The *Saccharomyces cerevisiae* *SPR1* Gene Encodes a Sporulation-Specific Exo-1,3- β -Glucanase Which Contributes to Ascospore Thermoresistance

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A number of genes have been shown to be transcribed specifically during sporulation in *Saccharomyces cerevisiae*, yet their developmental function is unknown. The *SPR1* gene is transcribed during only the late stages of sporulation. We have sequenced the *SPR1* gene and found that it has extensive DNA and protein sequence homology to the *S. cerevisiae* *EXG1* gene which encodes an exo-1,3- β -glucanase expressed during vegetative growth (C. R. Vasquez de Aldana, J. Correa, P. San Segundo, A. Bueno, A. R. Nebrada, E. Mendez, and F. del Ray, *Gene* 97:173–182, 1991). We show that *spr1* mutant cells do not hydrolyze *p*-nitrophenyl- β -D-glucoside or laminarin in a whole-cell assay for exo-1,3- β -glucanases. In addition to the absence of this enzymatic activity, *spr1* mutant spores exhibit reduced thermoresistance relative to isogenic wild-type spores. These observations are consistent with the notion that *SPR1* encodes a sporulation-specific exo-1,3- β -glucanase.

In *Saccharomyces cerevisiae*, sporulation is initiated when a/α diploids are deprived of glucose and nitrogen. This complex pathway, which includes premeiotic DNA synthesis, high levels of genetic recombination, and two meiotic divisions, culminates in the formation of an ascus containing four haploid ascospores (reviewed in reference 11). Molecular and genetic analyses of this pathway are important because sporulation can serve as a model system for eukaryotic differentiation, i.e., for the coordination of a complex series of events that result in the formation of a new cell type. Furthermore, the process of recombination and meiosis is fundamental to eukaryotic biology.

Genetic analysis has identified more than 30 complementation groups that are required for the completion of specific sporulation events (11, 45); several of these genes have been cloned either by direct complementation of *spo* mutants, e.g., *spo11* (1), or by chromosome walking from a closely linked locus, e.g., *spo13* (49). It has also been possible to isolate genes involved in sporulation by selecting sequences that are transcribed preferentially during sporulation (*SPR*, [6], *SPS* [32], and *SIT* [14] genes). The *SPR* (sporulation-regulated) genes are expressed at specific stages during sporulation but not as part of the cell's response to nitrogen starvation (6, 32); a large number of them are regulated at the level of transcription (16).

Most of the genetic analysis of sporulation has focused on early events such as entry into the meiotic pathway (21, 25, 29), recombination (26, 43), and chromosome separation (52), perhaps because these are the events which are common to most other eukaryotic organisms. Less attention has been paid to the genes that participate in events that occur during the late stages of sporulation (such as spore wall formation and development of resistance to environmental

stresses), even though this part of the developmental cycle is an excellent example of microbial cell differentiation. The *SPR* and *SPS* genes, isolated by virtue of their strong differential transcription during the middle and late periods of sporulation, are strong candidates for involvement in the processes of spore formation. Indeed, two of the *SPS* genes have clear sporulation-specific functions. *SPS1* is required for formation of spore walls (33), while *SPS100* confers ether resistance on the developing ascus (24). In addition, Briza et al. have isolated two sporulation-regulated genes (*DIT1* and *DIT2*) by complementation of dityrosine-deficient mutants (3). Ascospores that are mutant at either *DIT1* or *DIT2* lack the outermost layer of the spore wall and exhibit reduced resistance to environmental stresses (3). Other late sporulation genes may have more subtle roles. For example, Kao et al. have found that *spr3* mutants exhibit a slight reduction in sporulation efficiency (20), and Kallal et al. showed that strains defective for three genes, *SPR1*, *SPR3*, and *SPR6*, sporulate to only 60% of the wild-type rate (19).

By searching sequence data bases, we found that the *SPR1* gene had substantial homology to the *EXG1* gene from *S. cerevisiae*. *EXG1* encodes exo-1,3- β -glucanase isozymes I and II, designated EXG I and EXG II by Vasquez de Aldana et al. (46). These isozymes hydrolyze β -O-glycosidic linkage at nonreducing ends of glucan polymers; they also contain β -glucosidase activity since they can hydrolyze the glycosidic bond in synthetic glucosides such as *p*-nitrophenyl- β -D-glucoside (pNPG). Since they are expressed during a specific phase of the cell cycle, they may participate in modifications of the cell wall necessary for budding (8). Del Rey et al. have identified a third exo-1,3- β -glucanase which is expressed specifically during the later stages of sporulation (9). We have found that *spr1* mutants fail to express high levels of exo- β -glucanase activity during sporulation and are less thermoresistant than wild-type strains. These observations support the contention that *SPR1* encodes a sporula-

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TABLE 1. List of strains used

Strain	Genotype	Source or reference
DCO4 α	<i>MATα ade1 leu2-04 [<i>cir</i>⁰]</i>	5
YNN27	<i>MATα trp1-289 ura3-52 gal2</i>	5
YNN281	<i>MATα ura3-52 lys2-801 ade2-101 trp1-901 his3-2</i>	5
W66-8a	<i>MATα/MATα HO ade2-1 lys2-1 trp5-2 leu1-12 can1-100 ura3-1 met4-1</i>	36
WSRB1	Same as W66-8a, except <i>spr1-1::URA3/SPR1</i> ⁺	This study
W61A	Same as W66-8a, except <i>spr1-1::URA3/spr1-1::URA3</i>	This study
W61C	Same as W66-8a (sister spore to W61A)	This study
SCMS7-1	<i>MATα/MATα HO ade his4-519 leu2-3,112</i>	41
SPB1	Same as SCMS7-1, except <i>SPR1⁺/spr1-2::LEU2</i>	This study
S52A	Same as SCMS7-1 (sister spore to S52B)	This study
S52B	Same as SCMS7-1, except <i>spr1-2::LEU2</i>	This study
AP1 a/ α	A364A $\times$ α 131-20	18
AP1 α / α	MAT α /MAT α , derived from AP1a/ α	18
AP3 a/a	MAT α /MAT α , derived from AP1a/ α	18

tion-specific exo-1,3- β -glucanase and are consistent with the notion that this enzyme participates in the morphogenesis of ascospore walls.

MATERIALS AND METHODS

Strains and growth conditions. All strains used in this work are described in Table 1. Strains were induced to sporulate by using the following protocol: cells were grown in pre-sporulation (PSP₂) medium (34) to a density equal to 2×10^7 to 5×10^7 cells per ml, washed twice in sterile, distilled water, and then transferred to sporulation SP₂ medium (18). Strains W66-8a, SCMS7-1, and AP1 a/ α sporulate to 70 to 90% efficiency within 24 h.

Plasmid constructions and gene disruptions. Restriction endonucleases and other DNA-modifying enzymes were obtained from Bethesda Research Laboratories or Boehringer Mannheim and were used under the conditions recommended by the supplier. A 4.4-kb *Xho*I fragment from λ sp1 (6) was cloned into the *Sal*I site of pUC18 to yield plasmid pCG33 and into the *Sal*I site of the yeast-replicating vector YRp12 to yield plasmid pCG1. Two transplacement vectors were derived from pCG33; the structure of each transplacement is given in Fig. 1. (i) The *URA3* gene on a *Sal*I-*Cla*I fragment derived from YEp24 was inserted between the *Sal*I and *Cla*I sites of pCG33 to yield pSURA, which carried the *spr1-1* allele. (ii) The *LEU2* gene on a *Sal*I-*Sst*I fragment derived from YEp13 was inserted between the *Sal*I and *Sst*I sites of pCG33 to yield pSLEU, which carried the *spr1-2* allele. These transplacements were introduced into homothallic diploid strains (W66-8a or SCMS7-1) by the one-step gene disruption technique (35). Plasmid pSURA was targeted to the *SPR1* locus by digestion with *Sst*I and *Eco*RI; plasmid pSLEU was targeted by digestion with *Bam*HI and *Pst*I. Recipient strains were transformed by the spheroplasting method described in Sherman et al. (40). These two disruptions were used in order to test the effect of the *SPR1* mutations in different genetic backgrounds and to control for

secondary mutations that may have occurred following transformation.

The presence of each transplacement was confirmed by Southern blot analysis of genomic DNA from transformants and from homozygous spore cultures (data not shown). Since the 5' end of the *SPR1* gene (with coding capacity up to codon 112) was left intact in both mutant alleles, we also assessed the abundance of transcripts from the mutant strains. Total RNA from cells growing in PSP₂ medium or from sporulating cells (8 h in SP₂ medium; hereafter referred to as 8-h sporulation RNA) was isolated from strains W61A, W61C, S52A, and S52B and subjected to Northern (RNA) blot analysis. Both poly(A)⁺-enriched RNA and total RNA were fractionated on formaldehyde gels and probed with the 4.4-kb *Xho*I fragment. Normal *SPR1* transcripts were observed in poly(A)⁺-enriched RNA from sporulating cultures of wild-type strains S52A and W61C. No transcript was detected in either mutant strain, S52B (*spr1-2/spr1-2*) or W61A (*spr1-1/spr1-1*). Similar results were obtained with total RNA. Filters were washed and reprobed with radiolabeled plasmid pGAP, which carries the gene for glyceraldehyde-3-phosphate dehydrogenase, to ensure that equal amounts of RNA were loaded (data not shown). S1 analysis was performed in order to detect low levels of the *SPR1* transcript in mutant strain S52B. Vegetative RNA and 10-h sporulation RNA were hybridized to a 374-bp *Dde*I-*Sal*I fragment (−42 to +333) and digested with S1 nuclease. A full-length protected fragment was observed only in sporulation RNA from the wild-type strain (data not shown).

Plasmid pMP1 was constructed by inserting the 2.8-kb *Eco*RI fragment from λ sp1 (6) into the *Eco*RI site of pBR322; plasmid YIp515 was constructed by inserting an *Hpa*II fragment (nucleotides +890 to +1317) into the *Cla*I site of YIp5.

Diploids that were homozygous at the disrupted locus were obtained by sporulation of homothallic heterozygous disruptants followed by dissection and isolation of ascospores on YEPD medium (40) by micromanipulation. Cells that germinate from these spores rapidly diploidize as a result of mating-type switching.

Nucleic acid preparation. Large amounts of plasmid DNA were prepared either by an isopycnic centrifugation method described by Maniatis et al. (27) or by a polyethylene glycol method (10a). Yeast genomic DNA was prepared from a 10-ml suspension of stationary-phase cells as described by Sherman et al. (40). Southern blotting was performed as described by Maniatis et al. (27). Radiolabeled probes for filter hybridization were prepared by nick translation of gel-purified restriction fragments.

Total RNA was prepared from yeast by the method described by Zitomer et al. (54). Poly(A)⁺ RNA was isolated by the method described by Harper et al. (15). This RNA was fractionated on 2.2 M formaldehyde–1.0% agarose gels, blotted to nitrocellulose filters, and hybridized to radiolabeled probes according to procedures described by Thomas (44). After hybridization, filters were washed with 0.2% SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) for 1.5 h at 45°C.

For heat shock experiments, cells were grown at room temperature and then shifted to 30, 39, or 45°C for 1 h. Total RNA was prepared, blotted onto nitrocellulose (by using a dot blotting manifold), and probed with radiolabeled plasmid pCG33. Substantial induction of the *HSP26* transcript was noted at both 39 and 45°C in control hybridizations.

S1 protection for transcript mapping was based on the protocol of Favoloro et al. (12). DNA probes were labeled at

FIG. 1. (A) Restriction map of the *SPRI* locus. The boxed section indicates the position of the long open reading frame; the arrow indicates the position of the *SPRI* transcript. (B) DNA sequencing strategy. The arrows indicate the regions sequenced by either the dideoxy or Maxam-Gilbert method. (C) Genomic structure of *SPRI* disruptants *spr1-1* and *spr1-2*. Wide boxes indicate portions of the *SPRI* open reading frame; narrow boxes indicate sequences used in gene disruption. Restriction site designations: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hind*III; S, *Sal*I; Sc, *Sca*I; Ss, *Sst*I; X, *Xho*I.

the 5' end with polynucleotide kinase and [γ - 32 P]ATP. All probes were double stranded, except the 225-bp *Eco*RI-*Hind*III fragment, for which the strands were resolved by electrophoresis in a polyacrylamide gel with 0.1% bisacrylamide (28). The strand labeled at the *Hind*III site migrated as two separate species on these gels. Both species were used in S1 protection experiments. Total RNA (50 to 200 μ g) and probe DNA were dissolved in hybridization buffer, denatured by heating at 90°C, and then hybridized at 42°C for 3 h. Hybridization mixtures were treated with 5 to 20 U of S1 nuclease (Bethesda Research Laboratories) for 20 min at 37°C. S1 digestion was terminated by the addition of ammonium acetate and EDTA to final concentrations of 50 and 5 mM, respectively; the products were then precipitated with ethanol, resuspended in 90% formamide loading buffer, and analyzed on 4 to 6% polyacrylamide-7 M urea gels. Autoradiography was performed by using Kodak XAR-5 film.

Orthogonal-field-alternating gel electrophoresis. Intact yeast chromosomes were prepared from yeast strains YNN281, DCO4 α , AB972, and A364a by the agarose-embedding method of Carle and Olson (5); yeast chromosomes were resolved by orthogonal-field-alternating gel electrophoresis in 1.2% agarose gels at 300 V with a switching time of 60 s. Chromosomes were transferred to nitrocellulose filters by Southern blotting (5). Radiolabeled probes for Southern hybridization to orthogonal-field-alternating gel electrophoresis filters were prepared by nick translation of plasmid pCG33.

DNA sequencing. The region of the *SPRI* locus between *Bam*HI and *Sst*I was sequenced by the dideoxy method of Sanger et al. (37). Single-stranded templates were prepared from either M13mp18 or M13mp19 phage vectors by scaling up the procedure of Messing and Vieira (28). Two strategies

were used in generating the subclones for sequencing. First, several well-characterized restriction fragments were subcloned into M13mp18 and M13mp19 replicative forms (51). Second, a "shotgun" library was prepared by digesting the *Bam*HI-*Sst*I fragment with *Msp*I and subcloning these fragments into the *Acc*I site of M13mp18. Specific oligomers were synthesized and used as primers to fill in the remaining gaps in the sequence.

Chemicals and enzyme assays. The substrates for exo- β -glucanase, laminarin and pNPG, were obtained from Sigma Chemical Company.

Intact cells from 16-h sporulating cultures were assayed for exo-1,3- β -glucanase activity as described by Santos et al. (38). Either laminarin or pNPG was used as a substrate. Each reaction mixture contained 10^8 cells in a final volume of 1 ml of 50 mM acetate buffer (pH 5.5). Substrates were added to a final concentration of 0.25%, and incubations were performed at 37°C for 60 min. After the incubation period, the reaction mixture was boiled for 3 min and then centrifuged at $13,000 \times g$ for 5 min. Supernatant fluid was removed and assayed for the reaction products. The concentration of p-nitrophenol was determined by adding 4.5 ml of 4% sodium carbonate to 0.5 ml of supernatant fluid and measuring A_{415} . Glucose released in the laminarin assay was estimated by using glucose oxidase and peroxidase according to the method of Dahlquist (7). One unit of activity was defined as the formation of 1 nmol of reaction product (either glucose or p-nitrophenol) produced per h per 10^8 cells.

Germination and ascospore thermoresistance. For spore thermoresistance studies, after a defined incubation period in 10 to 20 ml of SP₂ medium, equal numbers of asci from wild-type and isogenic mutant strains (roughly 10^7 asci per ml) were mixed together; mixing asci from different strains

FIG. 2. Comparison of mRNA levels of *SPRI* and *URA3* genes. Twenty-five micrograms of vegetative (lanes 1, 3, and 5) or 8-h sporulation (lanes 2, 4, and 6) poly(A)⁺ RNA was fractionated in 1.0% agarose gels and transferred to a nitrocellulose filter. The radiolabeled probes used were YIp515 (*SPRI* and *URA3*, lanes 1 and 2), pMP1 (*SPRI* only, lanes 3 and 4), and YIp5 (*URA3* only, lanes 5 and 6).

minimized experimental variation that might occur during the preparation of free spores or during heat stress. Spores were freed by treating the ascus wall with β -mercaptoethanol and with glusulase (DuPont Biotechnology Laboratories); β -mercaptoethanol and glusulase were removed by being washed three times in sterile water. Spore clusters were then disrupted by sonication; three 20-s bursts converted 80% of all tetrad clusters to single spores. Spore preparations were incubated at 4, 30, or 42°C. At several time points (24, 48, 60, and 80 h), spore viability was determined by plating diluted samples in duplicate onto YEPD medium and incubating the samples at 30°C for 48 h; in experiments with mixtures of wild-type and mutant spores, colonies were replica plated to minimal plates without leucine in order to determine the genotype of the cells in the spore colony. The ability of spores to germinate was tested in a similar manner. Free spores were prepared as described above, plated immediately onto YEPD medium, and incubated at either 30 or 37°C; colonies were counted after 48 h.

RESULTS

Isolation and mapping of the *SPRI* locus. The *SPRI* gene was isolated from a genomic library of *S. cerevisiae* (prepared in λ phage Charon 28) by screening for genes transcribed preferentially during sporulation. Several phage clones, including λ sp1 and λ sp16, carry the *SPRI* locus (6). The *SPRI* gene encoded a 1.6-kb transcript that was expressed after 8 h in sporulation medium (Fig. 2, lanes 2 and 4); this transcript was not present during vegetative growth in presporulation (PSP₂) medium (Fig. 2, lanes 1 and 3). The same RNA preparations were probed with the *URA3* gene (plasmid YIp5; Fig. 2, lanes 5 and 6) to confirm the integrity of the RNA. Holaway et al. (16) have shown that during

sporulation the *SPRI* transcript is present at 130 copies per cell. This transcript was probably polyadenylated because it could be enriched by oligo(dT) chromatography (data not shown).

We used pulsed-field gel electrophoresis to determine the chromosomal location of *SPRI* (5). Plasmid pCG33, which carried the 4.4-kb *Xho*I fragment, was labeled by nick translation and hybridized to an electrophoretic separation of chromosomes of several yeast strains. This plasmid hybridized to a single band corresponding to chromosome XV in strain DCO4 α and to a doublet band corresponding to chromosomes XV and VII in strains AB972, A364a, and YNN281; identification of chromosome XV was confirmed by hybridizing the same karyotypes to a restriction fragment from *STE4*, a known chromosome XV locus (data not shown).

Transcript mapping and developmental regulation of *SPRI*. S1 nuclease protection methods were used to determine the direction of transcription of the *SPRI* transcript, its approximate 5' end, and its relative abundance throughout sporulation and in asporogenous diploids. The direction of transcription was determined by hybridizing total RNA from either vegetative cells (grown in PSP₂ medium) or sporulating cells (8 h in SP₂ medium) of strain AP1 a/ α to the 225-bp *Eco*RI-*Hind*III DNA fragment labeled at either 5' end (Fig. 3A). Protection (full length) of the *Hind*III-labeled strand by sporulation RNA (Fig. 3A, lanes 2, 5, and 6) indicated that transcription is from left to right, as shown in Fig. 1; no protection of the *Eco*RI-labeled strand was observed (Fig. 3A, lanes 8 to 11).

The 1,108-bp *Bam*HI-*Sal*I fragment was used to assess the abundance of the *SPRI* message and to approximate its 5' end. When this probe was hybridized to RNA from 6-, 8-, 10-, or 15-h sporulating cultures, a major fragment of 400 bases and several minor fragments (390, 450, and 500 bases in length) were observed after S1 digestion (Fig. 3B, lanes 4 to 7); this suggests that the major site of transcription is 65 bp upstream from the first ATG in the predicted *SPRI* open reading frame. No protected fragment of any size was observed when RNA from AP1 a/ α vegetative cultures or early sporulating cultures was hybridized to the probe (Fig. 3B, lanes 1 to 3). Kurtz et al. (23) have reported that the *HSP26* transcript is induced during sporulation. No induction of the *SPRI* transcript was observed during heat stress at either 30, 39, or 45°C (data not shown).

DNA sequence of the *SPRI* locus. In order to gain some insight into the function of the *SPRI* gene product, the region between the *Bam*HI and *Sst*I restriction sites was sequenced (Fig. 1). Within this sequence there was only one long open reading frame (ORF1 at positions +1 to +1338) which could encode a protein consisting of 445 amino acids (Fig. 4). Given a transcriptional start at -65, it seems likely that the ATG at +1 was the translational start, since the next in-frame ATG was located at +616. Translation of ORF1 predicted a protein with a hydrophobic leader that had three features in common with the signal peptide found in many secreted and some membrane proteins as follows (48, 50): (i) a basic residue (arginine) near the amino terminus, (ii) a stretch of hydrophobic amino acids (7 of 12 from amino acid 7 to amino acid 18), and (iii) an adjacent C-terminal polar region. The codon bias index of ORF1 was low (0.041), which is characteristic of poorly expressed yeast genes (2, 39). There was also one short open reading frame upstream of and within the same reading frame as ORF1 (ORF2 at positions -134 to -23).

We used both Genetics Computer Group (University of

FIG. 3. (A) Direction of transcription of *SPRI*. The 225-bp *Eco*RI (E)-*Hind*III (H) fragment was labeled at either of its 5' ends, indicated by an asterisk. Single-stranded fragments were isolated by electrophoresis on a 5% strand separation gel. The *Hind*III-labeled strand routinely migrated as a doublet on these gels. Both *Hind*III-labeled species (distinguished as upper and lower fragments) and the single *Eco*RI-labeled strand were used separately in protection experiments as follows: upper *Hind*III-labeled fragment (lanes 1 and 2), lower *Hind*III-labeled fragment (lanes 3 to 6), and *Eco*RI-labeled strand (lanes 7 to 11). These probes were hybridized to 200 μ g of either vegetative or 8-h sporulation total RNA at 42°C for 3 h and then digested with S1 nuclease. Lanes: 1, vegetative RNA (20 U of S1 nuclease); 2, sporulation RNA (20 U of S1 nuclease); 3, probe only (no S1 nuclease treatment); 4, vegetative RNA (20 U of S1 nuclease); 5, sporulation RNA (20 U of S1 nuclease); 6, sporulation RNA (100 U of S1 nuclease); 7, probe only (no S1 treatment); 8, vegetative RNA (20 U of S1 nuclease); 9, sporulation RNA (20 U of S1 nuclease); 10, sporulation RNA (50 U of S1 nuclease); 11, sporulation RNA (100 U of S1 nuclease). (B) Expression of the *SPRI* transcript during sporulation. The 1,108-bp *Bam*HI-*Sal*I fragment was hybridized to 200 μ g of total RNA prepared from sporulating cultures of strain AP1 α/α at 0 h (lane 1), 2 h (lane 2), 4 h (lane 3), 6 h (lane 4), 8 h (lane 5), 10 h (lane 6), and 15 h (lane 7). All hybridizations were digested with 20 U of S1 nuclease; S1 nuclease products were analyzed on a denaturing 4% polyacrylamide gel.

Wisconsin) (Devereux et al. [10]) and GENEPRO (version 5.0; Riverside Scientific) software to search DNA and protein sequence data bases (GenBank release 69, EMBL release 27, and PIR release 30) for homology to the *SPRI* gene. Only one sequence, the *EXG1* gene sequence (46), showed significant homology to the *SPRI* sequence. At the DNA sequence level, the coding regions of these two genes showed 67% homology under maximal alignment parameters.

Comparison of the predicted peptides from each sequence revealed several long stretches of exact matches and an overall 64% amino acid identity (288 matches of 447 aligned amino acids); another 8% of the aligned sequences represented conserved amino substitutions (Fig. 5). A few other genes encoding 1,3- β -glucanases, such as *BGL2* from *S. cerevisiae*, have been sequenced (22). Comparison of the *SPRI* peptide to the predicted *BGL2* peptide showed only limited identity (10%).

Three TATA boxes (TATAAA) were found upstream of the proposed transcription start site at positions -565, -471, and -441 (shown in Fig. 4); several degenerate TATA boxes (5 of 6 matches) at positions -341, -214, and -180 were found downstream of the canonical elements. The URS_{1H} sequence (TGGGCGGCT) is required for the developmental expression of several meiosis-specific genes (4, 47). A sequence (TGGGCGTTT) with good homology to URS_{1H} (7 of 9 matches) was located at -449.

The sequence AATAAA has been implicated in the addition of polyadenylic acid tracts (13, 53). Two such sequences were found in the region following the termination codon of ORF1 (positions +1438 and +1471 in Fig. 4).

Phenotype of the *spri* mutant strains. In order to confirm that *SPRI* encoded an exo-1,3- β -glucanase, we constructed

two diploid strains that were homozygous for mutant alleles (strains W61A and S51B) and assayed whole cells from sporulating cultures for the presence of the enzyme activity. Mutant transplacements were introduced into homothallic diploid strains (W66-8a and SCMS7-1) by the one-step gene disruption technique (35); the structure of the transplacements is shown in Fig. 1C. The presence of each transplacement was confirmed by Southern blot analysis of genomic DNA from transformants and from homozygous spore cultures (data not shown). No bands other than those expected from the *SPRI* locus were detected in these blots. Since filters were washed at high stringency, this observation is consistent with the limited DNA sequence homology between *SPRI* and *EXG1* and suggests that *SPRI* is a single-copy gene.

Either laminarin or pNPG was used as the substrate in exo-1,3- β -glucanase assays of whole cells from 16-h sporulating cultures. When pNPG was the substrate, strains W61A and S52B had less than 10% of the wild-type activity, whereas with laminarin as the substrate, the mutant strains had less than 0.1% of the wild-type activity (Table 2). The wild-type levels in our sporulating cultures are comparable to those found in early logarithmic phase cultures of *exg1*⁺ strains by Nebreda et al. (30).

Since exo-1,3- β -glucanases seem to be involved in wall morphogenesis, diploid strains homozygous for either mutant allele were tested for the ability to form mature ascospores, for the ability of the spores to germinate at various temperatures, and for the ability of the ascospores to survive heat stress. *spri* mutant strains S52B and W61A sporulated as efficiently as isogenic wild-type diploid strains S52A and W61C (70 to 80% tetranucleate cells at 24 h). The time at which four spore asci appeared was the same for both

<u>GGATC</u> CTGAAGTAAA ACAACTTGAA AAAGAAGGAG AGGATGGACT GGACTCATAA	-721	GTT ATT GGA ATC GAA TTG CTC AAT GAA CCG TTA GGT CCA GTT ATT GAC	720
GGTAATGTCA ACAGCTTAAA GTTGCTCTCA GGGCCCCACA CTTTCTAGTT TGGCAGCTCA	-661	Val Ile Gly Ile Glu Leu Leu Asn Glu Pro Leu Gly Pro Val Ile Asp	240
TTTATGAAT TCACAAATTG GTTACATTAA GTACTAAATC TGCTAGCCGA CCAAAAAAGA	-601	ATG GAA AGA TTG AAA AAT TTG CTT TTG AAG CCG GCT TAT GAC TAT TTG	768
ACAACAATA TACAATTGTA TGAGTAATA TGCAATATA ATCAAAATGTA TTTAGTTAAA	-541	Met Glu Arg Leu Lys Asn Leu Leu Lys Pro Ala Tyr Asp Tyr Leu	256
GGCCAGAAG TAAGGCTACA GACTGTAAAA GAAAATTAGA TGGAAACCCG CTTGAGGAAA	-481	AGA AAT AAA ATT AAT AGC AAC CAG ATC ATT GTA ATA CAT GAT GCT TTT	816
AAAGGACATT ATAAAGATA CAGCCAAGAA GTGGGCGTTT ATAAACAAT TATATAACAT	-421	Arg Asn Lys Ile Asn Ser Asn Gln Ile Ile Val Ile His Asp Ala Phe	272
GACCGTCGAT TATATTCTGA CAGGAATGGC GACGATTGCA TCGTTATATT TCATTTCATT	-361	CAA CCT TAC CAT TAT TGG GAT GGG TTT TTG AAT GAT GAA AAG AAC GAA	864
CCCAACTCAA CGATACTGGT ATCAAAAAAG CTCTCTGTTC ATTATTGTGC CCACTACAAG	-301	Gln Pro Tyr His Tyr Trp Asp Gly Phe Leu Asn Asp Glu Lys Asn Glu	288
CGACTCTCTT ATTTTGGCG GTCTCTGCCA GCTTAAGAAA AAGCGGTAC CAAACGACAC	-241	TAT GGC GTC ATA ATT GAC CAT CAT CAT TAT CAG GTG TTC TCG CAG GTG	912
TAAAGAAGC GGCGCGTCTG GTTCAATTAGA AATTGTGTCG TAATGCTGTC AATCTTTGTG	-181	Tyr Gly Val Ile Ile Asp His His His Tyr Gln Val Phe Ser Gln Val	304
TAAAGTCTT TCACATCAGT TGCCACCCCT TTTGTAGCAA CTTTCATGCG TAGAACAAAC	-121	GAA TTA ACA AGA AAA ATG AAT GAA CGT ATC AAA ATC GCC TGC CAA TGG	960
TCACATTCAT TATTTTTTTT ACCCTCTCTCT TTTAATGTT CAATTGCGAA CAGACCATCC	-61	Gln Arg Lys Arg Lys Met Asn Glu Arg Ile Lys Ile Ala Cys Gln Trp	320
AAAGATTGCA AAGAAAGACT GAGGTGCTCC GCATAAAATC TTTATAAATC TTAGAAGTAA	-1	GGG AAA GAT GCC GTA AGC GAG AAG CAT TGG TCC GTA GCA GGC GAA TTT	1008
ATG GTT TCG TTC AGA GGG CTG ACT ACA CTA ACA CTA CTT TTT ACC AAA	48	Gly Lys Asp Ala Val Ser Glu Lys His Trp Ser Val Ala Gly Glu Phe	336
Met Val Ser Phe Arg Gly Leu Thr Thr Leu Thr Leu Leu Phe Thr Lys	16	TCA GCG GCC TTA ACA GAT TGT ACA AAG TGG TTA AAT GGG GTT GGT CTA	1056
TTA GTA AAC TGT AAT CCT GTT TCC ACA AAA AAT AGG GAC TCT ATA CAG	96	Ser Ala Ala Leu Thr Asp Cys Thr Lys Trp Leu Asn Gly Val Gly Lys	352
Leu Val Asn Cys Asn Pro Val Ser Thr Lys Asn Arg Asp Ser Ile Glu	32	GGT GCC AGA TAT GAT GGA AGT TGG ACC AAA GAC AAT GAA AAA TCT CAT	1104
TTT ATT TAT AAA GAA AAG GAT AGT ATA TAC TCT GCC ATC AAC AAT CAA	144	Gly Ala Arg Tyr Asp Gly Ser Trp Thr Lys Asp Asn Glu Lys Ser His	368
Phe Ile Tyr Lys Glu Lys Asp Ser Ile Tyr Ser Ala Ile Asn Asn Gln	48	TAC ATA AAT ACG TGT GCA AAC AAC GAG AAT ATT GCC TTG TGG CCC GAA	1152
GCC ATC AAT GAA AAA ATT CAT GGA GTC AAT TTG GGT GGG TGG CTA GTA	192	Tyr Ile Asn Thr Cys Ala Asn Asn Glu Asn Ile Ala Leu Trp Pro Glu	384
Ala Ile Asn Glu Lys Ile His Gly Val Asn Leu Gly Gly Trp Leu Val	64	GAG AGA AAA CAG AAC ACT AGA AAG TTT ATC GAG GCT CAA TTA GAT GCT	1200
TTG GAG CCG TAT ATT ACA CCT TCT TTA TTC GAG ACT TTC CGT ACT AAT	240	Glu Arg Lys Gln Asn Thr Arg Lys Phe Ile Glu Ala Gln Leu Asp Ala	400
Leu Glu Pro Tyr Ile Thr Pro Ser Leu Phe Glu Thr Phe Arg Thr Asn	80	TTT GAA ATG ACT GGT GGA TGG ATA ATG TGG TGT TAC AAG ACA GAG AAT	1248
CCG TAC AAC GAT GAC GGT ATT CCT GTT GAT GAA TAC CAT TTT TGT GAA	288	Phe Glu Met Thr Gly Gly Trp Ile Met Trp Cys Tyr Lys Thr Glu Asn	416
Pro Tyr Asn Asp Asp Gly Ile Pro Val Asp Glu Tyr His Phe Cys Glu	96	AGT ATC GAA TGG GAT GTT GAA AAA TTG ATT CAA CTC AAC ATT TTT CCG	1296
AAA TTA GGC TAT GAA AAG GCA AAG GAA CGC CTT TAT AGT CAT TGG TCG	336	Ser Ile Glu Trp Asp Val Glu Lys Leu Ile Gln Leu Asn Ile Phe Pro	432
Lys Leu Gly Tyr Glu Lys Ala Lys Glu Arg Leu Tyr Ser His Trp Ser	112	CAA CCT ATC AAC GAT AGG AAA TAT CCT AAC CAA TGT CAT TGA	1338
ACG TTC TAT AAA GAG GAA GAC TTC GCG AAA ATT GCT TCT CAA GGC TTC	384	Gln Pro Ile Asn Asp Arg Lys Tyr Pro Asn Gln Cys His ***	445
Thr Phe Tyr Lys Glu Glu Asp Phe Ala Lys Ile Ala Ser Gln Gly Phe	128	AAATTTGTGG CATATATTAA AGGTCTTGGG AATGTGATGA TTATATTGG AGAAAAAAT	1398
AAT TTG GTT AGA ATT CCT ATT GGG TAT TGG GCC TTT ACA ACG TTG AGT	432	GCCATCTAAA ACTCTTATA TAACATGATG AGGTATACAA ATAAAAATA CTTTATATCT	1458
Asn Leu Val Arg Ile Pro Ile Gly Tyr Trp Ala Phe Thr Thr Leu Ser	144	TATAATTATG CAATATAAAG TGCTTCCATC AACCGCTACT TCACITTTTA TCTGAACAT	1518
CAT GAT CCC TAT GTT ACC GCC GAG CAG GAA TAT TTT CTA GAC CGG GCT	480	GAATGGGGAG CTCAATGATGA TTACTGTGTG ATATTACGTG AGCGCGAAGA	1568
His Asp Pro Tyr Val Thr Ala Glu Gln Glu Tyr Phe Leu Asp Arg Ala	160		
ATC GAT TGG GCT AGG AAA TAT GGT TTG AAA GTA TGG ATT GAT CTT CAT	528		
Ile Asp Trp Ala Arg Lys Glu Lys Val Trp Ile Asp Leu His	178		
GGA GCC GCT GGT TCA CAA AAC GGA TTT GAT AAC TCA GGG TTG AGG GAT	576		
Gly Ala Ala Gly Ser Gln Asn Gly Phe Asp Asn Ser Gly Leu Arg Asp	192		
TCA TAT AAG TTT CTG GAA GAT GAA AAT TTA AGC GCC ACC ATG AAA GCT	624		
Ser Tyr Lys Phe Leu Glu Asp Glu Asn Leu Ser Ala Thr Met Lys Ala	208		
TTA ACG TAT ATT TTA AGC AAA TAC TCA ACA GAC GTA TAC CTG GAC ACC	672		
Leu Thr Tyr Ile Leu Ser Lys Tyr Ser Thr Asp Val Tyr Leu Asp Thr	224		

FIG. 4. Nucleotide sequence of the *SPR1* locus. TATAAA elements are underlined, and polyadenylation signals are marked with asterisks. The amino acid sequence derived from ORF1 starts at position +1 and ends at +1338. *Bam*HI (−775), *Sal*I (+333), and *Ssr*I (+1526) sites are indicated by lines above the sequence.

wild-type and mutant strains. These mutant strains germinated on YEPD medium at 30 or 37°C as well as the isogenic wild-type strains.

Spore thermoresistance was tested by removing the ascus wall from asci at various stages in development and incubating separated ascospores at various temperatures. In order to ensure that spores from both wild-type and mutant strains were stressed equally and also to minimize plating errors, sporulating cultures of cells of the two types were mixed prior to heat stress; for strains S52A and S52B, survivors were distinguished by scoring for the leucine requirement. The *spr1-2* mutant spores survived as well as wild-type spores at 4°C, room temperature, or 30°C (data not shown); however, when spores were incubated at 42°C (as separate cultures in Fig. 6A or as mixed cultures in Fig. 6B), *spr1* mutant spores were killed much more rapidly than wild-type spores. Similar results were observed with mutant ascospores derived from strain W61A (*spr1-1/spr1-1*; data not shown). This phenomenon appears to be related to spore maturation because the increased killing rate is observed in

spores only from newly formed asci (up to 20 h in SP₂ medium) and not in mature (24 h or older) asci. In contrast, wild-type and mutant spores obtained by sporulation of a heterozygous (*SPR1/spr1-2*) diploid strain, SPB1, were killed at equal rates at 42°C (Fig. 6C). This observation suggests that the *SPR1* exo-1,3- β -glucanase functions before partitioning of nuclei or is distributed to developing ascospores prior to the completion of (pro)spore walls.

DISCUSSION

Three lines of evidence are presented to demonstrate that the late sporulation gene *SPR1* encoded an exo-1,3- β -glucanase as follows. (i) The peptide predicted by the *SPR1* sequence had 64% identity to the *EXG1* exo-1,3- β -glucanase. (ii) *SPR1* transcripts appeared at the same time (between 6 and 8 h) during sporulation that the sporulation-specific exo-1,3- β -glucanase (characterized by Del Rey and coworkers) is induced (8, 9). (iii) *spr1* mutant strains fail to produce an exo-1,3- β -glucanase activity during sporulation. It seems likely then that *S. cerevisiae* encodes at least two exo-1,3- β -glucanases: *EXG1*, which is expressed maximally during the transition from the S phase to the G₂ phase of the vegetative cell cycle (8), and a conserved homolog, *SPR1*, which is expressed specifically during ascospore wall formation. Given the limited DNA sequence homology between the two genes, it is not surprising that we failed to detect the

SPR1	MVSFRGLTTLTLLFTKLVNCPVSTKNRDSIQFIYKEKDSIYSAINNQAINKEHGVNLG	60
EXG1	MLSLKTLTC-TLLTVSSVLATPVPARDPSSIQFVHEENKKRYDYDHGSLGEPVIRGVNIG	59
SPR1	GWLVLPEYITPSLFETFRNTNPNYDDGIPVDEYHFCCKLGYEKAKERLYSHWSTFYKEEDF	120
EXG1	GWLLLEPYITPSLFEAFRTNDNDDEGIPVDEYHFCQYLKGLAKSRLQSHWSTFYQEQDF	119
SPR1	AKIASQGFNLVRPIGYWAFTTSLHSDPYTAEQYFLDRAIDWARKYGLKVVWDLHGAAG	180
EXG1	ANIASQGFNLVRPIGYWAFQTLDDDPYVSLQESYLDQAIGWARNNSLKVWDLHGAAG	179
SPR1	SQNGFDNSGLRDSYKFLDENLSATMKALTYILSKYSTVDVYLDTVIGIELLNEPLGPVID	240
EXG1	SQNGFDNSGLRDSYKFLDENLAVTTNVLNLYLKKYSAEYLDTVIGIELNEPLGPVLD	239
SPR1	MERLKNLLKPAYDYLRNKNINSNQLIIVHDAFPQYHYWDFLNDKNEY-GVIIDHHBYQ	299
EXG1	MDKMKNDYLAPAYEYLRNKNISDQVIIHDAFPQYNYWDDFMT-ENDGYWGVTDHBBYQ	298
SPR1	VFSQVELTRKMERIKIACQWGDVASEKHWSVAGEFSAALTDCTKWLNGVGLGARYDGS	359
EXG1	VFASDQLERSIDEHIKVACEWGTGVLNESHWTGCEFAAALTDCTKWLNSVFGARYDGS	358
SPR1	WTKDNEKSHYINTCANNENIALWPBPKQNTKRFIEAQLDAFEMTGGWIMWCYTENSIE	419
EXG1	WVNGDQTSYIGSCANNDDIATVSDERKENTRYVEAQLDAFEMRGGWIMWCYTSSLE	418
SPR1	WDVEKLIQLNIFPQIPINDRKYPNQCHO	446
EXG1	WDAQRMLMFLNGLFPQPLTDKRYPNQCGTISNO	449

FIG. 5. Alignment of SPR1 and EXG1 peptide sequences. Alignment was determined with GENEPRO sequence analysis software (version 5.0) and revealed 288 matches over a span of 447 amino acids (64% homology). Colons (:) indicate the positions of exact matches, while asterisks (*) indicate conserved matches.

EXG1 gene by probing genomic Southern transfers with the *SPR1* gene under conditions of high stringency. Nebreda et al. (30) isolated *EXG1* and a related gene called *EXG2* by complementation of an *exg1-2* mutant strain. Vasquez de Aldana et al. (46) reported that the predicted *EXG2* gene product has significant homology to that of *EXG1*. Since the restriction maps of the *EXG2* and *SPR1* genes are rather dissimilar, it seems unlikely that these two genes are identical.

The predicted *SPR1* gene product contains a hydrophobic leader sequence similar in length to the sequence found in the *EXG1* gene product. This leader may serve to target the *SPR1* gene product to an extracellular compartment. The presence of the leader sequence, the kinetics of appearance of the *SPR1* transcript, and the enzymatic activities of exo-1,3- β -glucanases suggest that the *SPR1* gene product modifies glucan linkages in the developing ascospore wall and these linkages might either strengthen or, alternatively, lend plasticity to the developing wall. If this is so, then we would expect *spr1* mutant strains to form defective ascospores. We found that while *spr1* mutant strains sporulated with the same efficiency as wild-type strains, isolated mutant spores lost viability at 42°C much more rapidly than similarly

FIG. 6. *spr1-2* ascospores are less thermoresistant than wild-type spores. (A) Free ascospores from 16-h sporulating cultures of strains S52A (○) and S52B (□) were separately prepared and incubated at 42°C. (B) Free ascospores were prepared from a mixture of sporulating cultures of S52A and S52B and incubated at 42°C; Leu⁻ (○) and Leu⁺ (□) survivors were scored on minimal medium supplemented with adenine and histidine. (C) Free ascospores were prepared from strain SPB1 (*SPR1*⁺/*spr1-2*) and incubated at 42°C. ○, Leu⁻ survivors; □, Leu⁺ survivors.

treated wild-type spores. Only spores harvested before 16 h display this increased thermosensitivity; mutant spores harvested after 20 h were indistinguishable from the wild type in thermoresistance. This phenomenon seems most likely attributable to a delay in spore wall maturation. Del Rey et al. have shown that the vegetative exo-1,3- β -glucanase persists during sporulation, although at levels lower than those of the sporulation-specific enzyme (9). The eventual acquisition of thermoresistance may be the result of the relatively weak activity of the vegetative enzyme. This hypothesis could readily be tested by constructing an *exg1 spr1* double mutant strain and determining the viability of its ascospores.

A relatively long incubation period (24 to 60 h) at 42°C was required in order to observe the effect on viability. We assayed spore thermoresistance at 50°C and found a more rapid rate of killing, but the killing rates were virtually the same for both wild-type and mutant spores (data not shown). At the lower temperature, spore death may in part be the result of spore lysis or spore wall leakiness; mutant spores may die more rapidly than the wild type because of defects in the cell wall. Spore death at the higher temperature may be the result of protein denaturation; the rapid rate of killing by this mechanism obscures the more gradual killing observed at the lower temperature.

As might be expected from the kinetics of *SPR1* transcript accumulation, heterozygotes containing one copy of the disrupted gene and one wild type produce normal spores of both genotypes. This fact indicates that the *SPR1* gene is maternally acting, in that its action in the ascus is not limited to those progeny with an intact copy. It is probably distrib-

TABLE 2. Level of exo-1,3- β -glucanase activity in *SPR1* and *spr* mutant strains

Strain	Exo-1,3- β -glucanase activity with the following substrate ^a :	
	Laminarin	pNPG
W61C (<i>SPR1</i> ⁺)	253.0 $\pm$ 4.6	51.0 $\pm$ 1.2
W61A (<i>spr1-1</i>)	0.07 $\pm$ 0.01	4.2 $\pm$ 0.3
S52A (<i>SPR1</i> ⁺)	249.0 $\pm$ 7.5	63.5 $\pm$ 1.3
S52B (<i>spr1-2</i>)	0.09 $\pm$ 0.01	3.9 $\pm$ 0.5

^a Activity is expressed as nanomoles of product formed per hour per 10⁸ cells.

uted to prospore walls at a time before the individual spore nuclei are completely delimited.

The experiments described here show that among the yeast genes isolated by differential hybridization with sporulation-specific cDNA is one, *SPR1*, which is involved in the development of heat-resistant ascospores. We originally suggested that *SPR1* is first transcribed at about 6 h in yeast strain SK1 (17), which sporulates almost twice as fast as do most yeast strains (18). The results in this paper show that the kinetics of *SPR1* transcript appearance in strain AP1 a/ α is delayed relative to that of strain SK1. In strain AP1 a/ α , transcripts reach maximal levels at 10 h, while in strain SK1 transcript abundance peaks between 7 and 8 h (17). This delay in *SPR1* transcript accumulation may (partly) explain the slower rate of spore formation in strain AP1 a/ α relative to strain SK1.

In *S. cerevisiae* and other eukaryotes, the TATA element determines the site at which transcription is initiated and is usually 40 to 120 bp upstream from this site (42). TATA elements (canonical and degenerate) were located at sites ranging from 115 to 500 bp upstream of the start site of transcription. These elements may be too distant to control initiation of *SPR1* transcription and other sequence elements may function in place of the TATA element, as in the case of the *PGK* gene (31). Finally, we have found a sequence in the *SPR1* promoter region that has good homology to the URS1_H sequence element (4). We plan to construct a set of deletions in the *SPR1* promoter to assess the significance of this element.

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