MOLECULAR AND CELLULAR BIOLOGY, Mar. 1995, p. 1479–1488 Vol. 15, No. 3
0270-7306/95/$04.00+0

The *sak1*$^+$ Gene of *Schizosaccharomyces pombe* Encodes an RFX Family DNA-Binding Protein That Positively Regulates Cyclic AMP-Dependent Protein Kinase-Mediated Exit from the Mitotic Cell Cycle

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Received 6 September 1994/Returned for modification 5 October 1994/Accepted 12 December 1994

In *Schizosaccharomyces pombe*, meiosis is initiated by conditions of nutrient deprivation. Mutations in genes encoding elements of the cyclic AMP-dependent protein kinase (cAPK) pathway interfere with meiosis. Loss-of-function alleles of genes that stimulate the activity of cAPK allow cells to bypass the normal requirement of starvation for conjugation and meiosis. Alternatively, loss-of-function alleles of genes that inhibit cAPK lead to the inability to undergo sexual differentiation. The *cgs1*$^+$ gene encodes the regulatory subunit of cAPK, and the *cgs2*$^+$ gene encodes a cyclic AMP phosphodiesterase. Thus, both genes encode proteins which negatively regulate the activity of cAPK. Loss of either *cgs1* or *cgs2* prevents haploid cells from conjugating and diploid cells from undergoing meiosis. In addition to these defects, cells are unable to enter stationary phase. We describe a novel gene, *sak1*$^+$, which when present on a plasmid overcomes the aberrant phenotypes associated with unregulated cAPK activity. Genetic analysis of *sak1*$^+$ (suppressor of A-kinase) reveals that it functions downstream of cyclic AMP-dependent protein kinase to allow cells to exit the mitotic cycle and enter either stationary phase or the pathway leading to sexual differentiation. The *sak1*$^+$ gene is essential for cell viability, and a null allele causes multiple defects in cell morphology and nuclear division. Thus, *sak1*$^+$ is an important regulatory element in the life cycle of *S. pombe*. Sequence analysis shows that the predicted product of the *sak1*$^+$ gene is an 87-kDa protein which shares homology to the RFX family of DNA-binding proteins identified in humans and mice. One member of this family, RFX1, is a transcription factor for a variety of viral and cellular genes.

A universal response of all organisms to environmental signals is to alter both the specificity and the level of gene expression. Frequently, extracellular signals act through second messengers to regulate the activity of a protein kinase cascade which then modulates transcription factor activity by phosphorylation. In many eucaryotic cells, cyclic AMP (cAMP) serves as a second messenger to activate cAMP-dependent protein kinase (cAPK). It accomplishes this by binding the regulatory subunit of cAPK to cause its dissociation from the catalytic subunit. The free and active catalytic subunit is then able to phosphorylate target proteins, some of which function to regulate transcription of cAMP-inducible or -repressible genes (5). In mammalian cells, phosphorylation of a transcription factor, CREB, causes its activation, thus allowing expression of unlinked genes (13, 14, 18).

Many elements of the cAPK pathway have been identified in *Schizosaccharomyces pombe*. cAMP is generated from ATP by the enzyme adenylate cyclase (*cyr1*$^+$ [41, 42]). A cAMP phosphodiesterase, which converts cAMP to AMP, is encoded by the *cgs2*$^+$ (also known as *pde1*$^+$) gene (9, 27). The cAPK holoenzyme consists of two catalytic subunits, encoded by the *pka1*$^+$ gene (24), and two regulatory subunits, encoded by the *cgs1*$^+$ gene (9). In *S. pombe*, the role of the cAMP signalling pathway is pleiotropic. It functions to inhibit exit from the mitotic cell cycle into either the G_0 stationary phase or the sexual differentiation pathway (9, 27). In addition to regulating exit from the mitotic cycle, cAMP levels regulate expression of the *S. pombe fbp1*$^+$ gene (20). The *fbp1*$^+$ gene, which encodes fructose-1,6-bisphosphatase, is transcriptionally repressed by glucose (19, 38). Exposure of cells to glucose results in a transient increase in intracellular cAMP levels (7), thus activating cAPK and preventing expression of *fbp1*$^+$.

During sexual differentiation, haploid cells of opposite mating types that are arrested in G_1 conjugate to form a diploid zygote (11). The diploid zygote formed will then undergo meiosis, ultimately producing an ascus containing four spores. Both conjugation and sporulation are regulated by nutritional limitation, particularly of nitrogen (10). Much evidence suggesting that the function of cAMP during sexual differentiation is to regulate expression of genes required for conjugation and meiosis has been obtained (9, 39). Nitrogen starvation of fission yeast cells results in a transient decrease in the intracellular concentration of cAMP, presumably inactivating cAPK (27). Consistent with this, conditions that activate cAPK, such as inclusion of high levels of cAMP in the growth medium or mutational inactivation of the regulatory subunit of cAPK, cause cells to be sterile and meiotically defective (4, 9). Under these conditions, expression of the *ste11*$^+$ gene is undetectable. The product of the *ste11*$^+$ gene is a DNA-binding protein essential for expression of other genes required for sexual differentiation (37). In particular, it is needed for expression of the *mei2*$^+$ gene, which is absolutely indispensable for meiosis (4, 6, 36, 37). No conditions that allow meiosis in the absence of the *mei2*$^+$ gene have been found.

Meiosis in *S. pombe* thus appears to be regulated by the level of cAMP and ultimately by the activity of cAPK. We have therefore focused on the role of cAMP in sexual differentiation of *S. pombe* as a means of identifying other important elements

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TABLE 1. Yeast strains used in this study

Strain	Genotype
SP66	*h*^{90} *leu1-32 ade6-M210*
SP491	*h*^{90} *leu1-32 cgs1-1 ade6-M210*
SP578	*h*^{90} *cgs2-1 leu1-32 ade6-M210*
SP659	*h*$^{-}$ *leu1-32 ura4-D18 ade6-M210*/*h*$^{-}$ *leu1-32 ura4-D18 ade6-M216*
SP826	*h*$^{+}$ *leu1-32 ura4-D18 ade6-M210*/*h*$^{+}$ *leu1-32 ura4-D18 ade6-M216*
SP871	*h*^{90} *leu1-32 ura4-D18 ade6-M210 ran1*$^{+}$::pRAN1.49
SP925	*h*^{90} *leu1 ste5*[a]
SP972	*h*$^{-S}$
SPB68	*h*^{90} *leu1-32 cgs1-1 ade6-M210 mei2*::*lacZ*
SPB88	*h*^{90} *leu1-32 cgs1-1 ade6-M210 sak1*$^{+}$ pSAK1.2
SPB89	*h*$^{+}$ *leu1-32 ura4-D18 ade6-M210*/*h*$^{-}$ *leu1-32 ura4-D18 ade6-M216 sak1*::*ura4*$^{+}$
SPB92	*h*^{90} *leu1-32 cgs1-1 ade6-M216 ura4*::*fbp-lacZ*
SPB110	*h*^{90} *leu1-32 ura4-D18 ade6-M210 ras*$^{Val-12}$

[a] *ste5* is *ras1*.

of the pathway in order to determine its role in a complex signal transduction cascade.

MATERIALS AND METHODS

Strains and growth media. All strains of *S. pombe* used in this study were derived from the original *h*^{90}, *h*$^{-S}$, and *h*$^{+N}$ isolates introduced by U. Leupold and are listed in Table 1. The *ura4*::*fbp1-lacZ* allele is a disruption of the *ura4* gene by a *fbp1-lacZ* translational fusion (19). The *mei2-lacZ* allele was constructed with plasmid pMEI2.22, which contains an in-frame translational fusion of the *mei2*$^{+}$ gene and the *lacZ* gene. For this construction, a *Bam*HI restriction site was created in the *mei2*$^{+}$ gene such that the first eight amino acids of *mei2*$^{+}$ were fused in frame to the product of the *lacZ* gene. A DNA fragment containing the fusion gene was transformed into SP66 along with a replicating plasmid containing the *LEU2* gene. This was necessary because the *mei2-lacZ* fusion fragment contains no selectable marker. Cotransformants obtained were screened to identify those that also exhibited the Mei2^{-} phenotype: that is, they were able to conjugate and unable to sporulate.

Yeast cells were cultured either in rich medium (YEA; 0.5% yeast extract, 3% glucose, 0.075 mg of adenine per ml) or minimal medium (MM [1]) buffered with 50 mM sodium phthalate (pH 5.6). In the nutritional shift experiments whose results are reported, cells were either shifted from MM to MM minus NH_4Cl or from MM to MM minus glucose plus 8% glycerol. Standard yeast methods were used in all experiments (1).

Measurement of survival after entry into stationary phase. The indicated strains were grown in the appropriate medium at 32°C to a density of 10^7/ml. The cultures were maintained at this temperature, and at daily intervals, a portion of each was removed, diluted, and plated onto YEA plates for cultivation at 32°C. The colonies formed were counted manually after 3 days.

Cytology. Photomicrographs of live cells were obtained with a Nikon Optiphot-2. For visualization of nuclei, cells of the indicated strains were stained with 4,6′-diamidino-2-phenylindole (DAPI; Sigma) as previously described (1).

Cloning of *sak1*$^{+}$. *S. pombe* SP491 (*h*^{90} *leu1-32 cgs1-1 ade6-M210*) was transformed by protoplast fusion (3) with gene banks generously constructed and provided by Paul Young (Queen's University, Kingston, Ontario, Canada). The fission yeast genomic library was constructed in the vector pWH5 (40) with chromosomal DNA partially digested with *Sau*3A. Transformants were selected on MM plates containing sorbitol and incubated at 32°C for 3 days. The plates were inverted over iodine crystals to identify colonies that had acquired the ability to sporulate. Plasmids were recovered from *S. pombe* transformants into *Escherichia coli* TG1. Each plasmid was retested after this procedure to determine that it contained sequences capable of complementing the *cgs* Spo^{-} phenotype. Two plasmids, each containing nonoverlapping fragments, were able to complement *cgs1-1*: pCGS1.1 and pSAK1.1. One of these, pSAK1.1, contained sequences unlinked to *cgs1-1* when integrated by homologous recombination into the chromosome. Only pCGS1.1 was linked to *cgs1-1*, and sequence analysis of this plasmid has been described (9).

Plasmids. Deletion and frameshift derivatives of pSAK1.1 were created in pIRT2 (1). pSAK1.12 was derived from pSAK1.4 by digestion with *Nhe*I and *Sph*I and creating flush ends with Klenow polymerase prior to ligation. pSAK1.13 was derived from pSAK1.4 by digestion and filling in the unique *Bgl*II site in *sak1*$^{+}$. pCGS1.1 contains sequences encoding the regulatory subunit of cAPK in the vector pWH5 (9).

Nucleotide sequence determination. The DNA sequence of *sak1*$^{+}$ was determined by the dideoxynucleotide chain termination method (35). Single-stranded DNA template was prepared from pUC118 and pUC119 plasmids, each carrying the *sak1*$^{+}$ gene as a 3.4-kb DNA fragment flanked by *Pst*I and *Xba*I restriction sites. The use of both pUC118 and pUC119 as vectors enabled us to determine the sequences of both DNA strands of the *sak1*$^{+}$ gene. The plasmids were obtained in single-strand form following superinfection of plasmid containing *E. coli* with phage M13KO7 (34). To obtain a series of overlapping clones for sequencing, unidirectional deletions were constructed by the method of Henikoff (17). The predicted protein sequence was analyzed by using the BLASTP program (2) and the PIR (Protein Identification Resource) data bank maintained by the National Biomedical Research Foundation, Washington, D.C.

Construction of a null allele. The 0.2-kb *Bam*HI-to-*Bgl*II fragment contained within the open reading frame (ORF) of *sak1*$^{+}$ was removed and replaced with a 1.8-kb DNA fragment flanked by *Bam*HI restriction sites and containing the *ura4*$^{+}$ gene. A linear DNA fragment containing the disrupted *sak1*$^{+}$ gene was obtained by PCR. PCR was performed for 30 cycles at 94°C (1 min), 50°C (1 min), and 72°C (3 min) with 100 pM concentrations of oligonucleotide L and oligonucleotide R and 10 ng of DNA. The 2.4-kb PCR fragment obtained was purified from a low-melting-temperature agarose gel and used for transformation of strain SP826. DNA was prepared from stable Ura^{+} transformants and digested with *Hin*dIII for Southern analysis. The probe was prepared with a 1.1-kb *Hin*dIII DNA fragment from *sak1*$^{+}$ and the randomly primed DNA labeling kit (Boehringer Mannheim).

Measurement of β-galactosidase activity. A permeabilized cell assay was used to measure β-galactosidase activity (16). A total of 10^7 cells were harvested and used in each reaction mixture.

Oligonucleotides used in this study. All oligonucleotides were synthesized by the phosphoramidite method with an Applied Biosystems 380A synthesizer. The sequence of each is as follows: oligonucleotide L, 5′-TACTGACAAATCGTTC CCAGTCG-3′; oligonucleotide R, 5′-TGCTCCACAAGCCCATCCCCTACC -3′; oligonucleotide 100, 5′-CCCTCCATAACCTCCGAATC-3′; and oligonucleotide 102, 5′-GAACCTAGCAGCAGCATGAG-3′.

RESULTS

Isolation and characterization of *sak1*$^{+}$. The gene encoding the regulatory subunit of cAPK is defined by the mutant allele *cgs1-1*. All available biochemical evidence indicates that cells containing this allele have cAPK activity that is unregulated by cAMP. Cells containing the *cgs1-1* allele are highly elongated, unable to enter stationary phase, sterile, and meiotically defective (9) (Fig. 1). To identify novel genes that could rescue the meiotic defect caused by loss of *cgs1*, we introduced an *S. pombe* library, contained on a multicopy vector, pWH5 (41), into a *leu*$^{-}$ *cgs1*$^{-}$ strain (SP491). Approximately 10,000 transformants were screened with iodine vapors to identify colonies containing cells that had acquired the ability to conjugate and sporulate (Fig. 1). Two transformants were identified in this manner, and each displayed cosegregation of the plasmid-borne *leu*$^{+}$ marker and the ability to conjugate and sporulate. Plasmids from each candidate were rescued into *E. coli*, and the two plasmids recovered were shown by restriction mapping to contain unique genomic DNA fragments (data not shown). One plasmid contains the gene encoding the regulatory subunit of cAPK, as previously reported (9). The second plasmid contains sequences unlinked to the *cgs1*$^{+}$ locus and was designated pSAK1.1 (suppressor of A-kinase).

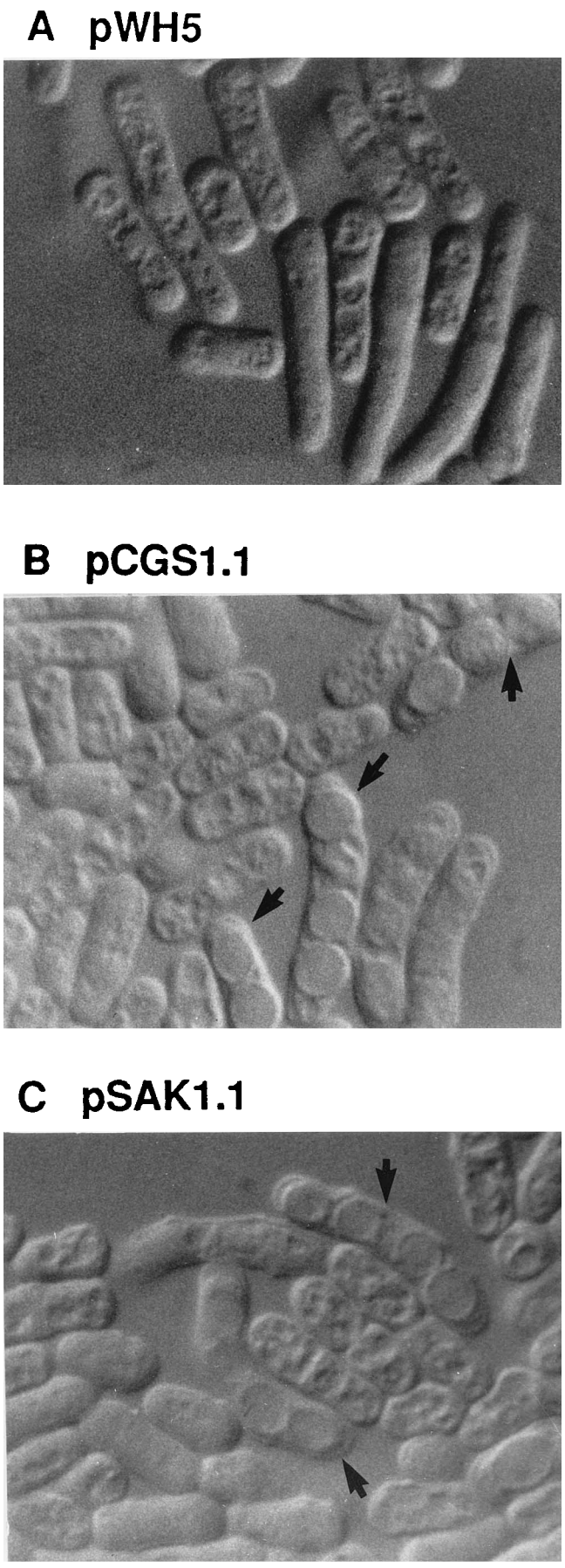

FIG. 1. Complementation of a *cgs1-1* mutant strain by the *sak1*$^+$ gene. Shown are photomicrographs of *cgs*$^-$ cells (SP491) grown on minimal medium at 30°C for 3 days and containing an empty vector (pWH5) (A), a plasmid carrying the *cgs1*$^+$ gene (pCGS1.1) (B), or a plasmid carrying the *sak1*$^+$ gene (pSAK1.1) (C). Arrows indicate cells which had undergone conjugation and meiosis to form spores.

Plasmid pSAK1.1 contains a 7,650-bp genomic DNA fragment. Various fragments of yeast DNA from this plasmid were cloned into fission yeast replicating vectors, and the ability of each to rescue the meiotic defect caused by loss of *cgs1-1* was tested (Fig. 2). The activity was localized to a 3,400-bp fragment flanked by *Xba*I and *Pst*I restriction sites (pSAK1.4 [Fig. 2]). In the course of further subcloning, it was found that all DNA fragments which lacked a unique *Bgl*II site located within the 3,400-bp fragment resulted in the loss of Spo$^+$ activity (Fig. 2).

***sak1*$^+$ encodes a novel protein.** The nucleotide sequence of the *Xba*I-to-*Pst*I fragment was determined (Fig. 3). Translation of the sequence in all three reading frames from both DNA strands revealed one large ORF capable of encoding a polypeptide of 734 amino acids (Fig. 3, nucleotides 358 to 2559). An in-frame methionine is present at position 358, and an in-frame termination codon is present 57 bases upstream, indicating that the methionine at position 358 is the true initiation codon. This ORF includes the unique *Bgl*II site required for activity (pSAK1.13 [Fig. 2]). A single-stranded probe complementary to the strand encoding the large ORF and encompassing nucleotides 1 through 1940 was used in Northern (RNA) analysis of RNA from vegetatively growing cells, and a 3,500-base transcript was detected (data not shown). On the other hand, a single-stranded probe corresponding to the strand encoding the large ORF detected no transcript from this region (Fig. 2, nucleotides 490 through 3394). Taken together, the above results show that the ORF extending from nucleotides 358 to 2559 encodes the *sak1*$^+$ gene.

A computer search revealed that the ORF is predicted to encode a novel protein; in addition, the search revealed significant homology between the predicted *sak1*$^+$ protein and

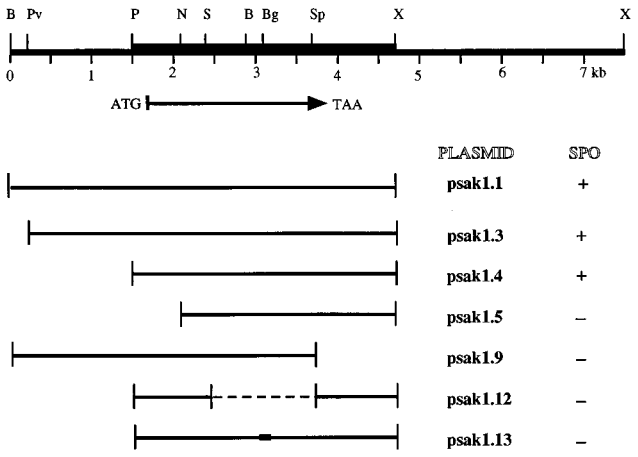

FIG. 2. Partial restriction map and subcloning of a 7.6-kb genomic fragment contained on plasmid pSAK1.1. The heavy line indicates the 3,413-bp region which was sequenced. An ORF of 734 amino acids is marked. The end points of deletions at the 5′ and 3′ ends of *sak1*$^+$ are shown. Each construction was scored as active (Spo$^+$) or inactive (Spo$^-$) according to the criteria described in the text. Plasmid pSAK1.12 contains a deletion, indicated by a broken line. pSAK1.13 contains a frameshift mutation, indicated by a block. The restriction sites are as follows: B, *Bam*HI; Pv, *Pvu*II; P, *Pst*I; N, *Nhe*I; Bg, *Bgl*II; Sp, *Sph*I; and X, *Xba*I.

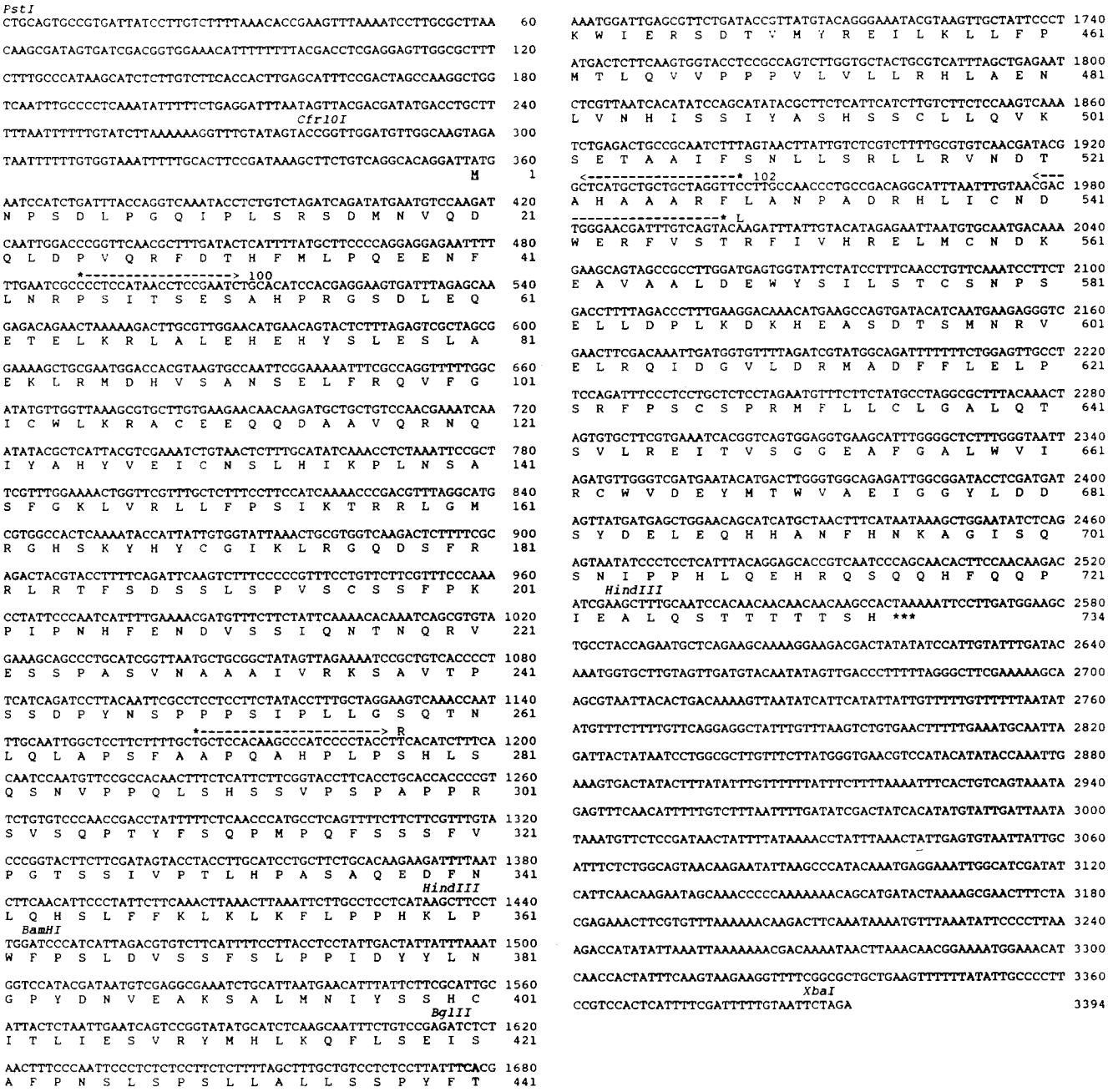

FIG. 3. Nucleotide sequence of the 3,413-bp *Pst*I-*Xba*I restriction fragment containing the *sak1*$^+$ gene. The predicted translational product of 734 amino acids is indicated. The initiating ATG is underlined. The unique *Bgl*II site is also indicated. Oligonucleotides used in this study are indicated by a broken arrow above the DNA sequence.

members of the RFX family of DNA-binding proteins identified in humans and mice. All proteins of this family contain both general features and specific signature regions that are highly conserved in all members (32). One area of homology between the *sak1*$^+$ protein and RFX1 (or any of the RFX proteins) is contained within the DNA-binding domain of RFX1 (31) and extends for 80 amino acids. Within this region, 68% of the amino acids are either identical or conserved between the two proteins (Fig. 4). The second region of homology is in a domain required for dimerization of RFX1 (31), and within this 114-amino-acid domain, 46% of the residues are either identical or conserved. The third region of homology is partially contained within the dimerization domain of RFX1, and within this region, the *sak1*$^+$ product and RFX1 contain 67% conserved or identical amino acids. The above results indicate that a class of proteins containing a unique DNA-binding motif exists and that this motif is conserved from yeasts to humans. We anticipate, on the basis of the computer search results, that *sak1*$^+$ encodes a DNA-binding protein.

***sak1*$^+$ is essential for viability.** We constructed a null allele of *sak1* by gene replacement to determine its role in *S. pombe*. Diploid fission yeast cells that are sporulation competent are

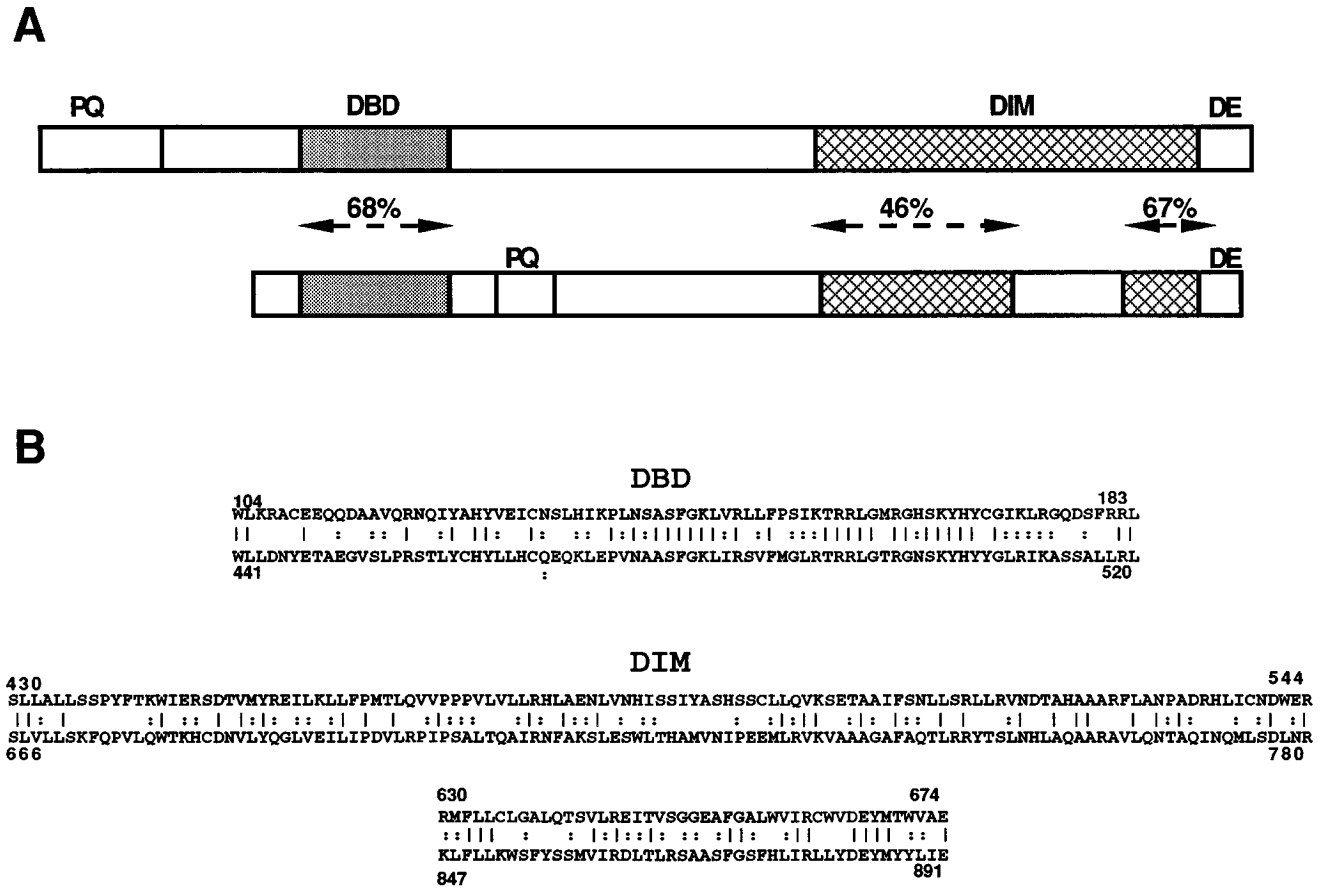

FIG. 4. The *sak1*$^+$ gene contains regions homologous to the RFX family of DNA-binding proteins. (A) Conserved regions between the RFX1 gene (top line) and *sak1*$^+$ (bottom line). Regions rich in a subset of amino acids are designated with the single-letter code (PQ and DE). DBD, DNA-binding domain; DIM, region required for dimerization. Arrows indicate positions, and numbers give the degrees of homology between RFX1 and *sak1*$^+$. (B) Amino acid sequence comparisons of the three homologous regions between *sak1*$^+$ (top sequence) and RFX1 (bottom sequence) proteins are shown. Identical amino acids are indicated by a line, and conserved amino acids are shown by a colon. The conserved amino acids are S, T, P, A, and G; N, D, E, and Q; H, R, and K; M, I, L, and V; and F, Y, and W.

difficult to maintain because of a tendency to sporulate when nutrients become limiting. Thus, the strategy used was to obtain a *sak1* disrupted allele in a diploid strain unable to sporulate because it is homozygous at the mating type locus. A homozygous *h*$^+$ *ura4-D18/h*$^+$ *ura4-D18* diploid strain (SP826) was transformed with a DNA fragment in which the *sak1*$^+$ coding region was removed and replaced by the *S. pombe ura4*$^+$ gene (15, 33) (see Materials and Methods). Stable Ura$^+$ yeast transformants were analyzed by Southern hybridization and shown to be heterozygous for the *sak1* gene disruption (data not shown). One of the diploid disruptants was mated to a homozygous Ura$^-$ diploid of the opposite mating type (SP659) and allowed to undergo meiosis. Diploid cells heterozygous at the mating type locus (and thus sporulation competent) that were also Ura$^+$ (and therefore contained one disrupted *sak1* allele) were identified. One of these (SPB89) was allowed to undergo meiosis, and four-spored asci were dissected. Each of the 15 complete tetrads examined produced only two viable spores, both of which were Ura$^-$ (data not shown). We infer that the two inviable segregants carry a replacement of *sak1* with *ura4*$^+$ and conclude that *sak1*$^+$ is essential for viability.

In order to characterize the nature of the phenotype caused by complete loss of *sak1*$^+$, germination of spores from the heterozygous diploid (SPB89) was monitored in liquid culture. Purified spores were inoculated into complete growth medium. After 12 h, when the cell number had doubled, cells were stained with DAPI to visualize DNA. Microscopic examination showed that the culture contained approximately 30% abnormal cells. The most predominant phenotypes were highly elongated cells, abnormal mitotic structures, and the presence of atypically placed fission plates (Fig. 5A and B). Later in the experiment, cell lysis became apparent.

To examine the phenotype of cells carrying a null allele of *sak1* in greater detail, a diploid cell line containing one disrupted *sak1* allele (SPB89) was transformed with a replicating plasmid carrying the *sak1*$^+$ gene (pSAK1.1 [Fig. 2]). After the cells had undergone meiosis, spores were obtained and allowed to germinate on selective medium such that haploid segregants carrying both the null allele of *sak1* and the autonomous pSAK1.1 plasmid were selected. In this experiment, cells containing the null allele of *sak1* are viable because the plasmid contains a functional copy of *sak1*$^+$. To observe the phenotype conferred by loss of *sak1*, haploid cells carrying the plasmid were allowed to grow in medium nonselective for the plasmid. A portion of the culture was removed at several time intervals, stained with DAPI to visualize the nucleus, and examined by microscopy. After 6 h of growth in nonselective medium, approximately 10% of the cells exhibited an aberrant morphology. These were highly elongated and irregularly shaped (Fig.

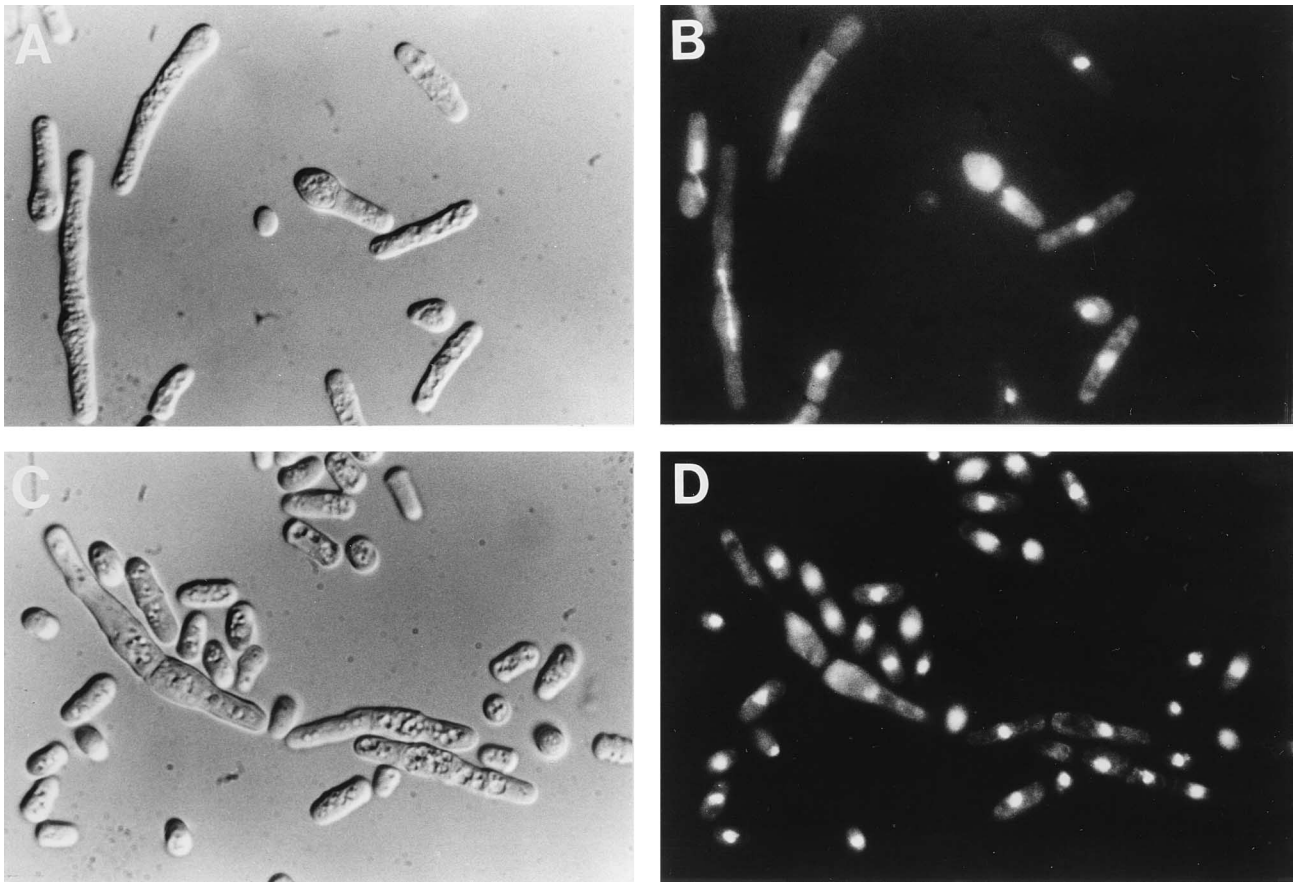

FIG. 5. Phenotype of cells containing *sak1*::*ura4*$^+$. Spores purified from a heterozygous diploid containing one *sak1*::*ura4*$^+$ allele were allowed to germinate in complete medium. A portion of the culture was stained with DAPI and photographed to visualize cells (A) or nuclei (B). Haploid cells containing both the *sak1*::*ura4*$^+$ allele and a plasmid encoding a functional *sak1*$^+$ gene were grown in nonselective medium to allow loss of the plasmid. Following 6 h of growth, a portion of the culture was stained with DAPI and photographed with DIC optics to visualize cells (C) or nuclei (D).

5C and D). Multiple fission plates which were frequently spaced at irregular intervals were often visible. Nuclei were observed within many, but not all, compartments separated by septa. At later times in the experiment, cell lysis became apparent. Taken together, the results of this and the previous experiment indicate that loss of *sak1* causes multiple defects in mitosis and regulation of cell size.

***sak1*$^+$ rescues *cgs2*.** To determine the extent of the suppression of defects in sexual differentiation by *sak1*$^+$, we investigated the effect of introducing a plasmid containing *sak1* (pSAK1.1) into a variety of strains containing mutations known to result in the inability of cells to undergo sexual differentiation.

In *S. pombe*, *ras*$^+$ is required for sexual differentiation. Unlike the function of *ras1*$^+$ in *Saccharomyces cerevisiae*, fission yeast *ras1*$^+$ does not appear to regulate the cAPK pathway (12). We wished to examine the genetic relationship between *sak1*$^+$ and *ras1*$^+$. Cells containing either a loss-of-function allele of *ras1* (SP925) or an activated allele of *ras1*, *ras*$^{Val\text{-}12}$ (SPB110), are sterile and meiotically defective (12, 28). The presence of pSAK1.1 in either strain does not suppress the inability of either to conjugate (Table 2). We conclude from this experiment that *sak1*$^+$ functions upstream of *ras1*$^+$ if it functions on this pathway at all.

Conjugation and meiosis in *S. pombe* are also regulated by *ran1*$^+$ (also known as *pat1*$^+$) protein kinase (21, 22, 25, 26, 29). During vegetative growth, *ran1*$^+$ is active and functions to prevent conjugation and meiosis (27). Nutrient deprivation results in inhibition of *ran1*$^+$. Partial inhibition of *ran1*$^+$ causes cells to conjugate, and complete inhibition of *ran1*$^+$ kinase leads to meiosis (4). Cells from a strain containing high levels of p52^{ran1} kinase (SP871) are sterile and meiotically defective (26). Thus, at least superficially, unregulated p52^{ran1} kinase causes the same defect as unregulated cAPK activity: cells are profoundly defective in both conjugation and meiosis. To determine if *sak1*$^+$ could suppress the conjugation and meiotic defect of strains containing high levels of p52^{ran1} kinase activity, pSAK1.1 was transformed into a strain expressing *ran1*$^+$ under the control of the strong, constitutive *ADH* promoter (SP871 [26]). pSAK1.1 weakly suppressed the conjugation defect (approximately 5% of the cells acquired the ability to conjugate), but no cells were able to sporulate (Table 2). Weak suppression of the conjugation defect observed in this strain is in sharp contrast to the strong suppression caused by expression of *sak1*$^+$ in *cgs1-1* strains. In *cgs1-1* strains, it is routinely observed that expression of *sak1*$^+$ causes greater than 90% of the cells to conjugate and sporulate (Table 2).

Only in the case of *cgs2-1* was suppression of all defects in sexual differentiation by *sak1*$^+$ observed. We have previously shown that *cgs2*$^+$ encodes a cAMP phosphodiesterase. Biochemical experiments demonstrate that *cgs2-1*-containing strains have elevated levels of cAMP compared with that in wild-type strains (9). The phenotype caused by loss of *cgs2* is indistinguishable from that caused by loss of *cgs1*: cells are

TABLE 2. Complementation of sterile mutants by *sak1*$^{+a}$

Strain	Relevant genotype	Phenotype	
		pIRT2	pSAK1-1
SP491	*cgs1-1*	C$^-$	C$^+$ S$^+$
SP578	*cgs2-1*	C$^-$	C$^+$ S$^+$
SP871	*ran1*$^+$ *O.P.*[b]	C$^-$	C^{+c} S$^-$
SP925	*ras*$^-$	C$^-$	C$^-$
SPB110	*ras*$^{Val-12}$	C$^-$	C$^-$

[a] Cells of each strain were transformed with either the control plasmid, pIRT2, or a plasmid containing *sak1*$^+$ (pSAK1-1). Transformants were grown for 3 days at 30°C on minimal medium. Conjugation (C) and sporulation (S) were scored by resuspending cells from a colony in H_2O and examining the cells in suspension, with a microscope. Conjugation produces a fused cell having a characteristic horseshoe shape, which normally immediately proceeds through meiosis to form a horseshoe-shaped ascus containing spores. Thus, the ability to conjugate was inferred from the shape of asci. At least 200 cells were counted from each sample. $^-$, fewer than 0.5% of cells formed zygotes or spores; $^+$, greater than 75% of the cells conjugated and underwent sporulation.

[b] Cells producing p52^{ran1} under the control of the *ADH* promoter are referred to as *ran1*$^+$ *O.P.* (26).

[c] A total of 5% of the cells conjugated, producing a horseshoe-shaped zygote, although no spore-forming cells were observed.

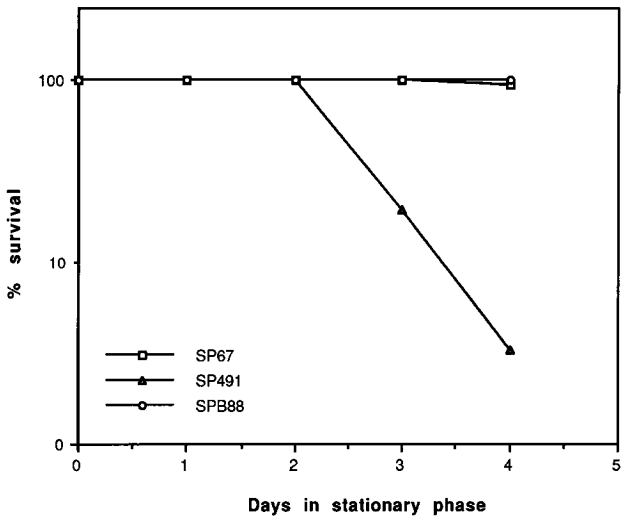

FIG. 6. Measurement of cell viability after entry into stationary phase. SP67 is a wild-type strain containing plasmid pIRT2. SP491 contains the *cgs1-1* allele and plasmid pIRT2. SPB88 contains the *cgs1-1* allele and plasmid pSAK1.1, which contains the *sak1*$^+$ gene. Cells maintained at G_0 for the indicated times were plated on complete medium to determine the number of viable cells.

profoundly sterile and unable to undergo meiosis. Transformation of cells containing a *cgs2-1* allele (SP519) with *sak1*$^+$ (pSAK1.1) allowed 90% of the cells to conjugate, and these subsequently underwent meiosis and formed four spores (Table 2). Therefore, suppression of defects in sexual differentiation by *sak1*$^+$ is not unique to strains containing a mutation in the regulatory subunit of cAMP-dependent protein kinase but is a property of episomally expressed *sak1*$^+$ in all cells containing mutations causing unregulated cAPK activity. Moreover, expression of *sak1*$^+$ does not induce sexual differentiation in a broad range of mutants that appear to be distinct from the cAPK pathway. Taken together, these results indicate that *sak1*$^+$ functions as a component of the cAPK pathway to induce meiosis. If this is true, then *sak1*$^+$ most likely exerts its effect downstream of the regulatory subunit of cAPK, although the above experiment does not rule out the possibility that *sak1*$^+$ acts upstream of cAPK to regulate its activity. Further experiments addressing this point are described below.

***sak1*$^+$ allows cells containing unregulated cAPK activity to enter G_0.** The close interaction between *sak1*$^+$ and the cAPK pathway prompted us to determine if the presence of a plasmid containing *sak1*$^+$ could suppress other defects known to be associated with unregulated cAPK activity, namely, the ability to enter stationary phase and the ability to mediate the effects of glucose repression.

When nutrients in the medium become limiting, wild-type fission yeast cells arrest in stationary phase as small cells, enter G_0, and are characterized by long-term survival (8). In contrast, *cgs*$^-$ cells remain elongated and are unable to survive periods of time in stationary phase (9). Survival in stationary phase was used as a criterion to compare the abilities of wild-type cells (SP67), cells containing *cgs1-1* (SP491), and cells containing *cgs1-1* plus multiple copies of *sak1*$^+$ (SPB88) to enter G_0. Cells were cultured to stationary phase, and at daily intervals thereafter, the viability of cells from each culture was determined by plating a portion of each onto fresh medium. The long-term viability of cells containing *cgs1-1* was less than 10% after 3 days in stationary phase, consistent with previous observations (9). In contrast, both wild-type cells and cells containing *cgs1-1* plus pSAK1.1 exhibited nearly 100% viability after 3 days in stationary phase (Fig. 6). Thus, *sak1*$^+$ suppresses not only the conjugation and meiotic defects observed in strains containing unregulated cAPK but also the observed inability of these cells to enter stationary phase.

***sak1* causes induction of *mei2*$^+$.** Meiosis in *S. pombe* requires expression of the *mei2*$^+$ gene, which is known to be transcriptionally induced by nutritional limitation (39). High levels of cAMP in the medium or unregulated expression of cAPK caused by loss of the regulatory subunit of cAPK in a *cgs1-1* mutant abolishes expression of *mei2*$^+$ (9, 39). Furthermore, failure to express *mei2*$^+$ is the sole reason that cells containing unregulated cAPK are unable to enter meiosis (9). We wished to determine if *sak1*$^+$ induced sexual differentiation in *cgs*$^-$ strains because it allowed induction of the *mei2*$^+$ gene or if meiosis caused by *sak1*$^+$ allowed cells to bypass the requirement for *mei2*$^+$. For this experiment, we constructed a *mei2-lacZ* translational fusion to replace the genomic copy of the *mei2*$^+$ gene (Materials and Methods). The *mei2-lacZ* allele is induced by starvation, as is the authentic *mei2*$^+$ gene. We wished to determine if a plasmid containing *sak1*$^+$ could allow transcription of *mei2-lacZ* in a strain containing *cgs1-1*. This strain (SPB68) was transformed with one of three plasmids: a control plasmid (pWH5), a plasmid containing the authentic *cgs1*$^+$ gene (pCGS1.1), or a plasmid containing *sak1*$^+$ (pSAK1.1). Cells were grown in complete medium containing a nitrogen source and then shifted to medium lacking nitrogen. Following the nutritional shift, a portion of each culture was removed and β-galactosidase activity was measured. Expression of the *mei2-lacZ* fusion protein was barely detectable in cells carrying the control plasmid. In contrast, cells containing either a plasmid carrying *sak1*$^+$ or a plasmid carrying *cgs1*$^+$ allowed expression of the *lacZ* fusion gene (Fig. 7). Thus, *sak1*$^+$ relieves the inability to allow transcription of the *mei2*$^+$ gene observed in a *cgs1-1* mutant. However, meiosis caused by expression of *sak1*$^+$ in *cgs1-1* cells does not bypass the requirement for *mei2*$^+$. No spore-forming cells were ever observed in cells containing the *mei2-lacZ* fusion allele in either the presence or the absence of episomally expressed *sak1*$^+$ (data not shown).

Since expression of the *sak1*$^+$ gene overrides the inability to allow transcription of *mei2*$^+$ caused by *cgs1-1*, we investigated

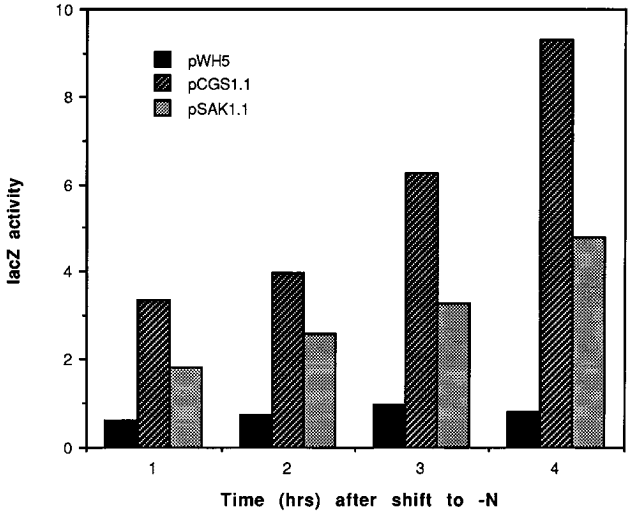

FIG. 7. Activation of the *mei2* reporter gene by *sak1*$^+$. Strain SPB68, which contains an in-frame translational fusion of *mei2*$^+$ and the *lacZ* gene, was transformed with a control plasmid (pWH5), a plasmid containing the regulatory subunit of cAPK (pCGS1.1), or a plasmid containing the *sak1*$^+$ gene (pSAK1.1). Cells of each culture were grown to a density of 10^7 cells per ml in minimal medium and shifted to a nitrogen-free medium. At the times indicated, 2×10^7 cells were removed, permeabilized with chloroform, and assayed for β-galactosidase activity. Five independent transformants were tested. The standard error was less than 1.0% for each sample.

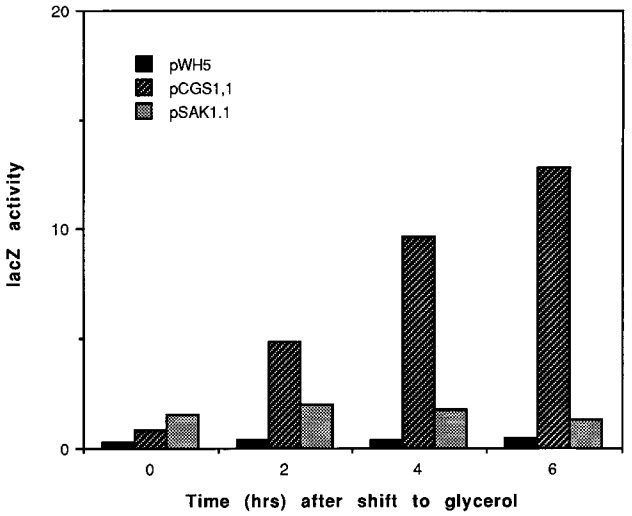

FIG. 8. Measurement of *lacZ* activity in cells shifted to glycerol-containing medium. Strain SPB92, which contains an in-frame translational fusion of *fbp1*$^+$ and the *lacZ* gene, was transformed with a control plasmid (pWH5), a plasmid containing the regulatory subunit of cAPK (pCGS1.1), or a plasmid containing the *sak1*$^+$ gene (pSAK1.1). Cells of each culture were grown to a density of 10^7 cells per ml and shifted to a glucose-free medium containing glycerol. At the indicated times, 2×10^7 cells were removed, permeabilized with chloroform, and assayed for β-galactosidase activity. Six independent transformants were tested, and the standard error was less than 3.0% for each sample.

the role of *sak1*$^+$ in expression of other genes regulated by cAMP but which have no role in sexual differentiation. Transcription of the fission yeast *fbp1*$^+$ gene (encoding fructose-1,6-bisphosphatase) is subject to glucose repression, and mutations causing elevated or unregulated levels of cAPK prevent induction of *fbp1*$^+$ even under derepressing conditions, such as growth on glycerol (19). We wished to determined if a plasmid containing *sak1*$^+$ would allow transcription of *fbp1-lacZ* in a strain (SPB92) containing *cgs1-1*. This strain was transformed with one of three plasmids: a control plasmid (pWH5), a plasmid containing the authentic *cgs1*$^+$ gene (pCGS1.1), or a plasmid containing the *sak1*$^+$ gene (pSAK1.1). Cells were grown in glucose-containing medium and then shifted to a medium lacking glucose but containing glycerol. These are conditions known to allow expression of *fbp1-lacZ* in wild-type cells (19). Following the nutritional shift, a portion of each culture was removed and β-galactosidase activity was measured. Expression of the *fbp1-lacZ* fusion protein was barely detectable in *cgs*$^-$ cells carrying the control plasmid or cells containing the plasmid carrying *sak1*$^+$. In both cases, *lacZ* activity was similar in cells shifted from glucose to glycerol. In contrast, *fbp1-lacZ* expression was derepressed in cells carrying the plasmid containing *cgs1*$^+$ and shifted to glycerol (Fig. 8). The results of this and previous experiments show that, while plasmid-borne *sak1*$^+$ can rescue most defects caused by loss of *cgs1*, it does not function to rescue the inability to allow expression of glucose-derepressible genes caused by loss of *cgs1*.

DISCUSSION

In this paper, we describe the isolation and sequence of a novel gene, *sak1*$^+$, from *S. pombe*. The *sak1*$^+$ gene was identified as a multicopy suppressor of mutations causing unregulated cAPK activity. The *sak1*$^+$ gene encodes a predicted protein of 87 kDa and is essential for cell growth. The *sak1*$^+$ protein shares homology with a known DNA-binding protein from human cells, RFX1, which functions to regulate expression of class II major histocompatibility complex antigens and as a transactivator of hepatitis B virus enhancer I (30, 32). RFX1 is one member of a family of homologous proteins that have been described for humans and mice.

Our results support a model in which *sak1*$^+$ is a component of the biochemical pathway regulated by cAMP. In addition to suppression of all defects in exit from the mitotic cell cycle observed in cells containing a loss-of-function allele of the gene encoding the regulatory subunit of cAPK, expression of *sak1*$^+$ also reverses these defects in strains containing loss-of-function alleles of a cAMP phosphodiesterase. The ability of *sak1*$^+$ to promote entry into the sexual differentiation pathway in cells that are meiotically defective appears to be specific to mutants encoding elements of the cAPK pathway and is not observed in any other meiotic mutant tested.

If, in fact, *sak1*$^+$ is a component of the cAPK pathway, then it most likely exerts its effects downstream of the cAPK catalytic subunit. A direct measurement of cAPK activity in *cgs*$^-$ cells and in *cgs*$^-$ containing *sak1*$^+$ shows that *sak1*$^+$ does not significantly alter cAPK activity (unpublished observation). However, the level of cAPK detected in *cgs*$^-$ cells is considerably lower than that observed in wild-type cells, presumably because of feedback inhibition mechanisms (9). Thus, experiments designed to ascertain the effect of *sak1*$^+$ on cAPK may not detect minor differences in activity. However, the genetic experiments reported here indicate that *sak1*$^+$ functions downstream of cAPK. Ectopic expression of *sak1*$^+$ reverses defects in sexual differentiation caused by mutations that activate cAPK but does not overcome the failure to induce *fbp1*$^+$ expression observed in these mutants. Thus, *sak1*$^+$ functions on the pathway leading to expression of *mei2*$^+$ but not on the pathway leading to expression of *fbp1*$^+$. These results indicate that *sak1*$^+$ does not function upstream of cAPK, because if this were the case, all effects of unregulated cAPK would be suppressed by the presence of *sak1*$^+$, including those of glucose-repressible gene expression. Therefore, *sak1*$^+$ defines a bifurcation in the pathway and must function downstream of cAPK.

The nutritional signals monitored by the cell to regulate the activity of cAPK activity have not been defined. However, induction of *fbp1*$^+$ is effected primarily by a glucose signal, while induction of *mei2*$^+$ is more sensitive to nitrogen concentration. While it may be the case that *sak1*$^+$ controls expression only of nitrogen-responsive genes, such as *mei2*$^+$, and not of glucose-responsive genes, we feel that this is probably not the case. Expression of *sak1*$^+$ also overcomes the inability of *cgs1-1* cells to enter G_0, and glucose availability mediates this response (9). An interesting possibility is that *sak1*$^+$ regulates only those pathways that participate in the exit of cells from the mitotic cell cycle.

Many genes encoding elements of the cAPK pathway in *S. pombe* have been identified and characterized, and loss of no single element is lethal (9, 23, 24). In contrast, the *sak1*$^+$ gene is essential for viability. This result suggests that if the *sak1*$^+$ gene is an element of the cAPK pathway, then it is involved in another pathway as well. The transition from vegetative cell growth to conjugation and meiosis is also mediated by *ran1*$^+$ protein kinase. We have observed that expression of *sak1*$^+$ in cells unable to conjugate because of unregulated *ran1*$^+$ kinase activity reverses this defect to some degree. Thus, it is possible that *sak1*$^+$ also functions on a pathway regulated by *ran1*$^+$ protein kinase.

Sequence similarities between the predicted *sak1*$^+$ translation product and members of the RFX family of DNA-binding proteins suggest that these proteins may be functionally related. In vitro, the characterized member of this family, RFX1, binds a specific DNA sequence and forms homodimers (31). In vivo, RFX1 is required for expression of major histocompatibility complex class II genes (32). All members of the RFX family of DNA-binding proteins from humans and mice contain several highly conserved motifs. The degree of conservation is particularly striking in the DNA-binding domain and the dimerization domain of each. In addition, all the RFX proteins contain distinct regions high in proline and glutamine or aspartic acid and glutamic acid. All these regions are also conserved in the *sak1*$^+$ gene product, leading us to speculate that *sak1*$^+$, like RFX1, might function as a transcription factor. The high degree of conservation between the DNA-binding domain of RFX1 and *sak1*$^+$ may indicate that *sak1*$^+$ binds to the same, or to a highly similar, DNA sequence as does RFX1.

Our results also suggest that if *sak1*$^+$ does in fact function to regulate gene expression, its ability to do so might be modulated by cAPK. Thus far, no substrates of cAPK in *S. pombe* that function to directly regulate gene expression have been identified. Genes required for meiosis in *S. pombe* are known to be regulated by nutritional conditions and specifically by cAPK. However, expression of meiotic genes cannot be the only function of *sak1*$^+$, since it is required for cell viability. We anticipate that *sak1*$^+$ might regulate expression of genes fundamental for growth. Further biochemical and genetic investigation of the *sak1*$^+$ gene should provide valuable insight into its role in exit from the cell cycle and in the cAPK signal transduction pathway.

ACKNOWLEDGMENTS

We thank Tao Chen for expert technical assistance and Li Peng for providing the *mei2-lacZ* fusion strain. C. Hoffman is thanked for many insightful discussions. In addition, we are grateful to J. Fields, F. Volkert, and M. McKowski for their suggestions.

This work was supported by grant MV-567 from the American Cancer Society.

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