, we recently have shown that at least one dominant suppressor of the *tpk2-63*(Ts) allele is unlinked to *SOK1* and may define yet another function of the A kinase pathway (33a). In addition, analyses of the physiological and morphological properties of conditional mutants with defects in each step of the pathway [i.e., *cdc25*(Ts), *ras1 ras2*(Ts), *cyr1*(Ts), and now *tpk1 tpk2*(Ts) *tpk3* mutants] are certain to contribute to our understanding of A kinase regulation (1, 25) and may help define the A kinase-independent function of the yeast Ras protein (1, 22, 33, 34).

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