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Characterization and Mutational Analysis of a Cluster of Three Genes Expressed Preferentially during Sporulation of *Saccharomyces cerevisiae*

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A differential hybridization screen of a genomic yeast DNA library previously identified 14 genes of *Saccharomyces cerevisiae* that are expressed preferentially during sporulation. Three of these sporulation-specific genes, *SPS1*, *SPS2*, and *SPS3*, have been shown to be closely linked. A mutational analysis has demonstrated that expression of the *SPS1* gene, but not the *SPS2* gene, is essential for the completion of sporulation. A diploid *MAT*a/*MAT*α strain homozygous for a disruption of the *SPS1* gene failed to form asci when subjected to sporulation conditions. The 3′ end of the transcript encoded by the *SPS1* gene was found to map only 185 base pairs from the 5′ end of the *SPS2* gene. The *SPS1*-*SPS2* intergenic region was shown to contain all of the regulatory sequences necessary for the sporulation-specific activation of the *SPS2* gene as assessed by expression of a translational *SPS2-lacZ* fusion gene present on a replicating, centromere-containing plasmid. The fusion gene was found to be expressed at the same time during sporulation as the chromosomal wild-type *SPS2* gene.

Sporulation in the yeast *Saccharomyces cerevisiae*, initiated upon starvation of *MAT*a/*MAT*α diploid cells, represents a regulated program of differentiation which includes meiosis and the encapsulation of the four haploid nuclei into ascospores. The ease of performing a variety of genetic manipulations in *S. cerevisiae* (29) makes sporulation in this organism an appealing eucaryotic system for studying developmentally regulated gene expression. The temporal program of genetic, biochemical, and morphological events occurring during sporulation has been described in detail (6), but little is known about the concomitant program of gene expression presumed to be essential for these events. Although a mutational analysis has identified several genes uniquely required for the successful completion of sporulation (6), the analysis of in vivo-labeled proteins revealed disappointingly few sporulation-specific proteins (11, 13, 24, 30, 33). In contrast, differential hybridization screening procedures have been successfully used to identify a large number of cloned genes which are expressed preferentially during sporulation (4, 23). The cloned genes identified in these studies are expressed only in *MAT*a/*MAT*α cells after transfer to sporulation conditions. They are not expressed during exponential growth or in nutrient-deprived *MAT*a/*MAT*a or *MAT*α/*MAT*α cells, which are asporogenous. More recently, an analysis of the in vitro coding capacities of RNAs isolated from sporulating cells has also clearly demonstrated the accumulation of an array of sporulation-specific mRNAs (14, 32).

In the present study we have characterized a cluster of three sporulation-specific genes previously identified in a differential hybridization screen (23). Ultimately, the elucidation of the manner in which these genes are activated will provide insights into the mechanisms involved in controlling development-specific gene expression. In this report, we have demonstrated by a mutational analysis that a gene which had been deemed sporulation specific on the basis of its expression pattern is indeed functionally required for the completion of sporulation, supporting the suggestion that its activation is directly dependent on sporulation-specific regulatory events.

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MATERIALS AND METHODS

Strains and culture conditions. *Escherichia coli* HB101 was used for the propagation of plasmids. The *S. cerevisiae* strains used were the AP3 *MAT*α/*MAT*α, *MAT*a/*MAT*a, and *MAT*a/*MAT*α diploids (10) previously employed (23), SC252 (*MAT*α *ade1 leu2-3 leu2-112 ura3-52*) (provided by J. Friesen), SR25-1A (*MAT*a *his4-912 ura3-52*) (provided by J. Friesen), and the diploid YHU101, which was obtained by mating SC252 and SR25-1A. The protocol for sporulation in liquid medium (23), used for all experiments except that in Table 1, resulted in 65% ascus formation within 24 h for AP3 (*MAT*a/*MAT*α) and in 15% ascus formation within 48 h for YHU101 as determined by light microscopy.

Plasmid constructions. p18 contains a 3.7-kilobase-pair (kbp) fragment of yeast DNA encompassing the entire *SPS1* gene and the 5′ end of the *SPS2* gene inserted into the *Bam*HI site of pBR322 (Fig. 1 and 2C) (23). p18-2 (Fig. 1), containing an *SPS1* gene which has been disrupted at its 3′ end by the insertion of a 2.6-kbp fragment containing the *URA3* gene, was derived from p18 as follows. A 1.1-kbp *Hin*dIII DNA fragment containing the yeast *URA3* gene was purified from pJH101 (provided by H. Himmelfarb). After the termini had been filled in with the Klenow fragment of *E. coli* DNA polymerase I, the fragment was ligated into the *Nru*I site of the polylinker of pDPL13 (7), generating pDPL13-*URA3* and allowing the *URA3* gene to be recovered on a 2.6-kbp *Bam*HI-*Bgl*II fragment for ligation with p18 DNA that had been digested with *Bgl*II. A plasmid denoted p18-1 (Fig. 1) was recovered in which the 0.8-kbp *Bgl*II fragment spanning the 3′ end of the *SPS1* gene and the 5′ end of the *SPS2* gene (Fig. 2D) had been replaced with the 2.6-kbp *URA3* fragment. p18-2 was then obtained by reintroducing the deleted 0.8-kbp *Bgl*II fragment, purified from p18, in the appropriate orientation into the unique *Bgl*II site of p18-1.

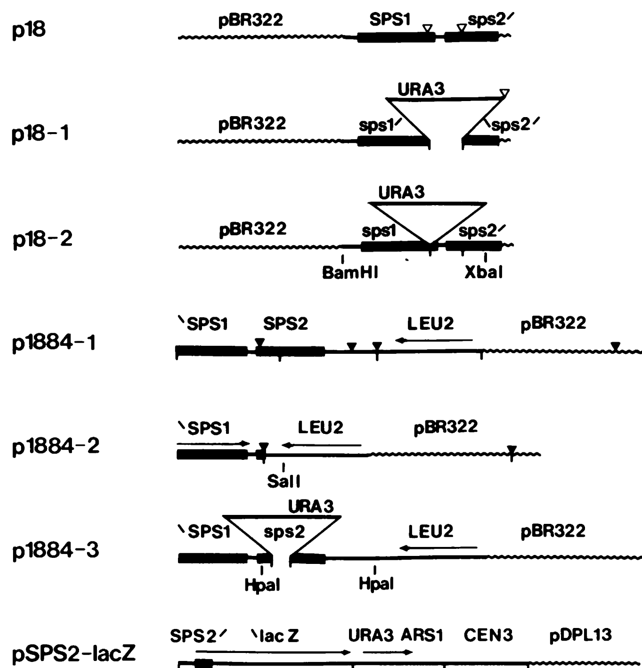

FIG. 1. Structure of the plasmids used in this study. The construction of these plasmids is described in the Materials and Methods. The maps have been linearized at the *Cla*I site of pBR322 and drawn so that the direction of transcription of the *SPS* genes is from left to right. The vertical lines denote the junctions between the various fragments joined in these constructs. The *Bgl*II sites in p18 and p18-1 and the *Pst*I sites in p1884-1 and p1884-2 are denoted by the open and closed arrowheads, respectively. The restriction sites indicated below the maps denote those at which the DNA was cut before being used for transformation of yeast cells.

p1884-1, which contains the *SPS1* gene truncated at the 5′ end (denoted *'SPS1*), the entire *SPS2* gene reconstituted from p18 and p84, and the yeast *LEU2* gene, was constructed as follows. A 3.2-kbp *Cla*I-*Sal*I fragment containing the 3′ portion of the *SPS2* gene was purified from p84 (Fig. 2) and inserted between the *Cla*I and *Sal*I sites of pBR322 to give pIntA. A 2.8-kbp *Xho*I-*Bgl*II fragment containing the yeast *LEU2* gene was purified from pJH44 (provided by B. Andrews) and inserted between the *Sal*I and *Bgl*II sites of pIntA to generate pIntB. A 2.7-kbp *Cla*I fragment extending from within the transcribed but nontranslated 5′ end of the *SPS1* gene into the 5′ portion of the *SPS2* gene was purified from p18 (Fig. 2A) and inserted into the appropriate *Cla*I site of pIntB to reconstitute the *SPS2* gene and generate p1884-1. For this last step, since pIntB contains two *Cla*I sites the plasmid DNA was digested with *Cla*I in the presence of ethidium bromide (40 μg/ml) to obtain singly cut molecules (22).

The *SPS2* gene was subsequently deleted from p1884-1 by digesting the plasmid DNA with *Pst*I and recovering and religating the two largest fragments in the appropriate orientation to yield p1884-2 (Fig. 1). p1884-1 was also used to generate p1884-3, in which a fragment containing the *URA3* gene replaces an internal portion of the *SPS2* gene (Fig. 1). A 2.6-kbp *Xba*I-*Bgl*II fragment containing the yeast URA3 gene was purified from pDPL13-*URA3* and inserted between the *Bgl*II and *Xba*I sites of the *SPS2* gene of p1884-1. Since p1884-1 has three *Bgl*II sites, the plasmid was digested with *Bgl*II in the presence of ethidium bromide (50 μg/ml) to obtain singly cut molecules (22).

An *E. coli* yeast shuttle vector containing the yeast *CEN3* sequence and an *SPS2-lacZ* fusion gene (p*SPS2-lacZ*, Fig. 1) was constructed as follows. A 2.1-kbp *Eco*RI-*Bam*HI fragment containing the yeast *URA3* gene and *ARS1* sequence was purified from pYRP10 (provided by H. Himmelfarb) and inserted between the *Eco*RI and *Bam*HI sites of pDPL13 (7) to generate pIntl. A 2.0-kbp *Bam*HI fragment containing the *CEN3* sequence was purified from pHF-2 (provided by H. Friesen) and inserted into the *Xho*I site of pIntl to generate pInt2. Before the ligation step, the *Bam*HI and *Xho*I ends had been made complementary by the addition of two nucleotides to the 3′ ends of the termini. A 3.5-kbp *Bam*HI-*Bgl*II fragment containing a *lacIZ* fusion gene lacking a translational start was purified from pRY64 (provided by R. Yocum) and inserted into the *Bam*HI site of pInt2 to generate pInt3. The unique *Bam*HI site in pInt3, at the 5′ end of the *lacIZ* gene, allowed an in-frame translational fusion with the *SPS2* gene to be readily constructed. The 0.8-kbp *Bgl*II fragment of the *SPS2* gene containing 358 base pairs (bp) of 5′ flanking sequence and extending to the codon for amino acid 120 was purified from p18 (Fig. 2D) and inserted into the *Bam*HI site of pInt3 to generate an *SPS2-lacZ* fusion gene. The fusion junction was sequenced to verify that the appropriate translational frame had been maintained. All DNA manipulations were carried out by previously described procedures (17).

Transcript mapping. After restriction enzyme maps had been deduced for plasmid 18 and plasmid 84 by standard procedures (17), the approximate positions of the sequences encoding the 1.9-, 1.6-, and 1.75-kilobase (kb) transcripts were determined by hybridization of purified, radioactively labeled DNA fragments to Northern blots of RNA isolated from sporulating cells. The genes were then mapped in more detail by hybridizing RNA that had been labeled in vitro to a Southern blot of plasmid DNA that had been restricted with a variety of enzymes (28). The RNA was labeled as follows. Polyadenylated RNA purified from *MAT***a**/*MAT*α cells harvested 10 h after transfer to sporulation medium (23) was incubated for 20 min at 90°C in a buffer containing 50 mM Tris hydrochloride (pH 9.5) and 50 μM spermidine (16). The fragmented RNA was then 5′ end labeled by T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. The ends of the transcripts were positioned by the S1 nuclease procedure of Berk and Sharp (1) as modified by Weaver and Weissman (31), with optimal hybridization temperatures (30 to 41°C) being determined empirically for each probe. The 3′ end of the transcript encoded by the *SPS2* gene was mapped by using an *Sau*3A-*Dde*I fragment (Fig. 2D) 3′ end labeled at the *Sau*3A terminus as a probe. The approximate position of the 5′ end of the *SPS3* gene was defined by using an *Xba*I-*Cla*I fragment (Fig. 2A) 5′ end labeled at the *Cla*I terminus as a probe. The mapping of the 5′ end of the *SPS1* gene is described in the legend to Fig. 8; the mapping of the 3′ end of the *SPS1* gene and the 5′ end of the *SPS2* gene is described in the legend to Fig. 4.

Transformation of yeast cells. Transformation of yeast spheroplasts generated by glusulase treatment (9) was performed as described by McNeil et al. (19).

RESULTS

Plasmids p18 and p84 represent a cluster of three sporulation-specific genes. We previously performed a differential

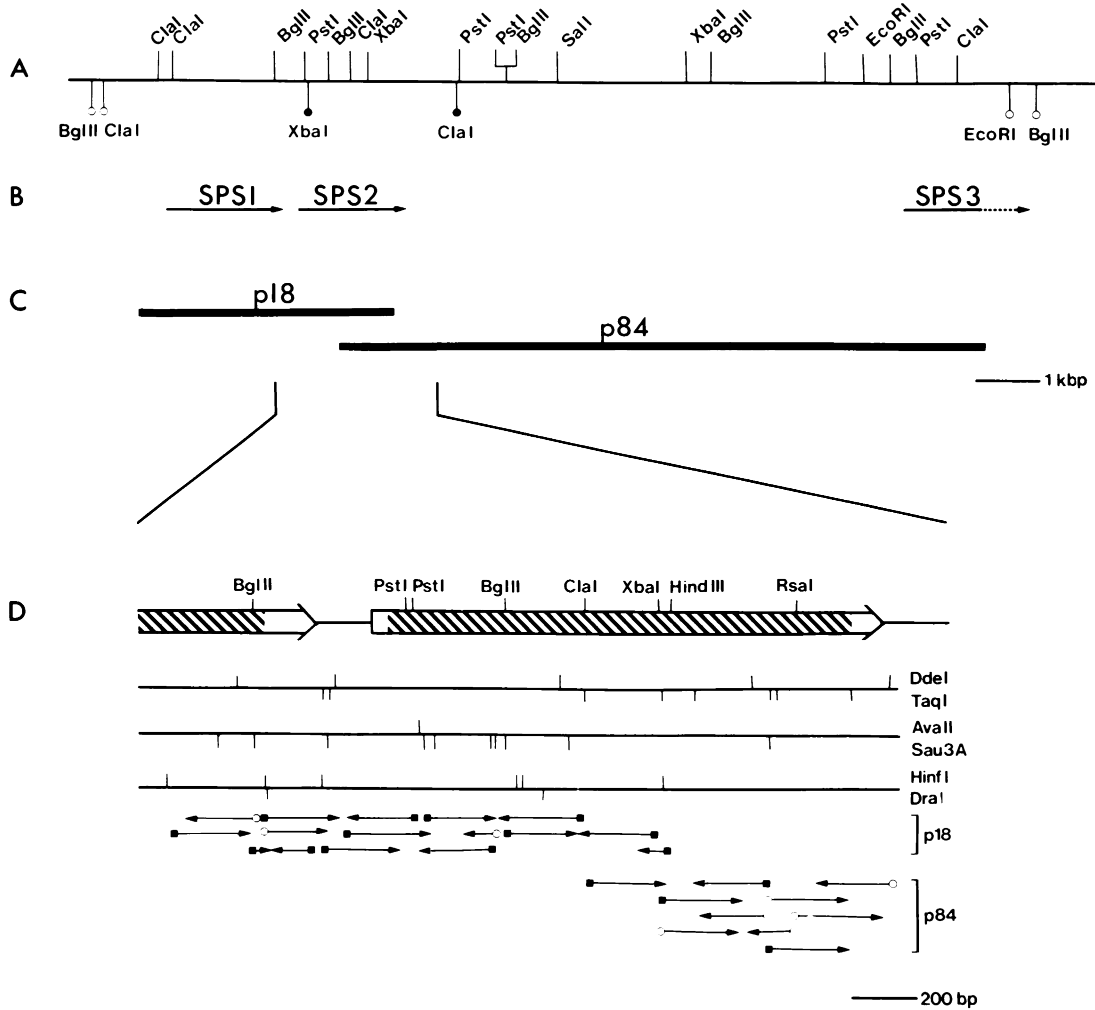

FIG. 2. Genomic organization of the sporulation gene cluster. (A) The restriction map of the gene cluster is shown for selected endonucleases. The sites denoted by the closed circles were restricted only in yeast DNA or in plasmid DNA that had been propagated in *E. coli* CGSC 5126, a Dam⁻ strain (provided by J. Friesen). The sites denoted by the open circles were mapped by analysis of genomic DNA rather than plasmid DNA. (B) The positions of the transcripts encoded by the *SPS1*, *SPS2*, and *SPS3* genes are indicated (see Materials and Methods). The arrowheads denote the direction of transcription. (C) Sequences represented by the yeast inserts of p18 and p84. (D) A more detailed endonuclease restriction map of the 3′ end of the *SPS1* gene and the entire *SPS2* gene. The large arrow depicts the transcriptional unit with the putative coding region hatched. The sequencing strategy is shown at the bottom of the figure. The upper and lower sets of arrows indicate the direction and extent of sequence determined for 5′ end-labeled (○) or 3′ end-labeled (■) fragments from p18 and p84, respectively.

hybridization screen of a yeast DNA library which identified 14 cloned genes that are expressed preferentially during sporulation in *S. cerevisiae* (23). We have now characterized the three sporulation-specific genes represented by the two plasmids p18 and p84 in more detail. Our preliminary characterization of these plasmids had indicated that p18 contained the sequences (or part thereof) encoding a 1.9-kb and a 1.6-kb transcript which accumulated preferentially in *MAT***a**/*MAT*α cells during sporulation (Fig. 3) (23). Plasmid p84, which appeared to overlap p18 within the sequence encoding the 1.6-kb sporulation-specific transcript, contained an additional sporulation-specific gene encoding a 1.75-kb transcript (Fig. 3) (23). An examination of the restriction enzyme maps of these two plasmids established that they do indeed contain overlapping sequences (Fig. 2A and C). A 227-bp *Cla*I-*Xba*I fragment was found to map adjacent to one end of the yeast DNA insert of both plasmids. A comparison of the nucleotide sequence of both plasmids through a portion of the predicted overlap confirmed the identity of the plasmids in this region (Fig. 2D). To ascertain that neither plasmid contained noncontiguous fragments of the yeast genome that had been inadvertently ligated together in the cloning procedure (23), p18 and p84 were used as probes in a Southern analysis of yeast DNA digested with a variety of restriction endonucleases. The pattern of hybridization obtained was completely consistent with p18 and p84 representing a contiguous and unique genomic sequence (data not shown). Therefore, the three sporulation-specific genes encoding the 1.9-, 1.6-, and 1.75-kb transcripts, which we have termed *SPS1*, *SPS2*, and *SPS3*, respectively, are linked with the *SPS2* gene being the central member of this gene cluster. The positions of the transcripts encoded by the three genes have been mapped onto the cloned DNA (Fig. 2B; data not shown; see Mate-

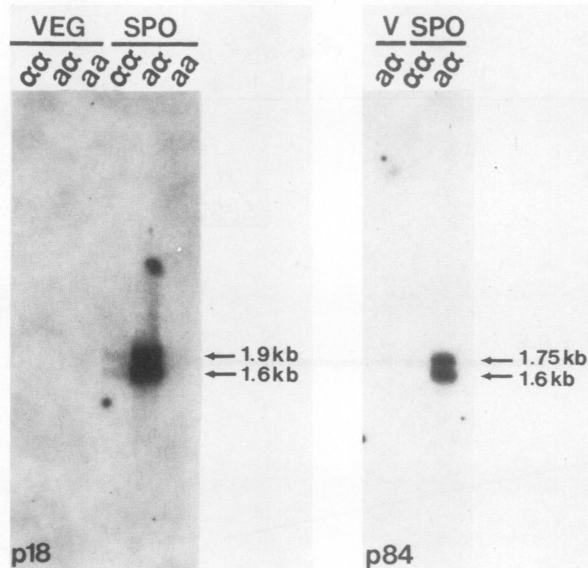

FIG. 3. Examination of the developmentally regulated synthesis of the transcripts encoded by p18 and p84. Polyadenylated RNA purified from either AP3 *MAT*α/*MAT*α, *MAT***a**/*MAT*α, or *MAT***a**/*MAT***a** cells (as indicated at the top of each lane) growing vegetatively (VEG, V) or at 10 h after transfer to sporulation medium (SPO) was denatured, separated by electrophoresis through a 1.5% agarose-formaldehyde gel, and then transferred to nitrocellulose paper. The filters were then hybridized with p18 or p84 DNA (as indicated) that had been radioactively labeled by nick translation (25). The sizes of the transcripts encoded by the plasmids are indicated on the right-hand side of each filter.

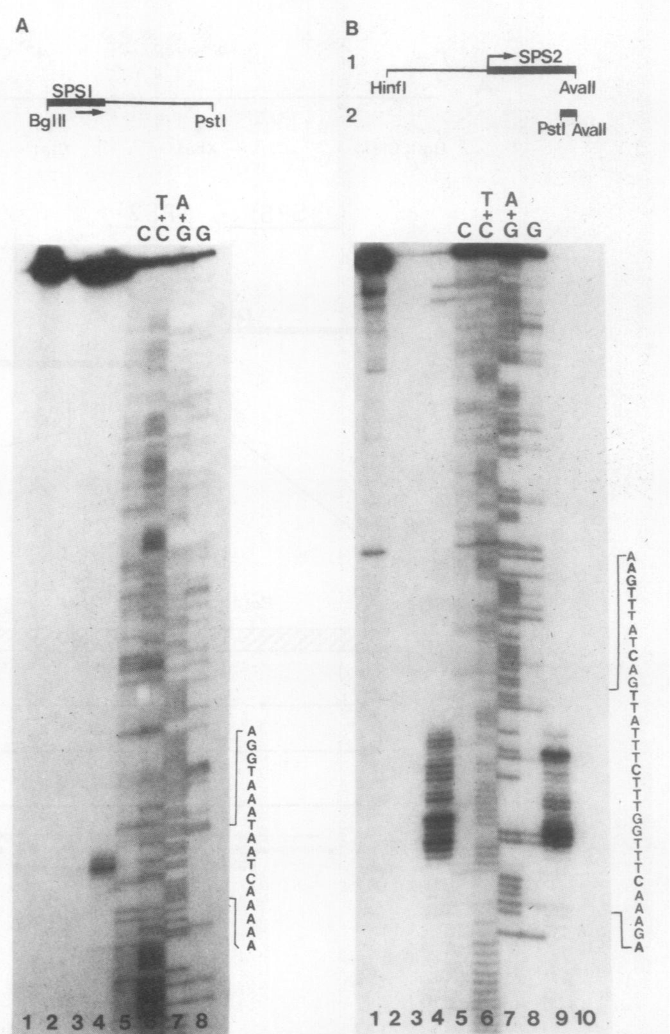

FIG. 4. Mapping of the 3′ end of the *SPS1* gene and the 5′ end of the *SPS2* gene. An S1 nuclease analysis was performed (see Materials and Methods) with the 3′ end-labeled *Bgl*II-*Pst*I fragment spanning the 3′ end of the *SPS1* gene (A) and the 5′ end-labeled *Hin*fI-*Ava*II fragment spanning the 5′ end of the *SPS2* gene (B, schematic 1) as probes (lanes 1 through 4). These DNAs were hybridized with 10 μg of total RNA purified from AP3 cells harvested at 10 h after transfer to sporulation medium (23). The reactions of lanes 1 and 4 contained RNA from *MAT***a**/*MAT*α cells; the reaction of lane 3 contained RNA from *MAT*α/*MAT*α cells; S1 nuclease was omitted from the reaction of lane 1; RNA was omitted from the reaction of lane 2. The 5′ end of the *SPS2* gene was also mapped by a primer extension analysis (lanes 9 and 10) performed as described by Ghosh et al. (8) with the 5′ end-labeled coding strand of the *Pst*I-*Ava*II fragment (B, schematic diagram 2) as a primer for reverse transcription of RNA from *MAT***a**/*MAT*α (lane 9) and *MAT*α/*MAT*α (lane 10) cells harvested at 10 h after transfer to sporulation medium. The S1-resistant RNA-DNA hybrids and the cDNA-RNA hybrids were heat denatured in sample buffer and subjected to electrophoresis on a 6% polyacrylamide-8 M urea gel adjacent to a chemical sequencing ladder of the labeled *Bgl*II-*Pst*I fragment (A, lanes 5 through 8) or the labeled *Hin*fI-*Ava*II fragment (B, lanes 5 through 8). The sequences are shown on the right.

rials and Methods); all three genes were found to be transcribed in the same direction.

Characterization of the *SPS2* gene. We have characterized in more detail the *SPS2* gene, the central gene of the cluster of three *SPS* genes. An S1 nuclease analysis was performed to precisely map the termini of the trancripts encoded by this gene. The 5′ ends of the transcripts were mapped by hybridizing the *Hin*fI-*Ava*II fragment indicated in Fig. 4B (schematic diagram 1) with RNA extracted from *MAT***a**/*MAT*α sporulating cells. This analysis revealed that there are 2 major and approximately 11 minor initiation sites over a 19-nucleotide sequence (Fig. 4B, lane 4). As a control, this analysis was also performed with RNA extracted from asporogenic *MAT*α/*MAT*α cells subjected to sporulation conditions. No S1-resistant hybrids were formed (Fig. 4B, lane 3). To verify that the transcriptional start sites mapped by the S1 nuclease procedure were authentic, the *Pst*I-*Ava*II fragment indicated in Fig. 4B (schematic diagram 2) was used as primer for reverse transcription of the *SPS2* transcripts. Analysis of the resulting cDNAs also revealed multiple initiation sites over a region coincident with that defined by the S1 nuclease procedure (Fig. 4B, lane 9); there was not, however, an exact correspondence between all of the major start sites.

Determination of the nucleotide sequence of the *SPS2* gene revealed a single long open reading frame which encodes a predicted protein of 469 amino acids (Fig. 5). The first ATG codon is 47 nucleotides downstream from the start of transcription. The translational termination codon of this open reading frame is followed by four additional termination codons within 90 bases. A TATA box sequence (3) is found 59 nucleotides upstream from the start of transcription. A sequence resembling the tripartite sequence implicated in transcription termination in yeast (35) begins 23 bp downstream from the translational termination codon of the *SPS2* gene. A comparison of the predicted *SPS2* protein with the proteins in the Protein Identification Resource protein

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          -595                -580                -565                -550
AAT GCT CAA GCA GAG ACC GAA ATA GTT CCT CTC TCT AAT CAT AAT AAG AAG CAT AAA AAG
Asn Ala Gln Ala Glu Thr Glu Ile Val Pro Leu Ser Asn His Asn Lys Lys His Lys Lys

          -535                -520                -505                -490
AAC GAT ATT CAG GCG TTG AAG ATA GAA AAG TTT GAC TAC TTG AAA AAC ATT GTA TCT CAT
Asn Asp Ile Gln Ala Leu Lys Ile Glu Lys Phe Asp Tyr Leu Lys Asn Ile Val Ser His

          -475                -460                -445                -430
ATT CTC AAC AGA ATG TAT GAT CGT GCG CGC GAC GAT GAA ACA AGA AAA TAC GTA AAC GTA
Ile Leu Asn Arg MET Tyr Asp Arg Ala Arg Asp Asp Glu Thr Arg Lys Tyr Val Asn Val

          -415                -400                -385                -370
AAT GAA ATG TTA AAG CAA TTC ATT AAA ACT GAG GCA AAC GTT CCT AGA TTT AAT GAA GTT
Asn Glu MET Leu Lys Gln Phe Ile Lys Thr Glu Ala Asn Val Pro Arg Phe Asn Glu Val

          -355                -340                -325
TTT ATA GAA GAG ATC TCA CTA AGA ATT GAA GCA ATA AAG AAA GGA TTC GTT TAA
Phe Ile Glu Glu Ile Ser Leu Arg Ile Glu Ala Ile Lys Lys Gly Phe Val

      -306       -296       -286       -276       -266       -256       -246
AAAAATAGAT ATATATATAT AATGTGTGTA TATGCATGAG TTTTTGTTTT CCATTCTCTT TCATCTTCAA

      -236       -226       -216       -206       -196       -186       -176
TAAATGAAGG ATGGATATAT TGAATTATAT AACAAACAGT AAAAAGTCAT GTGTTTTTGA TTATTTACCT

      -166       -156       -146       -136       -126       -116       -106
GCTTATTTCT CCCTTTTTTT TTCGATTCTT TTTTTCTGAT CGAGCGTTTA TGTCTTTACT GTTCCGTCAT

       -96        -86        -76        -66        -56        -46        -36
TGATGCGTCT CAAAAAAAAT ATTTTGTGGC ATTACTTAGA CTATAAAAGA AAATGCCAAT TTGGAAAACA

       -26        -16         -6          5         15         25         35
CAAACATTCT TCACTAGTAT TTCTGTTATT CAAATAGTCA ATAAAGAAAC CAAAGTTTCT ACAAAAAAAG

        45
AAAAGGATAG C

          61                  76                  91                 106
ATG TTG AAC CAG TTG AAC ACA ATC TTA AGA TTT TTA TTC TTG TTT CTG CAG TTA ATT AAA
MET Leu Asn Gln Leu Asn Thr Ile Leu Arg Phe Leu Phe Leu Phe Leu Gln Leu Ile Lys

         121                 136                 151                 166
TCA TCC GCT GCA GTT GAA CCT AAT GGT GGA CCG AAT ATT CTA GAT CAC AAC ATT ATG TTG
Ser Ser Ala Ala Val Glu Pro Asn Gly Gly Pro Asn Ile Leu Asp His Asn Ile MET Leu

         181                 196                 211                 226
GTT AAC ACT AAT GCA ACG ATC CCT AAA AAG GAA CAA ACT GAT TTT GAG GTG ATT TCT CCA
Val Asn Thr Asn Ala Thr Ile Pro Lys Lys Glu Gln Thr Asp Phe Glu Val Ile Ser Pro

         241                 256                 271                 286
ACA AAA CAA ACA CAA GTA GAT GAA GAC TGT AAA AAA GGC TTG TAT CAT ATT GAG AAT GCT
Thr Lys Gln Thr Gln Val Asp Glu Asp Cys Lys Lys Gly Leu Tyr His Ile Glu Asn Ala

         301                 316                 331                 346
GGA AAT TTG ATT GAA CTG CAA GCT AAA TGT TGG AAA GTG GTA GGA AAC ATT GAA ATA TCA
Gly Asn Leu Ile Glu Leu Gln Ala Lys Cys Trp Lys Val Val Gly Asn Ile Glu Ile Ser

         361                 376                 391                 406
AGT AAC TAC AGC GGA TCT CTT ATT GAT CTA GGG TTA ATA AGA GAG ATA GAG GGA GAT CTT
Ser Asn Tyr Ser Gly Ser Leu Ile Asp Leu Gly Leu Ile Arg Glu Ile Glu Gly Asp Leu

         421                 436                 451                 466
ATT ATC AAG AAT AAC AAA CAC ATT TTT AGA ATC CAG GGT TAT AAT TTA GAG TCT TTG GGA
Ile Ile Lys Asn Asn Lys His Ile Phe Arg Ile Gln Gly Tyr Asn Leu Glu Ser Leu Gly

         481                 496                 511                 526
AAG TTG GAA TTG GAT AGC CTA ACT TCC TTT GTA TCA TTA GAC TTT CCC GCT TTA AAA GAA
Lys Leu Glu Leu Asp Ser Leu Thr Ser Phe Val Ser Leu Asp Phe Pro Ala Leu Lys Glu

         541                 556                 571                 586
GTT GAA ACC GTT GAT TGG AGA GTT TTG CTT ATT CTA AGT AGT GTT GTC AGT AAC GGA AAT
Val Glu Thr Val Asp Trp Arg Val Leu Leu Ile Leu Ser Ser Val Val Ser Asn Gly Asn

         601                 616                 631                 646
ATT AAA AAG ATC AAA AAT ATT ATC ATA TCT GAT ACT GCA TTA ACC TCC ATC GAT TAC TTC
Ile Lys Lys Ile Lys Asn Ile Ile Ile Ser Asp Thr Ala Leu Thr Ser Ile Asp Tyr Phe

         661                 676                 691                 706
AAT AAC GTC AAG AAA GTG GAT ATT TTC AAT ATC AAC AAT AAC AGG TTT TTA GAA AAT TTA
Asn Asn Val Lys Lys Val Asp Ile Phe Asn Ile Asn Asn Asn Arg Phe Leu Glu Asn Leu

         721                 736                 751                 766
TTC GCA AGT TTA GAA AGT GTT ACT AAA CAA CTA ACC GTC CAC TCT AAT GCC AAA GAG CTG
Phe Ala Ser Leu Glu Ser Val Thr Lys Gln Leu Thr Val His Ser Asn Ala Lys Glu Leu

         781                 796                 811                 826
GAA CTT GAT TTG AGC AAC TTA CAC ACG GTT GAA AAT ATG ACC ATT AAG GAC GTT TCA GAA
Glu Leu Asp Leu Ser Asn Leu His Thr Val Glu Asn MET Thr Ile Lys Asp Val Ser Glu

         841                 856                 871                 886
ATT AAA CTA GCT AAA CTT TCC TCT GTG AAC AGT TCT CTA GAG TTC ATC GAG AAT CAA TTT
Ile Lys Leu Ala Lys Leu Ser Ser Val Asn Ser Ser Leu Glu Phe Ile Glu Asn Gln Phe

         901                 916                 931                 946
TCA AGC TTG GAA CTA CCA CTT TTG GCA AAA GGT CAA GGA ACG TTG GGG TTA ATA GAT AAT
Ser Ser Leu Glu Leu Pro Leu Leu Ala Lys Gly Gln Gly Thr Leu Gly Leu Ile Asp Asn

         961                 976                 991                1006
AAA AAT CTG AAA AAG CTA AAC TTT TCG AAT GCC ACC GAT ATT CAA GGG GGT CTA ATG ATT
Lys Asn Leu Lys Lys Leu Asn Phe Ser Asn Ala Thr Asp Ile Gln Gly Gly Leu MET Ile

        1021                1036                1051                1066
GCT AAC AAT ACA GAG CTT GCC AAA ATT GAT TTC TTC CCC AAG TTG AGG CAA ATT GGC GGT
Ala Asn Asn Thr Glu Leu Ala Lys Ile Asp Phe Phe Pro Lys Leu Arg Gln Ile Gly Gly

        1081                1096                1111                1126
GCA ATA TAT TTT GAA GGT AGC TTT GAT AAA ATT GAC CTC CCA GAG CTA AAG TTA GTA AAA
Ala Ile Tyr Phe Glu Gly Ser Phe Asp Lys Ile Asp Leu Pro Glu Leu Lys Leu Val Lys

        1141                1156                1171                1186
GGT AGC GCT TAT ATC AAA AGT TCA TCT GAG GAA TTA AAC TGT GAA GAA TTT ACA TCA CCA
Gly Ser Ala Tyr Ile Lys Ser Ser Ser Glu Glu Leu Asn Cys Glu Glu Phe Thr Ser Pro

        1201                1216                1231                1246
AAA GCC GGT CGT TCG ATC ATA AGA GGC GGT AAA ATC GAA TGC ACA TCT GGT ATG AAA AGT
Lys Ala Gly Arg Ser Ile Ile Arg Gly Gly Lys Ile Glu Cys Thr Ser Gly MET Lys Ser

        1261                1276                1291                1306
AAA ATG CTG AAT GTT GAT GAA GAG GGG AAT GTA CTA GGA AAG CAG GAA ACT GAC AAT GAT
Lys MET Leu Asn Val Asp Glu Glu Gly Asn Val Leu Gly Lys Gln Glu Thr Asp Asn Asp

        1321                1336                1351                1366
AAT GGA AAG AAA GAA AAA GGA AAA AAT GGT GCA AAA AGT CAA GGA AGT TCA AAG AAG ATG
Asn Gly Lys Lys Glu Lys Gly Lys Asn Gly Ala Lys Ser Gln Gly Ser Ser Lys Lys MET

        1381                1396                1411                1426
GAA AAC AGT GCT CCG AAG AAC ATT TTT ATT GAT GCT TTC AAA ATG TCA GTT TAT GCA GTT
Glu Asn Ser Ala Pro Lys Asn Ile Phe Ile Asp Ala Phe Lys MET Ser Val Tyr Ala Val

        1441                1456
TTC ACG GTA TTG TTC TCG ATA ATT TTT TAG
Phe Thr Val Leu Phe Ser Ile Ile Phe

      1466       1476       1486       1496       1506       1516       1526
GTTAGAAGAT TAACTTAAAT ACTAGCCATT TTATTTAGTT CTTATGAGAT ATAAATTTGC AGTAGAGATG

      1536       1546       1556
TAGTGTTCTG ATTATCTTCA CCTAGAGCAT AGCGT
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sequence database (20) revealed no significant amino acid homologies.

Regulated expression of an *SPS2-lacZ* fusion gene. Our preliminary transcript mapping had suggested that the *SPS1* gene terminated within several hundred base pairs of the start of the *SPS2* gene (Fig. 2B; data not shown; see Materials and Methods). To delineate the *SPS1-SPS2* intergenic region in more detail, the 3′ end of the *SPS1* gene was mapped more precisely by using the *Bgl*II-*Pst*I fragment indicated in Fig. 4A as a probe in an S1 nuclease analysis. This analysis indicated that the 3′ end of the transcripts encoded by the *SPS1* gene mapped only 185 bp upstream from the start of the *SPS2* gene (Fig. 4A and 5). This short distance between the 3′ end of the *SPS1* gene and the 5′ end of the *SPS2* gene suggested that the regulatory sequences directing sporulation-specific expression of the *SPS2* gene might be positioned relatively close to the 5′ end of the *SPS2* gene. Alternatively, expression of the *SPS2* gene could depend on a common control sequence which contributes to the activation of all three *SPS* genes and which is present elsewhere in the gene cluster. To distinguish between these possibilities an *SPS2-lacZ* translational fusion gene containing the immediate 5′ flanking sequence of the *SPS2* gene was constructed. The 762-bp *Bgl*II fragment spanning the 5′ end of the *SPS2* gene (from nucleotide −358 to +404, the 120th codon of the predicted *SPS2* protein; Fig. 5) was fused in frame to the β-galactosidase gene of *E. coli* (see Materials and Methods). The hybrid gene was inserted into a replicating, centromeric vector containing the *URA3* gene (p*SPS2-lacZ*, Fig. 1). A *MAT***a** *ura3*/*MAT*α *ura3* strain (YHU101) was then stably transformed to Ura^+ with this plasmid, and expression of both the episomal fusion gene and the wild-type chromosomal gene was monitored by S1 nuclease analysis. Probes specific for the transcript of the fusion gene (Fig. 6A) and for the transcript of the chromosomal gene (Fig. 6B) were hybridized with RNA extracted from vegetatively growing cells and from cells at 10, 15, and 20 h after transfer to sporulation medium. Neither the fusion gene nor the chromosomal *SPS2* gene was expressed in cells during exponential growth (Fig. 6, lanes 3 and 11) or in cells at 10 h after transfer to sporulation medium (Fig. 6, lanes 5 and 13). Expression of the fusion gene was first detected in cells at 15 h after transfer to sporulation medium (Fig. 6, lane 7). This coincided with the time at which the endogenous wild-type gene was activated (Fig. 6, lane 15). The estimated size of the S1 nuclease-resistant hybrids formed with the fusion transcript (1,250 bp) was in good agreement with the size expected (1,298 bp) for transcription initiating at the wild-type start site. This suggests that the sequences directing sporulation-specific expression of the *SPS2* gene are contained within the 185-bp *SPS1-SPS2* intergenic region; however, it is possible that regulatory sequences are present in the 3′ portion of the *SPS1* gene or the 5′ portion of the *SPS2*

FIG. 5. Nucleotide sequence of the nontranscribed strand of the *SPS2* gene. The sequence was obtained by the method of Maxam and Gilbert (18) as described in the legend to Fig. 2D. The unbroken underline indicates the sequence encompassing the multiple 5′ termini of the *SPS2* transcripts (Fig. 4B), with the most distal initiation site being denoted +1. The putative TATA box sequence preceding the *SPS2* gene is boxed. The arrows denote the positions of the termini of the transcripts of the *SPS1* and *SPS2* genes. Possible transcription termination signals of the *SPS1* and *SPS2* genes are underlined with a dashed line. Translation of the predicted open reading frames of the *SPS1* and *SPS2* genes is shown below the nucleic acid sequence.

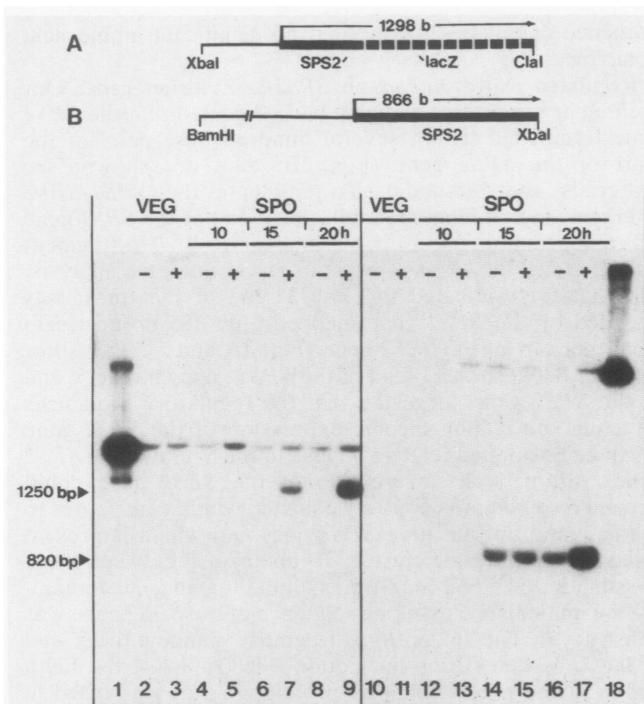

FIG. 6. Regulated expression of an *SPS2-lacZ* fusion gene. The replicating, centromeric plasmid depicted in Fig. 1 (p*SPS2-lacZ*) containing an *SPS2-lacZ* translational fusion gene with 358 bp of the 5' flanking yeast sequence was introduced into a diploid yeast strain (YHU101). Expression of the fusion gene was monitored by S1 nuclease analysis with the *Xba*I-*Cla*I fragment 5' end labeled at the *Cla*I terminus as a probe (A and lanes 1 through 9). (The *Xba*I site is within the pDPL13 polylinker region of the fusion gene plasmid.) Since the labeled end of the fragment is homologous to the *lacZ* gene, it will not hybridize with the transcript of the chromosomal *SPS2* gene. Expression of the chromosomal gene was monitored by using the *Bam*HI-*Xba*I fragment 5' end labeled at the *Xba*I terminus (B and lanes 10 through 18). As the portion of the *SPS2* gene extending to this *Xba*I site has been deleted in the fusion gene, this probe is specific for the wild type transcript. The probes were hybridized with 20 μg of RNA extracted (23) from nontransformed (lanes 2, 4, 6, 8, 10, 12, 14, and 16; indicated by the minus sign) and transformed (lanes 3, 5, 7, 9, 11, 13, 15, and 17; indicated by the plus sign) diploid cells growing exponentially (lanes 2, 3, 10, and 11) or at 10 h (lanes 4, 5, 12, and 13), 15 h (lanes 6, 7, 14, and 15), or 20 h (lanes 8, 9, 16, and 17) after transfer to sporulation medium. S1 nuclease was omitted from the reactions of lanes 1 and 18, and only one-fifth of these two reactions was analyzed. The S1 nuclease-resistant RNA-DNA hybrids were subjected to electrophoresis on a nondenaturing 2% agarose gel. The sizes of the hybrids (indicated at left) were estimated from the mobilities of size markers (λ DNA digested with *Eco*RI and *Hin*dIII) run in adjacent lanes (not shown).

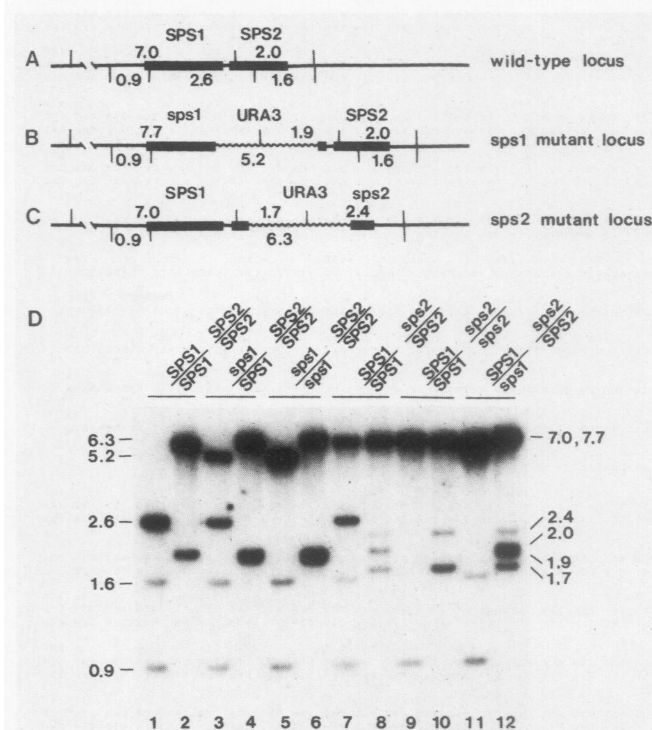

FIG. 7. Southern analysis of the mutant *sps1* and *sps2* loci. The *Pst*I and *Cla*I restriction sites in (A) the wild-type *SPS1*, *SPS2* locus, (B) the mutant *sps1* locus, and (C) the mutant *sps2* locus are denoted above and below the maps, respectively, with the sizes of the fragments indicated in kilobase pairs. (D) Southern analysis. DNA extracted by the rapid isolation procedure of Davis et al. (5) from the wild-type diploid strain (lanes 1 and 2), from the heterozygous (lanes 3 and 4) and homozygous (lanes 5 and 6) *sps1* mutant diploid strains, from the heterozygous (lanes 7 and 8) and homozygous (lanes 9 and 10) *sps2* mutant diploid strains, and from an *MAT***a** *SPS1 sps2*/*MAT*α *sps1 SPS2* strain (lanes 11, 12) was digested either with *Cla*I (lanes 1, 3, 5, 7, 9, and 11) or *Pst*I (lanes 2, 4, 6, 8, 10, and 12). The DNA fragments were separated by electrophoresis through a 1% agarose gel, and a Southern blot was probed with p18 DNA that had been radioactively labeled by nick translation (25). The sizes of the *Cla*I and *Pst*I fragments are denoted in kilobase pairs on the left and right side, respectively, of the autoradiogram. The 7.7- and 7.0-kbp *Pst*I fragments were not resolved in this gel. The genotypes denoted above the autoradiogram represent the diploid obtained by mating the *MAT***a** strain SR25-1A or a transformant of this strain (upper genotype) with the *MAT*α strain SC252 or a transformant of this strain (lower genotype).

gene included in the 762-bp yeast segment of the fusion gene. Nonetheless, these results indicate that the sequences immediately adjacent to the 5' end of the *SPS2* gene are sufficient to direct the sporulation-specific expression of the gene.

The *SPS1* gene, but not the *SPS2* gene, is essential for sporulation. The preferential expression of the *SPS* genes during sporulation (23) suggested that their gene products might serve functions essential for the successful completion of meiosis or ascus formation or both. This possibility has been investigated for the *SPS1* and *SPS2* genes by testing homozygous mutant diploid strains for their ability to sporulate.

As the first step in creating a *SPS1* mutant, a fragment containing the yeast *URA3* gene was inserted at the 3' end of the *SPS1* gene of p18 (p18-2 in Fig. 1). This insertion disrupts the gene 12 codons upstream from the predicted stop codon. After digestion of plasmid p18-2 with *Bam*HI and *Xba*I to separate the yeast sequence from the vector, the DNA was used to transform a *MAT***a** *ura3* strain and an *MAT*α *ura3* strain to Ura^+ (26). A *MAT***a** transformant was then mated with a *MAT*α transformant. After the genotype of the diploid had been confirmed by Southern analysis, the strain was tested for its ability to sporulate.

The Southern analysis depicted in Fig. 7 confirmed that integration of the fragment containing *URA3* had occurred at the *SPS1* locus, resulting in a disrupted gene. With p18 as a probe, 0.9-, 1.6-, and 2.6-kbp fragments were visualized on analysis of DNA that had been extracted from the wild-type diploid strain and digested with *Cla*I (Fig. 7, lane 1). The hybridization pattern with DNA from the *sps1*/*sps1* strain

demonstrated that insertion of the 2.6-kbp fragment containing *URA3* into the *SPS1* gene had enlarged the 2.6-kbp *Cla*I fragment to a 5.2-kbp *Cla*I fragment (Fig. 7, lane 5). Similarly, digestion of DNA with *Pst*I indicated that the insertion had introduced an additional *Pst*I site into the *SPS1* gene as visualized by the replacement of the 7.0-kbp fragment of the wild-type gene with 1.9- and 7.7-kbp fragments (Fig. 7, lanes 2 and 6).

Microscopic examination of cells subjected to sporulation conditions revealed that the homozygous mutant *MAT***a** *sps1/MATα sps1* diploid failed to form asci, whereas the heterozygous *MAT***a** *sps1/MATα SPS1* and *MAT***a** *SPS1/MATα sps1* strains sporulated as efficiently as the wild-type *SPS1/SPS1* strain (Table 1). The appearance of the asporogenous *MAT***a** *sps1/MATα sps1* cells in sporulation medium was similar to that of stationary-phase cells. However, staining the DNA of these cells with 33258 Hoechst (15) and visualization with fluorescent optics revealed the presence of tetranucleate cells (data not shown), indicating that the *SPS1* gene product is required for a late sporulation event.

The asporogenous phenotype of the *MAT***a** *sps1/MATα sps1* strain could be reversed by the integration of the *SPS1* gene at the *LEU2* locus in the *MAT***a** *sps1/MATα sps1* strain (Table 1). For this experiment, a plasmid containing a functional *SPS1* gene (denoted *'SPS1* and described below) and the *LEU2* gene was constructed (p1884-2, Fig. 1). The plasmid DNA, cut with *Sal*I to target integration to the *leu2* locus, was used to transform a *MATα leu2 sps1* strain to Leu^+. Appropriate integration was verified by Southern analysis of DNA extracted from the transformants (data not shown). A *MATα sps1 LEU2:'SPS1* transformant was then mated with a *MAT***a** *sps1* strain. The observation that this diploid strain had recovered the ability to sporulate excluded the possibility that the sporulation defect of the *MAT***a** *sps1/MATα sps1* strain was simply a fortuitous result of the transformation events (27).

The copy of the *SPS1* gene used to complement the sporulation defect of the *MAT***a** *sps1/MATα sps1* mutant in the experiment described above lacked the 5' end of the gene. Functional expression of this truncated gene (*'SPS1*)

TABLE 1. Sporulation efficiency of *SPS1* and *SPS2* mutants

Cross[a]		Sporulation efficiency (%)[b]	
*MAT***a**	*MATα*	Expt 1	Expt 2
SPS1 SPS2	*SPS1 SPS2*	23	16
sps1 SPS2	*SPS1 SPS2*	18	19
SPS1 SPS2	*sps1 SPS2*	25	17
sps1 SPS2	*sps1 SPS2*	0	0
sps1 SPS2	*sps1 SPS2 LEU2:'SPS1*	11	11
SPS1 sps2	*SPS1 SPS2*	26	20
SPS1 SPS2	*SPS1 sps2*	23	29
SPS1 sps2	*SPS1 sps2*	13	20

[a] The wild-type *MAT***a** (SR25-1A) strain and wild-type *MATα* (SC252) strain, or the transformants of these strains containing the mutant *sps1* or *sps2* locus described in the text, were mated as indicated in each line. Diploids were selected by prototrophic complementation.

[b] The diploids were assessed for their efficiency of sporulation by transferring patches of cells grown on presporulation plates (0.8% Bacto-yeast extract, 0.3% Bacto-peptone, 10% dextrose, 2% Bacto-agar; Difco Laboratories) for 2 days at 30°C to sporulation plates (1% potassium acetate, 0.1% Bacto-yeast extract, 0.05% dextrose, 2% Bacto-agar). After 3 days of incubation at 30°C at least 500 cells were examined microscopically for ascus formation. The two columns refer to the percentage of cells which had formed asci in two separate experiments. In each case, diploids obtained from additional independent haploid transformants gave similar results (data not shown).

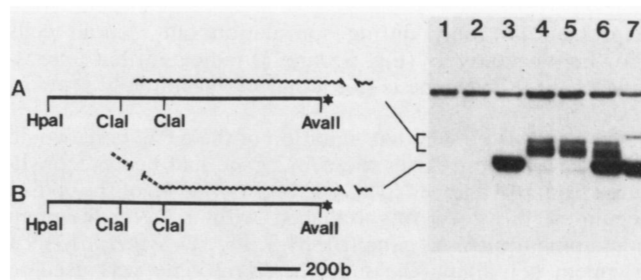

FIG. 8. S1 nuclease analysis demonstrating that transcription proceeds through the 5'-truncated *SPS1* gene integrated at the *LEU2* locus. The indicated *Hpa*I-*Ava*II DNA fragment spanning the 5' end of the *SPS1* gene was 5' end labeled at the *Ava*II terminus and used to probe for *SPS1*-specific transcripts in vegetatively growing cells (lanes 1 through 3) and in cells at 22 h of sporulation (lanes 4 through 6). RNA was extracted from *MAT***a** *SPS1/MATα SPS1* cells (lanes 1 and 4), from *MAT***a** *sps1/MATα sps1* cells (lanes 2 and 5), and from *MAT***a** *sps1/MATα sps1 LEU2:'SPS1* cells (lanes 3 and 6). The *sps1* allele refers to the *SPS1* gene disrupted at its 3' end by the insertion of a *URA3* containing DNA fragment (see the text). The *LEU2:'SPS1* locus refers to the insertion of a 5' truncated but functional copy of the *SPS1* gene at the *LEU2* locus (see the text). The DNA fragments resistant to S1 nuclease digestion after hybridization of the RNAs to the DNA probe were electrophoresed through a 5% polyacrylamide gel containing 7 M urea. The protected fragments denoted A resulted from hybridization of transcripts of the *SPS1* gene (〰) to the probe. The protected fragments denoted B resulted from hybridization of transcripts that initiated in pBR322 and extended through the 5' truncated *'SPS1* gene. The pBR322 portion of the fusion transcript is represented by the dashed line. Lane 7 shows the *Cla*I-generated partial digestion products of the *Hpa*I-*Ava*II probe. Note that in the *'SPS1* gene construct the *SPS1* gene was truncated at the *Cla*I site adjacent to the *Ava*II site.

can be accounted for by the following observations. The *Cla*I site used to transfer the *SPS1* gene from p18 to the rescue vector (p1884-2, Fig. 1; Fig. 2), although downstream from the transcriptional start sites, is upstream of the putative translational start codon of the gene (data not shown). Fortuitously, the pBR322 sequence adjacent to the *'SPS1* gene provides an efficient promoter (data not shown). An S1 nuclease analysis demonstrating that the integrated *'SPS1* gene is transcribed is shown in Fig. 8. This analysis was performed with RNA extracted from the *MAT***a** *SPS1/MATα SPS1* strain, the *MAT***a** *sps1/MATα sps1* strain, and the *MAT***a** *sps1/MATα sps1 LEU2:'SPS1* strain. The expected pattern of protection was obtained on analysis of the hybrids formed between RNA extracted from the wild-type strain or the homozygous mutant strain and a probe spanning the 5' end of the *SPS1* gene (Fig. 8A). No protection was observed with RNA from vegetatively growing cells (Fig. 8, lanes 1 and 2); the protected fragments obtained with RNA extracted from sporulating cells indicated that transcription had initiated between the two *Cla*I sites previously mapped to each side of the transcriptional start sites of the *SPS1* gene (Fig. 8, cf. lanes 4 and 5 with markers of lane 7). However, S1 nuclease treatment of the hybrids formed with RNA extracted from the *MAT***a** *sps1/MATα sps1 LEU2:'SPS1* strain either growing vegetatively or during sporulation generated an additional lower-molecular-weight fragment (Fig. 8, lanes 3 and 6). This fragment resulted from hybridization of the probe to the fusion transcript of the *'SPS1* gene (Fig. 8B, cf. lanes 3 and 6 with markers of lane 7) and indicated that transcription had initiated in the upstream pBR322 sequence and had proceeded through the *'SPS1* gene. Incidentally, the observation that the *'SPS1* gene was

expressed not only during sporulation but also in cells growing vegetatively (Fig. 8, lane 3) indicated that expression of the *SPS1* gene is not lethal to vegetatively growing cells.

We next tested whether mutation of the *SPS2* gene would affect sporulation. After the *SPS2* gene had been reconstituted from p18 and p84 (p1884-1, Fig. 1), 458 bp of the coding region of the gene was replaced with a DNA fragment containing the *URA3* gene (p1884-3, Fig. 1). An *Hpa*I DNA fragment containing the mutated *SPS2* gene was used to transform haploid strains, which were then mated to obtain a homozygous mutant diploid. Southern analysis of DNA extracted from the diploid strain verified that both copies of the *SPS2* gene had been mutated. The deletion-substitution event had expanded the *SPS2* gene, removing a *Cla*I site and introducing a *Pst*I site. This is visualized by replacement of the 2.6- and 1.6-kbp *Cla*I fragments of the wild-type gene (Fig. 7A, lane 1) by a single 6.3-kbp fragment in the mutated gene (Fig. 7C, lane 9) and by replacement of the 2.0-kbp *Pst*I fragment of the wild-type gene (Fig. 7A, lane 2) by 1.7- and 2.4-kbp *Pst*I fragments in the mutated gene (Fig. 7C, lane 10). When subjected to sporulation conditions, the homozygous mutant diploid strain was found to form asci as efficiently as the wild-type diploid strain (Table 1). Spore viability was also found to be similar for both strains (data not shown). Thus, in contrast to the *SPS1* gene, mutation of the *SPS2* gene did not lead to a readily detectable sporulation defect.

DISCUSSION

We had previously defined genes identifed in a differential hybridization screen of a yeast DNA library as sporulation specific on the basis of their preferential expression during sporulation. The genes, termed *SPS* genes, are expressed in *MAT***a**/*MAT*α cells of *S. cerevisiae* during sporulation but are not expressed during exponential growth or in asporogenous *MAT***a**/*MAT***a** and *MAT*α/*MAT*α strains after nutrient deprivation (23). We presume that the activation of these genes reflects regulatory events initiated in *MAT***a**/*MAT*α cells in response to nitrogen deprivation in the presence of an oxidative carbon source. However, the observation by Kaback and Feldberg that the products of genes preferentially expressed in *MAT***a**/*MAT*α cells during sporulation are not necessarily involved in the differentiation process (12) prompted us to test our presumption more directly by performing a mutational analysis of the *SPS1* and *SPS2* genes. Demonstration of a functional requirement for an *SPS* gene product during sporulation would support the idea that expression of the gene is controlled by development-specific regulatory mechanisms. Our mutational analysis has demonstrated that the product of the *SPS1* gene, which was identified on the basis of it expression pattern rather than function, is indeed essential for the completion of the sporulation process. A *MAT***a** *sps1*/*MAT*α *sps1* strain failed to form asci when subjected to sporulation conditions. This supports the suggestion that the activation of the *SPS1* gene reflects sporulation-specific regulatory events rather than fortuitous expression only incidentally influenced by sporulation-specific events.

In contrast to the *SPS1* gene, mutation of the *SPS2* gene had no apparent effect on the ability of a *MAT***a**/*MAT*α diploid strain homozygous for the mutation to form viable spores. Yamashita and Fukui (34) have recently shown that the *SGA* gene of *S. cerevisiae*, encoding a sporulation-specific glucoamylase activity, is also dispensable for the formation of viable spores. Since examination of ascus formation and spore viability only monitors the most significant meiotic and morphological events of sporulation, a more careful analysis of spore formation (such as monitoring the rate and frequency of recombination and measuring the degree of resistance of mature spores to various chemical agents) may be required to reveal a subtle defect in the developmental pathway caused by mutation of these genes. Alternatively, it is possible that an *SPS* gene product, although contributing to the sporulation process, may not itself be essential; for instance, another *SPS* gene product may have an overlapping function such that it can compensate for the absent gene product. Preliminary experiments support the idea that activation of the *SPS2* gene, although not essential for sporulation, is directly dependent on sporulation-specific regulatory events. The presence of the 5′ flanking region of the *SPS2* gene on a high-copy-number plasmid reduces the rate and efficiency of spore formation (A. Percival-Smith, unpublished observations). One interpretation of this observation is that the cloned *SPS2* DNA is sequestering a common transcriptional activator which is required not only for the expression of the *SPS2* gene but also for the expression of other sporulation-specific genes and that the products of at least some of these genes are required for key events in the sporulation process.

Although in general functionally related genes of yeast are not linked, our previous studies (23) and those of Clancy et al. (4) suggested that some sporulation-specific genes are contiguous. We have described in this report a cluster of three linked sporulation-specific genes. Since plasmids p18 and p84 do not extend beyond the 3′ end of the *SPS3* gene nor very far beyond the 5′ end of the *SPS1* gene, we do not yet know whether any additional *SPS* genes map to this cluster. Under the presporulation growth conditions that we use (a rich acetate medium), we do not find any genes expressed uniquely during exponential growth mapping within this cluster of sporulation-specific genes (Fig. 3). Interestingly, development-specific genes of several other organisms (e.g., *Aspergillus nidulans* [21] and *Neurospora crassa* [2]) have also been shown to be clustered. Although this suggests that there might be a global mechanism involved in the control of each cluster of genes, our study of an *SPS2-lacZ* fusion gene has indicated that the immediate 5′ flanking region of the *SPS2* gene is sufficient for its sporulation-specific expression. This does not exclude, however, the possibility that more distant regulatory sequences contribute to the developmental activation of the *SPS2* gene. A more detailed analysis of the regulation of expression of the *SPS2* gene will define the precise nature of the sequences controlling the sporulation-specific activation of this gene.

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