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The *SPS100* Gene of *Saccharomyces cerevisiae* Is Activated Late in the Sporulation Process and Contributes to Spore Wall Maturation

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We previously described the use of a differential hybridization screen of a genomic DNA library of *Saccharomyces cerevisiae* to identify sporulation-specific (*SPS*) genes (A. Percival-Smith and J. Segall, Mol. Cell. Biol. 4:142–150, 1984). This initial screen identified 14 *SPS* genes that are first expressed 6 to 8 h after transfer of cells to sporulation medium. Accumulation of transcripts corresponding to these genes becomes maximal at 8 to 12 h of sporulation, the time at which meiotic events are nearing completion, and by 15 h of sporulation, transcript levels are beginning to decrease. In the present study two additional *SPS* genes, first expressed at 12 h of sporulation, were isolated. The steady-state level of transcripts corresponding to these two genes, termed *SPS100* and *SPS101*, remains unchanged from 15 to 35 h, a time coincident with spore wall maturation. The nature of the putative 34.2-kilodalton protein encoded by the *SPS100* gene is consistent with its being a component of the glycoprotein matrix of the spore wall; the protein contains a potential signal sequence and cleavage site and numerous sites for potential glycosylation. A *MAT*a *sps100*/*MAT*α *sps100* strain was found to be indistinguishable from the wild-type strain when assessed for efficiency of ascus formation and spore viability. However, a more detailed analysis of the mutant strain revealed that the *SPS100* gene product serves a protective role during the early stages of spore wall formation. The time at which resistance to ether could first be detected in developing spores was delayed by 5 h in the mutant strain relative to the wild-type strain. This phenotype is presumably a reflection of a defect in spore wall maturation. This study has confirmed that temporally distinct classes of sporulation-specific genes are sequentially activated during the process of meiosis and spore formation and has shown that the *SPS100* gene, identified on the basis of its developmental-specific expression pattern, contributes to spore development.

Detailed studies of the mechanisms involved in controlling promoter selection in eucaryotes have been limited in part by the complexities of the eucaryotic transcriptional machinery and also by the difficulty of performing biochemical and genetic analyses. Each of these approaches has made key contributions towards the elucidation of the molecular processes regulating gene expression in procaryotes; the products of genes implicated in the regulation of gene expression on the basis of their mutant phenotypes were shown by elegant in vitro experiments to be intimately involved in directing promoter selection. Considering the valuable insights gained in these studies by mutational analyses, it is not surprising that *Saccharomyces cerevisiae*, a yeast in which a variety of genetic manipulations can be readily performed (37), has emerged as a favored organism for studying eucaryotic gene expression. Moreover, *S. cerevisiae* also provides an opportunity to investigate the control of sequential gene expression during differentiation of a eucaryotic cell; appropriate nutritional cues cause diploid *MAT*a/*MAT*α cells to enter a well-characterized developmental program leading to haploid spore formation.

The process of sporulation in *S. cerevisiae* consists of a series of meiotic events including DNA replication, recombination, and chromosome segregation, followed by the formation of spore walls around the four haploid nuclei (for a review, see references 7 and 11). Entry into the sporulation pathway is dependent on the *MAT*a/*MAT*α genotype (15). Several different approaches have been pursued to demonstrate that the subsequent program of genetic and morphological events reflects the sequential expression of sporulation-specific genes. Although analyses of in vivo-labeled proteins by one- and two-dimensional gel electrophoresis revealed disappointingly few sporulation-specific proteins (17, 20, 31, 38, 45, 46), mutational analyses identified a class of genes, termed *SPO*, dispensable for mitotic growth but essential for the completion of sporulation (10, 39). Expression of the *SPO13* gene has recently been shown to be developmentally regulated (41). In an alternative approach, genes expressed preferentially during sporulation have been directly identified by differential hybridization screening procedures (4, 13, 28). The transcripts encoded by these genes accumulate before the completion of meiosis (13, 16, 28). In complementary studies, analyses of the in vitro coding capacities of RNAs isolated from sporulating cells demonstrated the accumulation of an array of sporulation-specific mRNAs most of which also appear before the completion of meiosis (21, 44). Two of the sporulation-specific transcripts identified in a study performed by Kurtz and Lindquist, however, begin to accumulate only after spore wall synthesis has begun (22). Interestingly, these two late transcripts, but not the sporulation-specific transcripts which accumulate earlier during meiosis, are present in mature asci (21). This observation prompted us to perform a differential hybridization screen of an *S. cerevisiae* DNA library using probes prepared with RNA from spores. As described in this report, this screen identified two sporulation-specific genes which are expressed very late during the sporulation process. A mutational analysis revealed that one of these genes contributes to spore wall maturation.

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MATERIALS AND METHODS

Strains and culture conditions. The *S. cerevisiae* strains used were the AP-3 *MAT***a**/*MAT***a**, *MAT***a**/*MAT*α, and *MAT*α/*MAT*α diploids previously employed (28) and strains W3031A-H (*MAT***a** *can1-100 leu2-3,112 trp1-1 ura3-1 ade2-1*) and W3031B-T (*MAT*α *can1-100 his3-11 leu2-3,112 ura3-1 ade2-1*), kindly provided by D. Fields and A. Finkelstein, respectively. The latter two strains were derived by integrative transformation of the isogenic haploids W3031A and W3031B (constructed by R. Rothstein and provided by S. Lindquist) (32) with DNA fragments containing the wild-type *HIS3* and *TRP1* alleles, respectively. These alleles were introduced to allow prototrophic selection of diploids. Sporulation of AP-3 and the diploid obtained by mating W3031A-H and W3031B-T, termed LP-HT, was as described previously (28) except that LP-HT was grown to 2.5×10^6 to 5×10^6 cells per ml in YEPA medium (1% yeast extract, 2% peptone, 1% potassium acetate, pH 5.5) and then transferred to sporulation medium (1% potassium acetate) at 10^7 cells per ml. Under these conditions, 40 to 60% of the cells formed asci. Ascus formation was monitored by light microscopy using phase-contrast optics.

The *MAT***a** *sps100 sps101*/*MAT*α *sps100 sps101* strain was obtained by standard genetic techniques by mating the appropriate spores from a *MAT***a** *HIS3 trp1-1 ura3-1 sps100 SPS101*/*MAT*α *his3-11 TRP1 ura3-1 SPS100 sps101* diploid. The latter strain was obtained by mating a *MAT***a** *sps100* strain and a *MAT*α *sps101* strain, derived from W3031A-H and W3031B-T, respectively, as described below.

Differential hybridization screen. A differential hybridization screen of a partial *Sau*3A genomic DNA library of *S. cerevisiae* (28) was performed as described (28) using cDNA probes prepared with poly(A)$^+$ RNA extracted from AP-3 *MAT***a**/*MAT*α and *MAT*α/*MAT*α cells at 35 h after transfer to sporulation medium. DNA dot-blot analysis and determination of the sizes of the transcripts were done as previously described (28).

Plasmid constructions, integrative transformations, and DNA sequence determination. To sequence the *SPS100* gene, the 3.0-kilobase-pair (kbp) fragment of pB8 that extends from the *Cla*I site in the vector sequence to the *Kpn*I site downstream of the *SPS100* gene (see Fig. 4A) was cloned in both orientations at the *Sma*I site of the polylinker of pEMBL18 to generate pE18-B8a and pE18-B8b. A set of plasmids containing overlapping deletions of the *SPS100* gene was generated for each of these plasmids by restriction of the plasmid DNA with *Xba*I and *Pst*I, followed by treatment with exonuclease III and S1 nuclease, as described by Henikoff (14). The sequence of both strands of the *SPS100* gene was determined by the method of Sanger et al. (35).

The plasmid pE18-Δ*SPS100* was used to delete the chromosomal *SPS100* gene by integrative transformation. This plasmid contains the *SPS100* gene with the entire transcribed region deleted and replaced with a DNA fragment containing the *URA3* gene and was constructed from deleted versions of the gene prepared as templates for sequencing (see above) as follows. A 600-bp *Hin*dIII fragment containing the 5′ flanking region of the *SPS100* gene to nucleotide −7 was purified from a pE18-B8a-derived plasmid. This fragment extends from the *Hin*dIII site in the portion of pBR322 that was subcloned with the *SPS100* gene into pEMBL18 in the construction of pE18-B8a, through the 5′ flanking region of the *SPS100* gene, to the *Hin*dIII site in the polylinker of pEMBL18. This fragment was inserted into the *Sph*I site of a pE18-B8b-derived plasmid that contained the 3′ flanking region of the *SPS100* gene beginning at nucleotide 1942. The resulting plasmid contained the 5′ and 3′ flanking regions of the *SPS100* gene oriented in the same direction. A unique *Sph*I site, introduced with the *Hin*dIII fragment from pE18-B8a, was present in the polylinker region between the two portions of the gene. A 1.2-kbp *Hin*dIII fragment containing the *URA3* gene was inserted into this *Sph*I site to generate pE18-Δ*SPS100*. The *SPS100* genes of W3031A-H and W3031B-T were deleted by integrative transformation (34) using the *Eco*RI fragment of pE18-Δ*SPS100* indicated in Fig. 7A. The construction of a replicating plasmid containing the *SPS100* gene as well as *LEU2*, *URA3*, *ARS1*, and *CEN3* sequences will be presented elsewhere.

pC2::*URA3*, the plasmid used to introduce a disruption into the chromosomal copy of the *SPS101* gene, was constructed by introducing a 1.2-kbp *Hin*dIII fragment containing the *URA3* gene into the unique *Sac*I site of pC2. This site is within the transcribed portion of the gene approximately 320 bp from the 3′ end of the gene. The *SPS101* genes of W3031A-H and W3031B-T were disrupted by integrative transformation using the *Eco*RI fragment of pC2::*URA3* shown in Fig. 7C.

DNA manipulations were carried out by previously described procedures (25). Transformation of yeast spheroplasts generated by glusulase treatment was performed as described previously (28).

Transcript mapping. The approximate positions of the sequences on pB8 and pC2 encoding the *SPS100* and *SPS101* transcripts, respectively, were determined by hybridizing RNA that had been radioactively labeled in vitro (24, 28) to Southern blots (36) of plasmid DNA that had been restricted with a variety of restriction enzymes. The ends of the transcripts were then positioned using the S1 nuclease procedure of Berk and Sharp (2) as modified by Weaver and Weissmann (43), with optimal hybridization temperatures being determined empirically for each probe. The 5′ ends of the transcripts encoded by the *SPS101* gene were mapped using a fragment extending from a *Hin*dIII site in the vector sequence upstream of the gene to the *Kpn*I site within the gene (Fig. 4B). The *Kpn*I end was 5′-end labeled by treatment with alkaline phosphatase and T4 polynucleotide kinase after removal of the 3′ overhanging nucleotides with the Klenow fragment of DNA polymerase. The 3′ ends of *SPS101* transcripts were positioned using a *Mlu*I-*Bam*HI fragment (Fig. 4B) 3′-end labeled at the *Mlu*I site. The 5′ ends of the transcripts encoded by the *SPS100* gene were initially mapped using an *Eco*RI-*Sal*I fragment (Fig. 4A) 5′-end labeled at the *Sal*I end. The 3′ ends of the transcripts encoded by the *SPS100* gene were mapped using a *Sal*I-*Eco*RI fragment (Fig. 4A) 3′-end labeled at the *Sal*I site. Detailed S1 nuclease mapping of the ends of the *SPS100* transcripts is presented in Fig. 6.

Assay for ether resistance. Resistance of cells to diethyl ether was assessed according to the procedure of Dawes and Hardie (8). Briefly, cells were grown vegetatively and transferred to sporulation medium as described above. At various times, 2×10^7 cells were collected by centrifugation and suspended in 100 μl of 0.2 M potassium acetate (pH 5.5) in a 1.5-ml plastic tube. A 5-μl sample of cells was diluted and plated to determine the viability of cells before exposure to ether. A 100-μl volume of ethyl ether (Caledon) was then added, and the sample was mixed by rotation on an Adams Nutator platform (Fisher Scientific) for 12 min at room temperature. After the phases had separated, duplicate portions of cells were immediately diluted and then plated to

determine cell survival. Under these conditions, survival of vegetative cells was less than 10^{-6} (the limit of detection) and survival of mature spores was approximately 10^{-2}.

RESULTS

Identification of genes encoding transcripts that accumulate at a very late stage of sporulation. We previously performed a differential hybridization screen of an *S. cerevisiae* DNA library, using as probes radioactively labeled cDNAs prepared with RNA purified from cells harvested before 15 h of sporulation (28). This screen identified 14 sporulation-specific (*SPS*) genes that are expressed at 6 to 15 h after transfer of *MAT***a**/*MAT*α cells to sporulation medium (28; D. T. S. Law, unpublished data; see below). This is the time at which meiotic events are occurring (17). We refer to these genes as middle sporulation genes. In the present study, a similar screen was performed using probes prepared with poly(A)$^+$ RNA isolated from *MAT***a**/*MAT*α and *MAT*α/*MAT*α cells at 35 h after transfer to sporulation medium. The goal of this screen was to identify sporulation-specific genes first expressed as the sporulation process is nearing completion. Duplicate sets of nitrocellulose filters containing the DNA print of 14,000 bacterial colonies from an *S. cerevisiae* genomic DNA library (28) were hybridized with the probes (see Materials and Methods). A total of 260 colonies gave an enhanced hybridization signal with the *MAT***a**/*MAT*α probe relative to the *MAT*α/*MAT*α probe. Upon rescreening, 68 of these clones hybridized preferentially with the *MAT***a**/*MAT*α cDNA probe. For a more quantitative comparison, plasmid DNA purified from each of these clones was hybridized with the two probes. In this DNA dot-blot analysis, 19 DNAs consistently hybridized preferentially with the *MAT***a**/*MAT*α probe (data not shown). Before analyzing the putative sporulation-specific genes present in these 19 clones in more detail, we compared the plasmid DNAs to determine whether the same yeast sequence had been identified more than once.

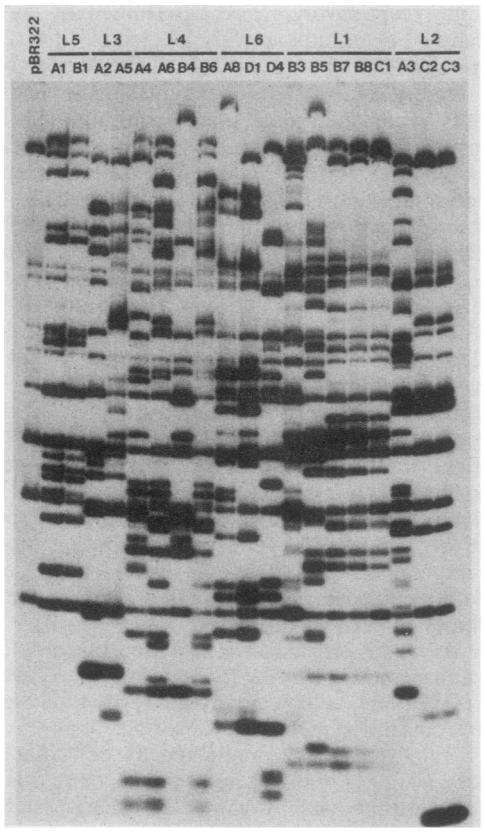

FIG. 1. Comparison of the fragments generated by treatment of plasmid DNAs with *Hin*fI. The various plasmid DNAs, as indicated along the top of the autoradiogram (L, group number; A1-C3, plasmid name), were digested with *Hin*fI, and the resulting fragments were radioactively labeled and separated by electrophoresis through an 8% polyacrylamide gel as described previously (28).

TABLE 1. Plasmids containing sequences complementary to transcripts present preferentially in 35-h spores

Group	Plasmids	Transcript (kilobases)
L1	B3, B5, B7/C1,[a] B8	1.6
L2	A3, C2/C3	2.2
L3	A2, A5	0.8
L4	A4, A6/B6, B4	1.6
L5	A1, B1	2.0
L6	A8, D1, D4	1.6

[a] The shill indicates that the two plasmids are identical.

The purified plasmid DNAs were restricted with *Hin*fI, and the resulting fragments were radioactively end labeled and separated by polyacrylamide gel electrophoresis. A comparison of the DNA fragments suggested that the 19 plasmids represented six distinct yeast sequences. Plasmids presumed to represent the same yeast DNA sequence did not necessarily have exactly the same array of *Hin*fI-generated fragments, but they did contain several comigrating fragments, indicating that the yeast DNA inserts contained overlapping sequences (Fig. 1). The plasmids were also compared by determining the sizes of the transcripts encoded by the cloned genes; radioactively labeled plasmid DNAs were hybridized with filters containing poly(A)$^+$ RNA that had been isolated from *MAT***a**/*MAT*α cells at 35 h after transfer to sporulation medium. Representative plasmid DNAs which had been presumed to contain similar yeast sequences hybridized with transcripts of identical size (data not shown). We therefore classified the 19 plasmids into six groups, termed L1 through L6, presuming that each group represented a distinct late gene (Table 1). Although the genes represented by groups L1, L4, and L6 all encoded a 1.6-kilobase transcript, the three genes appeared to be distinct on the basis of the partial sequence comparison described above and a comparison of their expression patterns (see below).

To verify that the genes identified in the present screen were distinct from the middle sporulation-specific genes that we had identified previously (28), we compared the temporal patterns of expression of the two groups of genes. For this analysis, various plasmid DNAs were initially used to probe a filter containing RNA extracted from *MAT***a**/*MAT*α cells growing vegetatively and at 7.5, 10, 12, and 15 h after transfer of cells to sporulation medium. Transcripts corresponding to the previously identified middle genes began to accumulate over a 4-h period; for example, the sporulation-specific gene represented by p74 was actively expressed at 7.5 h of sporulation, whereas active expression of one of the genes represented by p94 began between 10 to 12 h of sporulation (Fig. 2A, G14 and G11, respectively). Most of the middle genes, however, were first expressed between 6 and 8 h after transfer of cells to sporulation medium (Fig. 2A; data not shown). This is the time at which meiosis I is nearing completion (17).

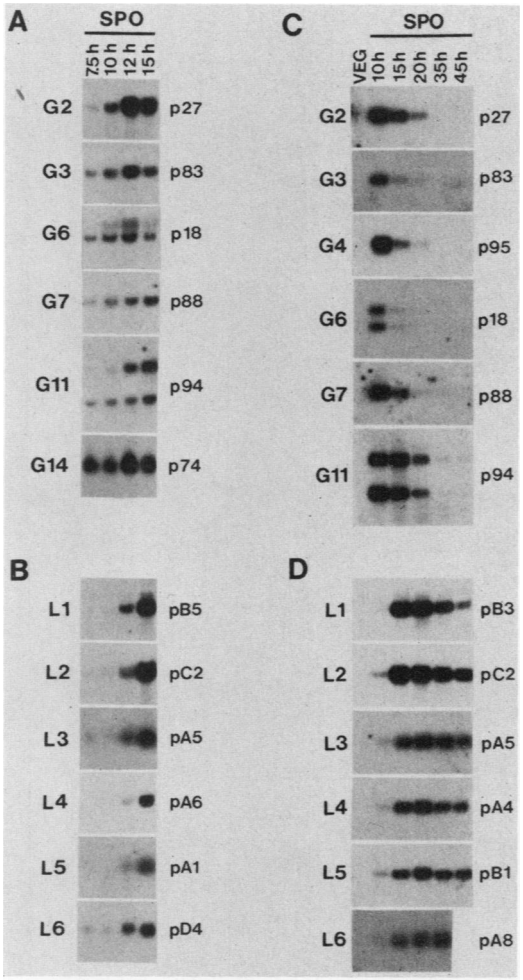

FIG. 2. Temporally distinct patterns of expression for middle genes and late genes. RNA purified from AP-3 *MAT***a**/*MAT*α cells growing vegetatively (VEG) and from cells at the indicated times after transfer to sporulation medium (SPO) was separated on a 1.5% agarose-formaldehyde gel and then transferred to a nylon membrane. The RNA blots were hybridized (28) with the indicated plasmid DNAs (group name given on the left, plasmid name given on the right) radioactively labeled by nick translation (33). Only the portions of the membranes containing transcripts which hybridized with the probes are shown. The 45-h lane for L6 in panel D is absent. The RNAs used for the blots of A and B were prepared from cells grown and sporulated at a separate time than the cells used to prepare RNA for the blots of C and D. Total RNA was used for A and B, and poly(A)$^+$ RNA was used for C and D. The plasmids representing the groups G2, G3, G4, G6, G7, G11, and G14 contain middle sporulation genes isolated previously (28).

Significant expression of the newly identified late genes did not begin until 12 h after the transfer of cells to sporulation medium (Fig. 2B). At this time, expression of the middle genes was maximal (Fig. 2A). The steady-state level of transcripts corresponding to the late genes increased from 12 to 15 h of sporulation (Fig. 2B). This corresponded to the time at which asci could first be detected by light microscopy (data not shown).

In a separate experiment, transcript accumulation was assessed at 10, 15, 20, 35, and 45 h after transfer of cells to sporulation medium (Fig. 2C and D). In this latter experiment, the sporulation process progressed slightly faster than in the experiment described above (Fig. 2A and B). The display of the transcript levels present at these later times clearly depicted the distinct expression patterns of the two sets of genes. Transcripts corresponding to the middle genes decreased in abundance from 10 to 20 h of sporulation (Fig. 2C; data not shown). As expression of these genes was being turned down, expression of the late genes was being activated (Fig. 2D). Transcripts encoded by the late genes increased from a barely detectable level at 10 h to near maximal levels by 15 h (Fig. 2D). The late transcripts remained abundant in cells that had been in sporulation medium for 35 h (Fig. 2D), whereas transcripts corresponding to the middle genes were almost undetectable in these cells (Fig. 2C). Transcripts corresponding to the late genes were also present in 45-h spores. This may reflect continued transcription of these genes until the time at which the spores became metabolically quiescent, or, alternatively, these transcripts may be unusually stable.

This comparison of the temporal patterns of expression of the middle and late genes clearly demonstrated that activation of the late genes occurred several hours after activation of the middle genes. This suggests that transcription of the two classes of genes occurs in response to distinct regulatory mechanisms. It should be noted that this description of gene expression was based on monitoring the steady-state levels of transcripts. We have assumed that the time at which transcripts began to accumulate reflected the time at which transcription of the corresponding gene was activated.

Sporulation-specific expression of the *SPS100* and *SPS101* genes. We next examined the developmental specificity of expression of the newly identified late genes. Filters containing poly(A)$^+$ RNA purified from *MAT***a**/*MAT***a**, *MAT***a**/*MAT*α, and *MAT*α/*MAT*α cells during vegetative growth and at 15, 20, and 35 h after transfer of cells to sporulation medium were hybridized with labeled plasmid DNAs. This analysis confirmed that expression of two of the genes was restricted to sporulating cells; the gene represented by the group L1 plasmids (Fig. 3A) and the gene represented by the group L2 plasmids (Fig. 3B) hybridized with transcripts present only in *MAT***a**/*MAT*α cells after transfer to sporulation medium. These two genes have been termed *SPS100* and *SPS101*, respectively. Restriction maps of the overlapping plasmids pB3 and pB8 containing the *SPS100* gene and of pC2 containing the *SPS101* gene are shown in Fig. 4. The approximate positions of the transcripts encoded by these genes are indicated (Fig. 4; see Materials and Methods).

Transcripts encoded by the four other late genes (represented by the plasmids of group L3 through L6) also accumulated preferentially in *MAT***a**/*MAT*α cells at 35 h after transfer to sporulation medium and were absent from vegetatively growing cells. However, at 20 h after transfer of cells to sporulation medium, significant accumulation of transcripts corresponding to the L3, L4, L5, and L6 genes could be detected not only in *MAT***a**/*MAT*α cells but also in the asporogenous *MAT***a**/*MAT***a** and *MAT*α/*MAT*α cells (Fig. 3C through F). Thus, the expression of these latter genes is not sporulation specific.

The hybridization pattern obtained with the control plasmid pC4, which contains an uncharacterized gene expressed in all cell types both during vegetative growth and after transfer to sporulation medium, indicated that similar amounts of intact RNAs had been used for the comparisons described above (Fig. 3G).

Characterization of the *SPS100* gene. We characterized the *SPS100* gene in more detail. The sequence of the gene is shown in Fig. 5. The only long open reading frame present in this sequence encodes a predicted protein of 326 amino acids

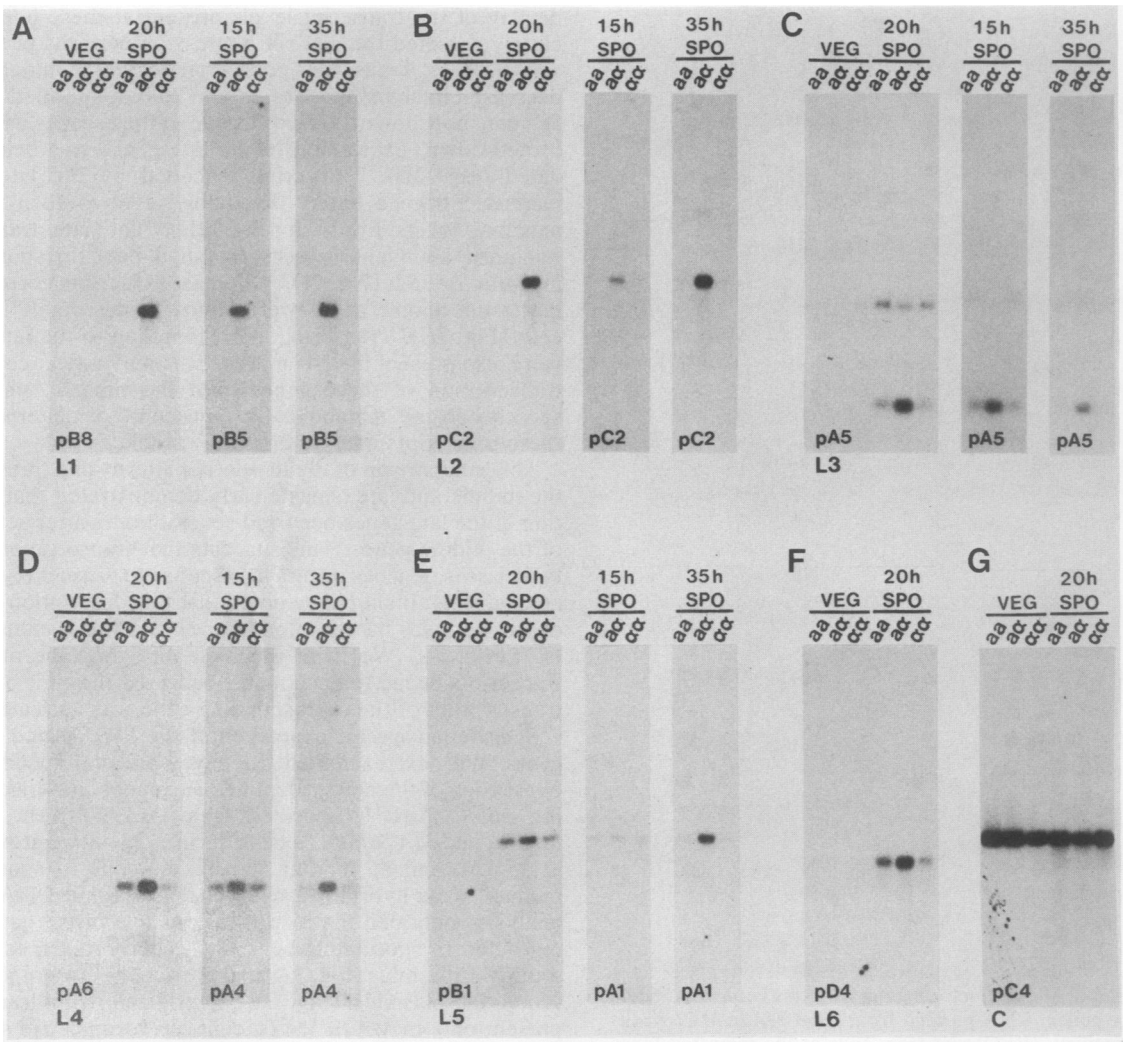

FIG. 3. Examination of the developmental specificity of the transcripts encoded by the cloned DNAs. Poly(A)$^+$ RNA, purified from AP-3 *MAT***a**/*MAT***a**, *MAT***a**/*MAT*α, and *MAT*α/*MAT*α cells (as indicated above each lane) growing vegetatively (VEG) and at 15, 20, and 35 h after transfer to sporulation medium (SPO), was denatured, separated by electrophoresis through a 1.5% agarose-formaldehyde gel, and then transferred to a nylon membrane. The RNA blots were then hybridized with plasmid DNAs (as indicated at the bottom left-hand corner of each filter) that had been radioactively labeled by nick translation.

with a molecular weight of 34,222. As a prerequisite for studies aimed at elucidating the regulatory mechanisms responsible for the activation of the *SPS100* gene, we performed an S1 nuclease analysis to map the exact start of transcription. The *Eco*RI-*Nhe*I DNA fragment indicated in Fig. 6A was hybridized with RNA extracted from *MAT***a**/*MAT*α cells at 20 h after transfer to sporulation medium. Analysis of the S1 nuclease-resistant hybrids indicated that there were multiple transcriptional initiation sites spanning a 42-nucleotide sequence 18 to 59 bp upstream of the putative translational start codon (Fig. 5 and Fig. 6A, lane 5). A putative TATA box was found 49 to 54 nucleotides upstream from the first transcriptional start site (Fig. 5). Preliminary mapping of the 3′ ends of the transcripts encoded by the *SPS100* gene had indicated that the 3′ ends mapped to three separate sites over a 260-bp sequence (data not shown). Interestingly, the transcripts of the *SPS101* gene also had multiple 3′ ends that mapped to two distinct sites 150 bp apart (data not shown). Positioning the 3′ ends of the longest *SPS100* transcripts onto a *Sau*3A-*Hin*dIII fragment (Fig. 6B) indicated that these transcripts contained a rela-

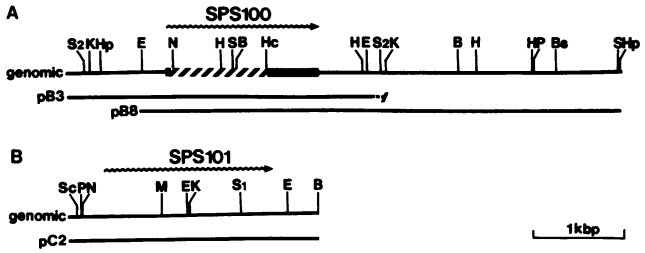

FIG. 4. Restriction map of the *SPS100* and *SPS101* genes. The restriction maps of (A) the *SPS100* gene as determined from the overlapping plasmids pB3 and pB8 and (B) the *SPS101* gene as determined from the plasmid pC2 are shown for selected endonucleases. The positions of the transcripts encoded by these genes are indicated (⟿), with the arrowhead marking the direction of transcription. The open reading frame of the *SPS100* gene is indicated by the hatched line. The yeast DNA inserts present in pB3, pB8, and pC2 are shown. pB3 contains two noncontiguous yeast DNA fragments; only the fragment from the *SPS100* locus is shown. The direction of transcription of the *SPS* genes in pB8 and pC2 is the same as that of the *tet* gene of the vector. B, *Bam*HI; Bs, *Bst*EII; E, *Eco*RI; H, *Hin*dIII; Hc, *Hin*cII; Hp, *Hpa*I; K, *Kpn*I; M, *Mlu*I; N, *Nhe*I; P, *Pst*I; S, *Sal*I; S1, *Sac*I; S2, *Sac*II; Sc, *Sca*I.

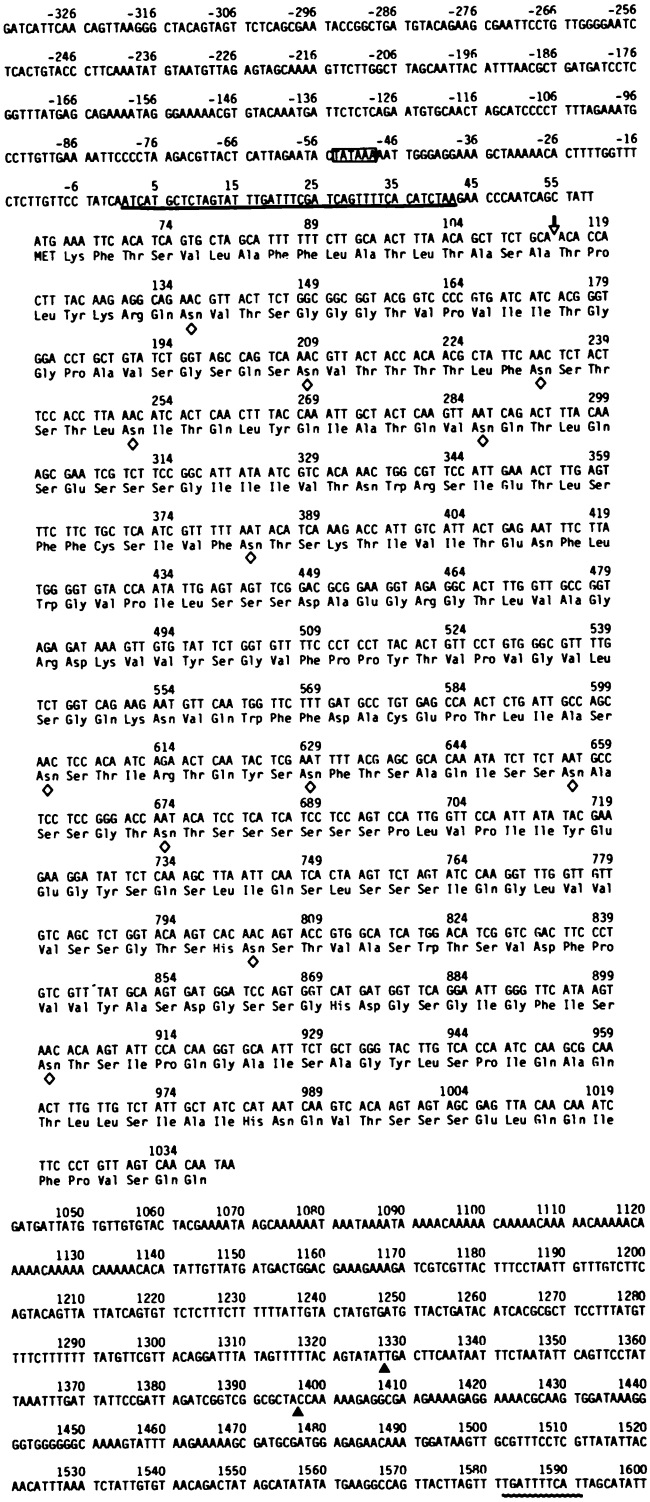

FIG. 5. Nucleotide sequence of the nontranscribed strand of the *SPS100* gene. The nucleotide sequence was obtained for both strands by the method of Sanger et al. (35) on progressive deletions of the *SPS100* gene cloned in pE18-B8a and pE18-B8b (see Materials and Methods). The sequence encompassing the multiple transcriptional initiation sites is indicated with the unbroken underline, with the most distal initiation site being denoted +1. The putative TATA box is boxed. Translation of the predicted open reading frame of the *SPS100* gene is shown below the nucleic acid sequence. The arrow indicates the putative signal sequence cleavage site. Potentially glycosylated asparagine residues are marked by the diamonds. The 3′ ends of the two shortest transcripts encoded by the gene map to the sites indicated by the solid triangles, and the 3′ ends of the longest transcripts map to the sequence underscored with a wavy line.

tively long (540-bp) 3′ untranslated sequence (Fig. 5 and Fig. 6B, lane 5). Within this untranslated sequence, the sequence A_5C was noted to be tandemly repeated eight times. As a control, the S1 nuclease analyses were also performed with RNA purified from cells growing vegetatively (Fig. 6A and B, lane 3) and from asporogenous *MATα/MATα* cells at 20 h after transfer to sporulation medium (Fig. 6A and B, lane 4). No S1-resistant RNA-DNA hybrids could be detected.

Delayed spore wall maturation in a *MAT*a *sps100/MATα sps100* strain. The most rigorous definition for a developmental-specific gene demands not only that expression of the gene be restricted to the appropriate cells, but also that the gene product serve a developmental-specific function. Therefore, to lend further support to our presumption that the activation of the *SPS100* gene is the consequence of a developmental-specific transcriptional control mechanism, we sought to demonstrate that the *SPS100* gene product is required for completion of the sporulation process.

In speculating on a possible function for the *SPS100* gene product, we conjectured that the protein was involved in the process of spore wall maturation for the following reasons. First, the *SPS100* gene was first expressed approximately 12 h after transfer of cells to sporulation medium, a time by which meiotic events have been completed and spore wall formation is beginning. Second, the characteristics of the predicted *SPS100* gene product are consistent with the protein having a role in spore wall biogenesis. The first step in the development of the spore wall is the coalescence of small lipid vesicles to generate the prospore wall, a double membrane surrounding each nucleus. The spore wall then forms by the deposition of glucans and mannoproteins between the two membranes, followed by the appearance of a glucosamine-containing, outer spore coat (reviewed in references 1 and 3). The predicted *SPS100* gene product contains a potential signal sequence; an amino-terminal hydrophobic sequence extends to a potential cleavage site at residue 18 (Fig. 5) (40, 42). The protein could be highly glycosylated; there are numerous sites for possible O-linked carbohydrates due to the unusually high number of serine (18%) and threonine (10%) residues, and 12 asparagine residues conform to sites for possible N-linked glycosylation [Asn-X-Ser(Thr)] (Fig. 5) (19). Therefore it seemed possible that the leader sequence of the *SPS100* gene product would allow translocation of the protein into the spore wall with the extensive glycosylation of the protein contributing to the matrix of the spore wall polysaccharides.

To test the presumption that the *SPS100* gene product plays a role in spore wall development, we performed a mutational analysis using the one-step gene disruption procedure described by Rothstein (34). *MAT*a and *MATα* strains with the *SPS100* gene deleted were obtained as follows. A plasmid was constructed that contained the sequences upstream and downstream of the *SPS100* gene but that had the entire transcribed region of the gene deleted (from nucleotide −7 to 350 nucleotides beyond the 3′ end of the gene) and replaced with a 1.2-kbp DNA fragment containing the *URA3*

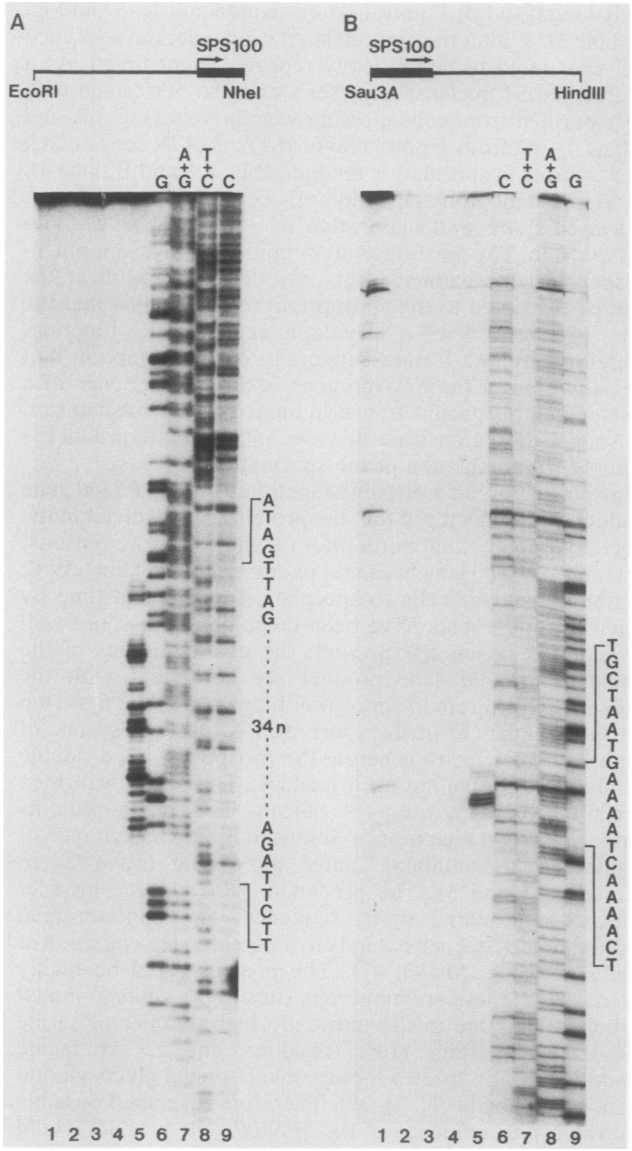

FIG. 6. Mapping the ends of the transcript encoded by the *SPS100* gene. (A) The 5′ end and (B) the 3′ end of the gene were mapped by an S1 nuclease analysis using the indicated *Eco*RI-*Nhe*I fragment 5′-end labeled at the *Nhe*I end and the *Sau*3A-*Hin*dIII fragment 3′-end labeled at the *Sau*3A end, respectively, as probes (lanes 1 through 5). The DNAs were hybridized with 10 μg of total RNA purified from AP-3 *MAT***a**/*MAT*α cells growing vegetatively (lane 3), *MAT***a**/*MAT*α cells 20 h after transfer to sporulation medium (lanes 1 and 5), or *MAT*α/*MAT*α cells 20 h after transfer to sporulation medium (lane 4). S1 nuclease was omitted from the reaction of lane 1; the reaction of lane 2 contained *E. coli* tRNA. The S1-resistant hybrids were heat denatured and subjected to electrophoresis on an 8% (A) or 6% (B) polyacrylamide–8 M urea gel adjacent to a chemical sequencing ladder (27) of the *Eco*RI-*Nhe*I fragment (A, lanes 6 through 9) or the *Sau*3A-*Hin*dIII fragment (B, lanes 6 through 9).

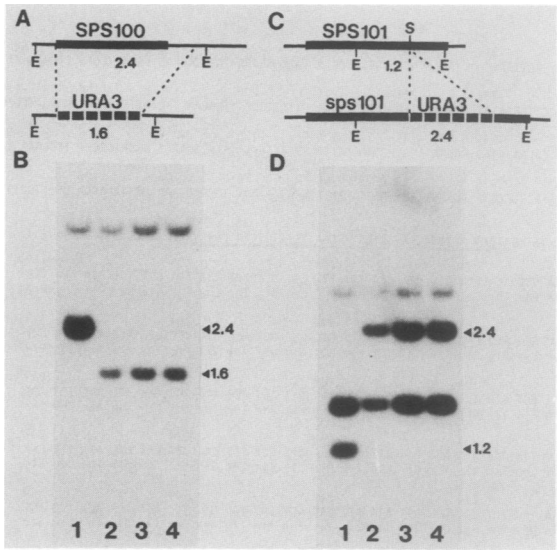

FIG. 7. Southern analysis of the mutant *sps100* and *sps101* loci. (A) *Eco*RI restriction map of the wild-type *SPS100* locus and the locus at which the entire *SPS100* gene had been replaced with a DNA fragment containing the *URA3* gene (see Materials and Methods). (C) *Eco*RI restriction map of the wild-type *SPS101* locus and the locus disrupted by insertion of a DNA fragment containing the *URA3* gene (see Materials and Methods). (B and D) Genomic DNA extracted by the rapid isolation procedure of Davis et al. (6) from the wild-type diploid strain LP-HT (B and D, lane 1), from a *MAT***a** *sps100*/*MAT*α *sps100* strain (B, lane 2), from a *MAT***a** *sps101*/*MAT*α *sps101* strain (D, lane 2), and from two independent *MAT***a** *sps100* *sps101*/*MAT*α *sps100* *sps101* strains (B and D, lanes 3 and 4) was digested with *Eco*RI, separated on a 1% agarose gel, and transferred to a nylon membrane. The blots were hybridized with (B) pE18-B8a DNA, which contains the *SPS100* gene (see Materials and Methods), and (D) pC2 DNA, which contains the *SPS101* gene, that had been radioactively labeled by nick translation. The sizes of the *Eco*RI fragments are given in kilobase pairs.

gene (Fig. 7A; see Materials and Methods). This plasmid, pE18-Δ*SPS100*, was treated with *Eco*RI to separate the yeast sequence from the vector sequence, and the DNA was then used to transform a *MAT***a** *ura3* strain (W3031A-H) and a *MAT*α *ura3* strain (W3031B-T) to Ura$^+$. A *MAT***a** transformant was then mated with a *MAT*α transformant, and the genotype of the diploid strain was verified. The Southern analysis depicted in Fig. 7B confirmed that both chromosomal copies of the *SPS100* gene had been replaced by the *URA3* gene; the deletion-substitution resulted in the 2.4-kbp *Eco*RI fragment encompassing the *SPS100* gene being replaced with a 1.6-kbp *Eco*RI fragment. The *MAT***a** *sps100*/*MAT*α *sps100* strain formed asci as efficiently as did the wild-type diploid strain, as assessed by the refractility of the spores on examination by light microscopy (data not shown). This suggested that the *SPS100* gene product was not an essential structural component of the spore wall, but did not exclude the possibility that the protein was present in the spore coat (see below). For comparison, it is interesting that mutation of genes encoding spore coat proteins of *Bacillus subtilis* revealed that the absence of any one of four spore coat proteins does not prevent spore formation (9). Viability of the spores of the *MAT***a** *sps100*/*MAT*α *sps100* strain and of the wild-type strain was also similar (data not shown).

It is possible that certain spore wall components contribute to the protective nature of the spore coat without being essential for spore wall formation. We therefore tested whether the *SPS100*-encoded protein contributed in a more subtle manner to spore wall development than could be detected by light microscopy. Dawes and Hardie (8) noted that ascospores of *S. cerevisiae* are considerably more resistant to killing by exposure to diethyl ether than are vegetative cells. The resistance of spores to ether may reflect

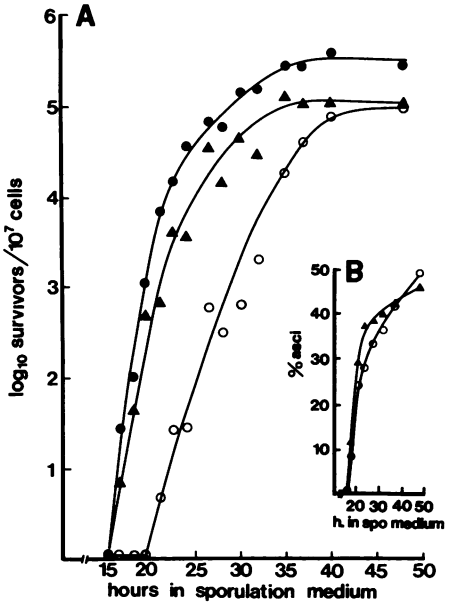

FIG. 8. Onset of diethyl ether resistance during sporulation of a wild-type strain and a *MATa sps100/MATα sps100* strain. (A) Cells were harvested at various times after transfer to sporulation medium and tested for resistance to exposure to diethyl ether as described in Materials and Methods. Symbols: ▲, *MATa SPS100/MATα SPS100* strain; ○, *MATa sps100/MATα sps100* strain; ●, *MATa sps100/MATα sps100* strain containing a plasmid with a copy of the *SPS100* gene. (B) The appearance of asci was monitored by light microscopic examination of cells. The experiment presented here has been performed three times. In each instance, there was a 5-h delay in the onset of ether resistance in the mutant strain relative to the wild-type strain.

the protective nature of the carbohydrate components of the spore wall. We therefore tested the resistance of *MATa sps100/MATα sps100* spores to ether. An analysis of the time at which the developing asci first became resistant to ether revealed a significant difference between the mutant and wild-type strains. Survival of vegetative cells to exposure to ether was less than 10^{-6}. Ether resistance began to develop in cells of the wild-type strain at 16 h after transfer of cells to sporulation medium (Fig. 8A). This was the time at which asci first became visible by light microscopy (Fig. 8B) and the time at which expression of the *SPS100* gene was maximal. The resistance to ether then increased to 10^{-2} over the next 20 h. In contrast, ether resistance could not be detected in the developing asci of the mutant strain until 21 h of sporulation, 5 h after ether resistance was first detected in the wild-type strain (Fig. 8A). Despite this lag in the onset of ether resistance in the mutant strain, by the time spore formation was completed the mutant cells had become as resistant to ether as had the wild-type cells. At 21 h of sporulation, cells of the mutant strain were 1,000-fold less resistant to ether than were cells of the wild-type strain. At 25 h of sporulation, the mutant strain was 100-fold less resistant to ether than was the wild-type strain, and 15 h later, when ascus formation was nearing completion, the ether resistance of the mutant strain was identical to that of the wild-type strain (Fig. 8).

In contrast to the 5-h delay in the onset of ether resistance observed for the mutant strain relative to the wild-type strain, the time course of ascus formation, as detected by light microscopy, was similar for both strains (Fig. 8B; data not shown). Thus, the major events of spore wall formation occurred with similar kinetics in the wild-type and mutant strains.

To confirm that the phenotype of the *MATa sps100/MATα sps100* mutant was truly due to the *SPS100* null mutation, we constructed a replicating, centromeric plasmid containing a copy of the *SPS100* gene. *MATa sps100/MATα sps100* cells containing this plasmid developed ether resistance at the same time as did the wild-type strain (Fig. 8A), indicating that the phenotype of the *MATa sps100/MATα sps100* strain was due to the absence of the *SPS100* gene product. Interestingly, in the plasmid-containing strain the level of ether resistance of the mature spores was enhanced approximately twofold relative to the wild-type strain.

This mutational analysis has indicated that the *SPS100* gene product serves a protective function during the early stages of spore wall development and has demonstrated that the time at which the gene product acts correlates with the time of expression of the gene. This analysis has therefore confirmed that a gene identified on the basis of its developmental-specific expression pattern contributes to a sporulation-specific event.

We also tested whether mutation of the *SPS101* gene would affect spore formation; the gene was disrupted within the transcribed sequence by insertion of a DNA fragment containing the *URA3* gene (Fig. 7C and D). A *MATa sps101/MATα sps101* strain formed viable ascospores as efficiently as did the wild-type diploid strain, and the development of resistance to ether was also similar for both strains (data not shown). We constructed a *MATa sps100 sps101/MATα sps100 sps101* strain to test whether the double mutant would have a more severe phenotype than the *MATa sps100/MATα sps100* strain; the phenotype of the double mutant, however, was similar to that of the single mutant (data not shown). As disruption of the *SPS101* gene occurred approximately 320 bp from the 3′ end of the gene, it is possible that the insertion did not affect the function of the gene or that it was not within the coding region of the gene.

DISCUSSION

Our previous differential hybridization screen of an *S. cerevisiae* DNA library, performed with probes prepared with RNA purified from cells harvested before 15 h of sporulation, identified a class of 14 sporulation-specific genes (28). Most of these genes are first expressed 6 to 8 h after transfer of cells to sporulation medium, the time at which meiosis I is nearing completion. Transcript accumulation becomes maximal several hours later, during meiosis II. The present screen, performed with probes prepared with RNA from 35-h spores, identified two additional sporulation-specific genes, termed *SPS100* and *SPS101*. These genes are first expressed at 12 h after transfer of cells to sporulation medium, just before the appearance of asci. Determination of the nucleotide sequence of the *SPS100* gene led to the prediction that the gene product might be involved in spore wall maturation. A mutational analysis then demonstrated that the *SPS100* gene product serves a protective role during the early stages of spore wall formation. We therefore presume that the *SPS100* gene contributes to spore wall development. However, such a role has not yet been directly demonstrated. We have previously shown that one of the middle sporulation genes, the *SPS1* gene, is required for sporulating cells to progress past meiosis II (29). These examples demonstrate that differential hybridization screen-

ing is a useful approach for the identification of genes that serve developmental-specific functions.

The *SPS100* and *SPS101* genes define a new class of temporally distinct sporulation-specific genes. Our observations, taken in conjunction with the studies of other investigators (16, 21, 41, 44), suggest that the program of events of meiosis and spore formation reflects the sequential expression of at least four temporally distinct classes of sporulation-specific genes. The *SPO13* gene is the prototype gene for the earliest class of sporulation-specific genes. This gene is required for proper chromosome separation during meiosis I and is expressed shortly after transfer of cells to sporulation medium (41). The sporulation-specific genes identified in the differential hybridization screen performed by Clancy et al. (4) have been grouped into three classes, early, middle, and late, based on their temporal expression pattern (16). Transcripts encoded by this late class of genes (16), as well as the transcripts of the *SIT* genes described by Gottlin-Ninfa and Kaback (13), begin to accumulate at the time of meiosis I (13, 16). Therefore, the genes which we have termed middle genes fall into this temporal class, and the *SPS100* and *SPS101* genes define a distinct class of very late sporulation genes.

The pattern of sporulation-specific gene expression observed for the middle and late genes analyzed in this study is remarkably consistent with the pattern of gene expression observed by Kurtz and Lindquist (21) in an analysis of the in vitro coding capacity of RNA from sporulating cells. In fact, the *SPS4* gene (12) identified in our previous study (28) has been shown to encode one of the 8-h transcripts identified in the study of Kurtz and Lindquist (12, 21, 22). It is possible that the *SPS100* gene characterized in this study specifies the 16-h sporulation-specific transcript described by Kurtz and Lindquist as encoding a 34-kilodalton protein (21). The transcript encoding this protein was found to be present in prospores and to be absent from the surrounding ascal cytoplasm, suggesting that the corresponding gene is transcribed only after the prospore walls have formed (22). If the *SPS100* gene does encode this transcript and if our presumption that the *SPS100* gene product is a spore wall component is correct, then the latter observation on transcript localization (22) supports the suggestion that at least some spore coat components are translocated into the spore wall through the endoplasmic reticulum of the prospore (23).

Previous mutational analyses of several genes that have a sporulation-specific expression pattern failed to reveal defects in sporulation (12, 13, 16, 29, 47). Possible reasons for the lack of an apparent mutant phenotype for these genes have been previously discussed (12, 13, 16, 29, 47). In our ongoing characterization of *SPS* genes identified by differential hybridization screens, we have demonstrated that three of these genes contribute to the sporulation process: mutation of the *SPS1* gene prevents ascospore development (29); the *SPS2* gene has been shown indirectly to play a role in the completion of sporulation (30); and in this study we have shown that the *SPS100* gene contributes to a sporulation-specific event. The phenotype of *MAT***a** *sps100*/*MAT*α *sps100* cells, a delay in the onset of ether resistance in developing spores, was not apparent by simple assessment of the formation of viable spores. This provides an example of a gene whose product serves such a subtle role in the developmental program that the mutant phenotype will escape notice unless the appropriate test is performed. This may account for the apparent lack of phenotype for at least some of the previously studied sporulation-specific genes and also for the *MAT***a** *sps101*/*MAT*α *sps101* strain. A more detailed analysis of spore wall formation, long-term spore viability, or rate of spore germination may reveal a function for the *SPS101* gene product.

The observation that the mature spores of the *MAT***a** *sps100*/*MAT*α *sps100* strain are as resistant to ether as are wild-type spores suggests that the *SPS100* gene product accelerates the synthesis or assembly of components leading to ether resistance or, more likely, that a separate component(s), which also leads to ether resistance, is synthesized 5 h after the *SPS100*-dependent component. The latter idea is consistent with the suggestion that in some instances the failure to readily detect a mutant phenotype for sporulation-specific genes is due to the presence of two or more gene products that serve complementary functions such that the cell can compensate for the absence of one of the gene products. In such instances, double mutations would generally be required in order to observe a mutant phenotype. As sporulation is a response of *S. cerevisiae* to adverse nutritional conditions, redundant sporulation functions would enhance the ability of cells to complete sporulation and survive nutrient limitation. Complementary functions contributing to the survival of cells on exposure to adverse growth conditions have been previously observed. For example, strains of *S. cerevisiae* containing a mutation in either the *YG100* or *YG102* gene can grow under the stress of elevated temperature, but a *yg100 yg102* strain cannot (5). The *ssp* genes of *B. subtilis*, responsible for UV light resistance of the bacterial endospores as well as contributing to spore outgrowth, have been shown to serve redundant functions (26). Functional redundancy may also account for the failure to observe a Spo$^-$ phenotype in spore coat mutants of *B. subtilis* (9).

From the above discussion, it is apparent that classical genetic analyses and differential hybridization screens provide useful complementary approaches for identifying sporulation-specific genes. For instance, the level of transcript accumulation from the *SPO13* gene, a gene that was isolated from a natural meiotic variant of *S. cerevisiae* (18), is probably too low to allow for its detection in a differential hybridization screen (41). On the other hand, sporulation genes that encode functionally redundant products or products that are not essential for meiosis or spore wall formation would not be uncovered in genetic screens which depend on a major developmental defect to identify mutants. However, such genes could be identified as developmental-specific genes in a differential hybridization screen.

As plasmids containing the *SPS100* gene and *SPS101* gene were identified several times, we presume that the class of late *SPS* genes is very small and may only include these two genes. However, genes belonging to this class and encoding transcripts that are not very abundant would most probably have escaped detection in this type of differential hybridization screen.

In addition to identifying the two sporulation-specific genes, *SPS100* and *SPS101*, the screen described in this study also identified four other late genes, represented by the plasmids of groups L3, L4, L5, and L6, expressed preferentially in *MAT***a**/*MAT*α cells at 35 h after transfer to sporulation medium. These latter genes, however, showed significant expression in asporogenous *MAT***a**/*MAT***a** and *MAT*α/*MAT*α strains at 20 h after transfer to sporulation medium (Fig. 3). It is possible that these genes are stationary-phase specific and that the signal responsible for their activation occurs only after several hours of exposure to nutrient limitation. A reduced stability of such transcripts in asporogenous cells relative to developing spores could ac-

count for the apparent enhanced expression of the group L3 through L6 genes in *MATa*/*MATα* cells at 35 h after transfer to sporulation medium. Alternatively, although only *MATa*/*MATα* cells can complete meiosis and spore formation in response to nutrient deprivation (15), it is possible that some minor developmental genes are activated in a temporally controlled sequence in response to the appropriate nutritional cues irrespective of the *MAT* genotype. Interestingly, expression of the developmentally regulated *SPO13* gene has been shown to be induced to a very low level shortly after transfer of *MATa*/*MATa* and *MATα*/*MATα* cells to sporulation medium (41); a very low level of expression of the *SPS100* and *SPS101* genes can also be detected in *MATa*/*MATa* and *MATα*/*MATα* cells, especially after 20 h in sporulation medium (Law, unpublished data).

We anticipate that elucidation of the elements responsible for the activation of the *SPS100* and *SPS101* genes will provide useful insights into the regulation of expression of developmental-specific genes.

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