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SSG1, a Gene Encoding a Sporulation-Specific 1,3-β-Glucanase in *Saccharomyces cerevisiae*

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In *Saccharomyces cerevisiae*, the meiotic process is accompanied by a large increase in 1,3-β-glucan-degradative activity. The molecular cloning of the gene (*SSG1*) encoding a sporulation-specific exo-1,3-β-glucanase was achieved by screening a genomic library with a DNA probe obtained by polymerase chain reaction amplification using synthetic oligonucleotides designed according to the nucleotide sequence predicted from the amino-terminal region of the purified protein. DNA sequencing indicates that the *SSG1* gene specifies a 445-amino-acid polypeptide (calculated molecular mass, 51.8 kDa) showing extensive similarity to the extracellular exo-1,3-β-glucanases encoded by the *EXG1* gene (C. R. Vazquez de Aldana, J. Correa, P. San Segundo, A. Bueno, A. R. Nebreda, E. Mendez, and F. del Rey, Gene 97:173–182, 1991). The N-terminal domain of the putative precursor is a very hydrophobic segment with structural features resembling those of signal peptides of secreted proteins. Northern (RNA) analysis reveals a unique *SSG1*-specific transcript, 1.7 kb long, which can be detected only in sporulating diploids (*MAT*a/*MAT*α) but does not appear in vegetatively growing cells or in nonsporulating diploids (*MAT*α/*MAT*α) when incubated under nitrogen starvation conditions. The meiotic time course of *SSG1* induction indicates that the gene is transcribed only in the late stages of the process, beginning at the time of meiosis I and reaching a maximum during spore formation. Homozygous *ssg1*/*ssg1* mutant diploids are able to complete sporulation, although with a significant delay in the appearance of mature asci.

The meiotic process in the yeast *Saccharomyces cerevisiae*, triggered upon starvation of *MAT*a/*MAT*α diploid strains, represents a regulated program of cellular differentiation in a relatively simple eukaryote, which includes genetic recombinations, meiotic divisions, and the encapsulation of the four haploid nuclei into progeny ascospores. As a model system for studying development, research has been directed largely toward identifying the cytological, biochemical, and genetic events that occur during sporulation and evaluating the extent to which such events are important to the process. Some of them may merely be a physiological response to changes in the conditions needed to induce sporulation and perhaps play no role at all in meiosis or spore formation. Such changes would probably occur in nonsporulating cells under the same changing conditions. Other events of more interest are those that are necessary for sporulation to occur; these events could also occur in nonsporulating cells or could be specific to the meiotic program (15).

Quite a few studies have now established that meiosis and ascosporogenesis are complex processes involving a large number of specific gene products. One class of genes that are uniquely concerned with sporulation has been identified by the isolation of asporogenous mutants (*spo*), and the analysis of these genes has provided significant insight into the nature of the process and its control as well as a sound basis for more detailed molecular genetic studies (1, 22, 23). In an alternative approach, a large number of genes expressed preferentially during sporulation (*SPR*, *SPS*, and *SIT* genes) have been directly identified by different hybridization screening procedures (11, 29, 55). Transcriptional studies of the cloned genes demonstrate that the elaboration of sporulation-specific messages is tightly programmed, and the correlation of the appearance of these messages with certain landmark events of sporulation has provided some indication of the role exerted by the encoded products. Thus, on the basis of the times at which increases in RNA are first seen, it has been proposed that genes whose expression is triggered during sporulation can be grouped into at least three induction classes of early, middle, and late genes (32, 36). More recently, a fourth class (very late) has been created to accommodate those genes whose transcripts appear during the final stages of sporulation (46). While early and middle genes are presumably involved in the meiotic events at the first stages of the differentiation process (DNA replication, recombination, and chromosome segregation), the genes included in the late and very late classes are assumed to be responsible for the synthesis and assembly of the cell wall components of nascent spores as well as for the maturation process which renders them more resistant to environmental stress. In most cases, however, the precise biochemical lesion in the asporogenous mutants and/or the physiological function of the products encoded by the cloned sporulation-specific genes remain to be established.

For a number of years, we have been involved in the characterization of 1,3-β-glucanases from *S. cerevisiae*, enzymes whose activity is thought to be necessary in morphogenetic events that require controlled hydrolysis of the cell wall, since 1,3-β-glucan is the main structural polysaccharide responsible for the strength and integrity of the yeast cell envelope. Current knowledge of the 1,3-β-glucanase complement of *S. cerevisiae* reveals that this is a quite complex system, with several forms differing not only in

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structural properties and substrate specificities but also in their fate and appearance through the cell cycle (54). Thus, during vegetative growth the presence of several endo- and exo-1,3-β-glucanases has been reported, although only two have been studied in detail (9, 24, 31, 63). The major form is a glycoprotein of about 56 kDa with exoglucanase activity (EXG II), which hydrolyzes both 1,3-β- and 1,6-β-linkages and even has β-glucosidase activity since it acts on synthetic glucosides such as *p*-nitrophenyl-β-D-glucoside and 4-methylumbelliferyl-β-D-glucoside. The second form is also a nonspecific exoglucanase (EXG I) that shares the same protein fraction with the major exo-β-glucanase but differs in a higher content of the carbohydrate moiety. Both forms are produced constitutively and are first secreted to the periplasmic space and then released into the culture medium, where they accumulate (60, 63). With regard to meiosis, the induction of the sporulation process significantly increases the level of 1,3-β-glucan-degradative activity. This alteration is due mainly to the induction of a new 1,3-β-glucanase which appears in cells committed to sporulation and is not detected in vegetatively growing cells (17, 18).

In recent years, our work has focused on the characterization of the system governing 1,3-β-glucanase synthesis in yeast. Genetic approaches were initiated by the isolation of *S. cerevisiae* mutants with highly reduced exo-β-glucanase activity by a screening method based on the capacity of these enzymes to hydrolyze synthetic derivatives, such as the fluorogenic substrate 4-methylumbelliferyl-β-D-glucoside. By functional complementation of the chromosomal mutation harbored by these strains (*exg1*), we cloned and identified two different fragments that were able to restore the capacity to hydrolyze 4-methylumbelliferyl-β-D-glucoside (51). One of the fragments contains a structural gene (*EXG1*) coding for a protein whose differential glycosylation accounts for the two main extracellular exo-1,3-β-glucanases (EXG I and EXG II) in culture supernatants of *S. cerevisiae* (50, 51, 72). The second fragment identified produces only partial complementation of the Exg^- phenotype of *exg1* strains and contains a gene (*EXG2*) that codes for a minor exo-1,3-β-glucanase that we have designated EXG III; this enzymic form is a high-molecular-weight protein exhibiting a high carbohydrate content but showing a significant degree of similarity in the protein fraction to those of the EXG I and EXG II exo-1,3-β-glucanases (19). This article reports on the molecular cloning and characterization of the gene coding for the sporulation-specific exo-1,3-β-glucanase.

MATERIALS AND METHODS

Strains and culture conditions. *Escherichia coli* DH5α and MV1190 were used as hosts for transformations and plasmid propagation. Most of this work was done with the sporulation-proficient *S. cerevisiae* strain AP1 *MAT***a**/*MAT*α and its isogenic asporogenous derivative AP1 *MAT*α/*MAT*α; the AP1 genotype is *ade1*/+ *ade2*/*ade2* *ura1*/+ *his7*/+ *lys2*/+ *tyr1*/+ *gal1*/+ +/*ura3* +/*cyh2* +/*can1* +/*leu1* (33). Other yeast strains used were EG123 (*MAT***a** *leu2 his4 ura3 trp1*), 80 (*MAT***a** *leu2 lys1 trp1*), α131-20 (*MAT*α *ade2 ura3 cyh2 can1 leu1*), 23a20 (*mat***a**1 *leu2 his4 ura3 trp1*), 23a7-12 (*mat***a**2 *leu2 his4 ura3 trp1*), 23α113 (*mat*α1 *leu2 his4 ura3 trp1*), 23α182 (*mat*α2 *leu2 his4 ura3 trp1*), X2180-1A (*MAT***a**), TD28 (*MAT***a** *ura3 ino1*), W3031A (*MAT***a** *ura3 leu2 his3 trp1 ade2*), and L839 (*MAT*α *ura3 leu2 his1*).

Bacteria were grown in Luria-Bertani, M9, or 2×YT medium (48), supplemented with ampicillin (50 μg/ml) or tetracycline (15 μg/ml) when required. Yeast cells were grown vegetatively in YEPD (1% yeast extract, 2% peptone, 2% glucose) or YEPA (0.5% yeast extract, 0.6% yeast nitrogen base, 0.5% peptone, 1% potassium acetate, 1.02% potassium biphthalate, pH 5.5) medium. For sporulation, cells were harvested from presporulation YEPA medium at 1 × 10^7 to 2 × 10^7 cells per ml, washed twice with sporulation medium (1% potassium acetate), and resuspended at 1.5 × 10^7 cells per ml in the same sporulation medium. Both pregrowth and sporulation cultures were aerated at 30°C on a gyratory shaker in flasks in which no more than 1/10 of the capacity was filled with medium. Ascus formation was determined by light microscopy using phase-contrast optics. In some experiments, progress through meiosis was monitored by staining the cells with the nuclear stain 4′,6-diamidino-2-phenylindole as described previously (43) and using a Nikon Optiphot microscope equipped for epifluorescence.

Recombinant DNA manipulations. Transformation of *E. coli*, plasmid preparation, restriction mapping, DNA ligations, colony hybridization, Southern blotting, and other DNA manipulations were done by standard techniques (48). Transformation of yeast cells was carried out by the lithium acetate procedure (35). Yeast genomic DNA for Southern blots was prepared as described by Struhl et al. (69). DNA fragments to be used as probes were labeled by the random priming procedure (25) using Klenow enzyme and hexamers from Amersham and were subsequently purified on Sepharose CL-6B columns.

Nucleotide sequences were determined by the enzymatic dideoxy chain termination method (64). Unidirectional deletions of DNA fragments, subcloned into the Bluescript KS^+/SK^+ vectors, were generated with exonuclease III and S1 nuclease (30), and single-stranded DNA sequencing was carried out by using the Sequenase version 2.0 kit, as described by the manufacturer (U.S. Biochemical Corporation). Occasionally, specific oligonucleotides synthesized according to the complementary strand were used as primers.

PCR analysis. Polymerase chain reaction (PCR) amplification was performed in a Thermal Cycler (Perkin-Elmer Cetus). The reaction mixture (100 μl total) contained PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 1.5 mM $MgCl_2$, 0.001% gelatin), 0.2 mM (each) deoxynucleoside triphosphate, 50 pmol (each) of the oligonucleotide primers, 2.5 U of *Taq* polymerase, and 50 ng of genomic DNA template prepared from *S. cerevisiae* X2180-1A. Amplification was carried out in 25 sequential cycles of denaturation of the DNA at 94°C for 30 s, primer annealing at 50°C for 30 s, and elongation at 72°C for 30 s.

RNA preparation, Northern analysis, and transcript mapping. RNA was prepared from sporulating cells by a method similar to that described by Percival-Smith and Segall (55). Cells harvested from 150 ml of sporulation cultures were disrupted by vortexing (8 min in 1-min bursts interrupted by 30-s cooling in an ice-salt slurry) in a mixture containing 1 ml of lysis buffer (50 mM Tris-HCl [pH 6.8], 10 mM EDTA, 100 mM NaCl), 2 ml of phenol-chloroform (1:1), 0.25 ml of 20% sodium dodecyl sulfate (SDS), and 5 g of acid-washed 0.5-mm glass beads. The aqueous phase, separated by centrifugation, was reextracted five times with phenol-chloroform (1:1), and the RNA was precipitated by the addition of 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ethanol. After precipitating at −20°C, the RNA was collected by centrifugation, resuspended in 2 ml of water, and reprecipitated by the addition of an equal volume of 5 M

LiCl and 2 volumes of ethanol. Poly(A)$^+$ RNA was isolated by affinity chromatography on oligo(dT)-cellulose.

In Northern (RNA) blot analyses, RNA was denatured by incubation at 50°C for 1 h in a mixture (24 μl) containing 1 M deionized glyoxal, 50% dimethyl sulfoxide, and 10 mM sodium phosphate (pH 7.0). The samples were then electrophoresed in a 1% agarose–10 mM sodium phosphate (pH 7.0) gel and transferred to a nylon membrane (Zeta-probe) overnight by capillary action with 10 mM NaOH. Blots were prehybridized for 4 to 5 h at 50°C in a buffer containing 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50 mM sodium phosphate (pH 7.0), 0.5% nonfat milk powder, 1% SDS, and 0.5 mg of salmon sperm DNA per ml. The filters were then placed in fresh buffer supplemented with 10% dextran sulfate, hybridized to the appropriate probe for 15 h at the same temperature, and washed at room temperature (15 min) with 2× SSC–0.1% SDS and then with 0.2× SSC–1% SDS at 68°C (15 min).

Primer extension analysis was carried out essentially as described by Maniatis et al. (48) by using a synthetic oligonucleotide (5′-GTTAGTGTAGTCAGCCCTCTGAAC-3′) complementary to nucleotide positions +32 to +9 of the *SSG1* coding sequence.

Assay for 1,3-β-glucanase activity. β-Glucanase activity was assayed in crude extracts from cells at different stages of the sporulation process. For this purpose, cells from 25-ml aliquots of sporulation cultures were harvested by centrifugation, washed with 50 mM sodium acetate buffer (pH 5.5), and suspended in 2 ml of the same buffer. Ballotini glass beads (3 g; diameter, 0.5 mm) were added, and the mixture was vortexed for 5 min with regular chilling on ice. The homogenate was dialyzed overnight against 50 mM acetate buffer (pH 5.5) and used for enzyme assay.

The assays were based on the release of reducing sugar groups from laminarin. Reaction mixtures contained, in 0.6 ml of 50 mM acetate buffer (pH 5.5), the substrate at a concentration of 0.25% and the corresponding enzyme preparation. Incubations were carried out at 37°C for periods ranging from 20 to 60 min, and the reaction was stopped by heating in a boiling-water bath for 3 min. The precipitated materials were removed by centrifugation, and the supernatant fluids were used to determine reducing sugars by the methods of Somogyi (66) and Nelson (52). The results were compared with glucose as a standard. Specific activity was expressed as units per milligram of protein, with a unit defined as the amount of enzyme that released reducing sugars equivalent to 1 μmol of glucose per h. Protein was quantitated by the method of Peterson (57), with bovine serum albumin as a standard.

Protein purification and N-terminal sequence determination. The starting material for purification of the sporulation-specific 1,3-β-glucanase was a culture of the AP1 *MAT***a**/*MAT*α strain maintained for 15 h under sporulation conditions. Cells from 10 liters of culture were harvested by low-speed centrifugation, broken down in a Braun homogeneizer, and centrifuged first at 40,000 × *g* for 20 min and then at 105,000 × *g* for an additional 40 min. The supernatant, considered the initial extract, was applied first to a DEAE-Bio-Gel A ion-exchange column equilibrated with 10 mM succinate buffer (pH 5.0), which retained the vegetative *EXG1*-encoded exo-1,3-β-glucanases. The eluate was dialyzed against 10 mM succinate buffer (pH 4.5) and applied to a CM-Bio-Gel A column equilibrated with the same buffer; the column was washed with the equilibrium buffer, and the proteins retained were eluted with a linear NaCl gradient (0 to 0.5 M in succinate buffer). Fractions showing β-glucanase activity were pooled, concentrated, dialyzed against 50 mM sodium acetate buffer (pH 4.5), and filtered through Sephacryl S-200. Finally, fractions containing 1,3-β-glucanase activity were subjected to fast-performance liquid chromatography (FPLC system; Pharmacia) first on a Mono S HR5/5 column equilibrated with 50 mM acetate buffer (pH 4.5) and then on a Mono Q HR5/5 column equilibrated with 20 mM piperazine buffer (pH 6.0); in both cases, the columns were developed with a linear NaCl gradient (0 to 0.3 M) in the corresponding buffer. After the different purification steps, progress was monitored by electrophoresing aliquots on 0.1% SDS–12% acrylamide gels according to the method of Laemmli (45); proteins were stained with silver nitrate (49).

Amino-terminal sequencing of the purified 1,3-β-glucanase was done on a liquid-phase Beckman sequencer (model 890-D) according to the method of Edman and Begg (21) and was followed by reverse-phase high-performance liquid chromatography analysis of the resulting phenyl-thiohydantoin derivatives on a Waters PTH amino acid analyzer.

Assay for spore thermotolerance. Resistance of cells to heat was assessed essentially as described by Briza et al. (7). Cells from sporulating cultures 37 h after transfer to sporulation medium were harvested by centrifugation and suspended in sterile water at a density of 2×10^7 cells per ml. One milliliter of this suspension was placed in an Eppendorf tube, which was then immersed in a 50°C water bath. At several time points (20, 40, 60, and 80 min), spore viability was determined by plating appropriate aliquots on YEPD medium and incubating at 30°C for 48 h.

Nucleotide sequence accession number. The sequence data reported here have been assigned EMBL accession number X59259.

RESULTS

Molecular cloning of the *SSG1* gene. We have previously reported that sporulation in *S. cerevisiae* is accompanied by a large increase in 1,3-β-glucan-degradative activity, an increase that is due mainly to the synthesis of a 1,3-β-glucanase not detected during vegetative growth (18). We took advantage of the abundance of this protein in sporulating cells in order to design a strategy for cloning the corresponding gene. Thus, the sporulation-specific exo-1,3-β-glucanase was isolated from *MAT***a**/*MAT*α diploid cells maintained for 15 h under glucose and nitrogen starvation conditions (35% asci), and the purified protein, with an estimated molecular mass of 44 kDa, was subjected to Edman degradation to obtain the first 23 N-terminal residues of the mature product (Fig. 1A).

Degenerate oligonucleotides based on the peptide sequences AINEKI (coding strand, mixture a) and EPYITP (noncoding strand, mixture b) were synthesized, with restriction endonuclease sites added at their 5′ termini (*Pst*I and *Sal*I, respectively). These oligonucleotide mixtures were used as primers in PCR to amplify the region of the yeast genomic DNA flanked by them. A major band, approximately 80 bp in length, was detected upon electrophoresis of the PCR products. This size coincided with the expected one deduced from the amino acid sequence data, and accordingly, this PCR product was digested with *Pst*I and *Sal*I, cloned into a Bluescript vector, and sequenced. The amino acid sequence deduced from its nucleotide sequence perfectly matched the N terminus of the exo-1,3-β-glucanase, and we therefore concluded that the PCR product was derived from the gene coding for this protein.

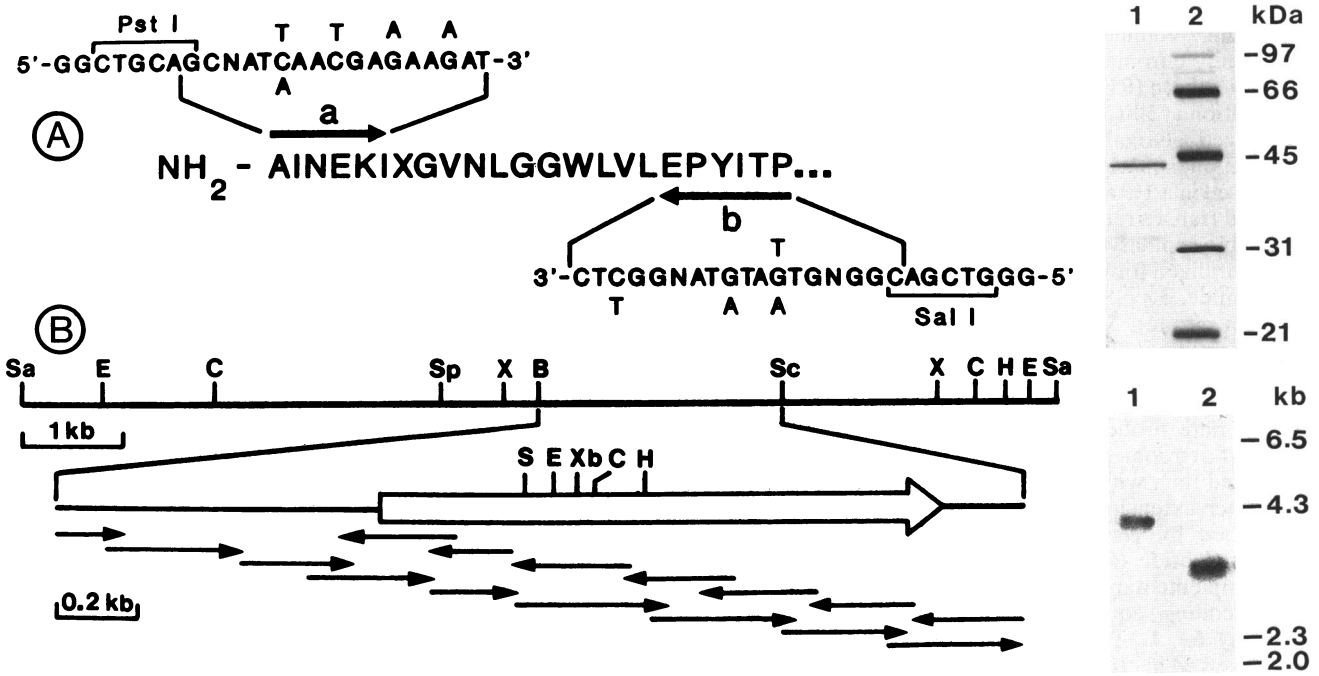

FIG. 1. Cloning and structure of the *SSG1* gene. (A) N-terminal amino acid sequence of the sporulation-specific 1,3-β-glucanase (X represents a residue that could not be identified), purified from cell extracts of an *S. cerevisiae* AP1 *MAT***a**/*MAT*α sporulating culture. The two stretches of the sequence indicated by horizontal arrows were chosen, and oligonucleotide mixtures a and b were synthesized as indicated. Electrophoretic behavior of the purified 1,3-β-glucanase (lane 1) in a 0.1% SDS–12% acrylamide gel, stained with silver nitrate, is shown on the right. Marker proteins (lane 2) are phosphorylase *b* (97 kDa), seroalbumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21 kDa). (B) Restriction map and sequencing strategy for the *SSG1* gene. The position of the *SSG1*-coding region and the direction of transcription are indicated (open arrow). Arrows at the bottom indicate the direction and extent of sequencing. Restriction sites: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; Sa, *Sau*3A; Sc, *Sac*I; Sp, *Sph*I; X, *Xho*I; Xb, *Xba*I. A Southern blot of chromosomal DNA from *S. cerevisiae* strain TD28, digested with *Xho*I and hybridized to a 1.2-kb *Sal*I-*Sac*I *SSG1*-containing probe (lane 1) or to a 2-kb *Xho*I-*Nae*I *EXG1*-containing probe (lane 2) (72), is shown on the right. *Hin*dIII-digested lambda DNA was run in adjacent lanes as a size standard.

Subsequently, the cloned segment was isolated by digestion with *Pst*I and *Sal*I, labeled, and used as a probe to screen a genomic *S. cerevisiae* GRF88 DNA library consisting of partially *Sau*3A-cleaved fragments ligated into the *Bam*HI site of the centromeric vector YCp50 (61). Of 8,000 clones screened by colony hybridization, 11 gave a positive signal, with all of them containing plasmids with an identical *Sau*3A insert of about 10 kb, as shown by restriction mapping. Sequence analysis of this fragment, using as primers oligonucleotides designed in accordance with the known sequence from the original PCR product, confirmed that the exo-1,3-β-glucanase-encoding gene, which we designated *SSG1* (sporulation-specific glucanase), was present in the insert.

Nucleotide sequence and transcription of the *SSG1* gene. The restriction map of the 10-kb *Sau*3A *SSG1*-containing fragment is outlined in Fig. 1B. Hybridization experiments using the PCR-derived probe showed that the coding region for the exo-1,3-β-glucanase was contained within the 4.2-kb *Xho*I fragment, and in view of this, the nucleotide sequence of this region was determined. Southern blot analysis, using different probes encompassed within the 4.2-kb *Xho*I fragment to hybridize genomic DNA digested with different restriction enzymes, indicated that no rearrangement of the DNA structure occurred during the cloning of the fragment and, additionally, that the *SSG1* gene exists as a single copy in the yeast genome (shown in Fig. 1B is an *Xho*I digestion product probed with the 1.2-kb *Sal*I-*Sac*I fragment). Experiments performed in parallel but using an *EXG1* gene-containing probe did not, under stringent conditions, reveal cross hybridization with the sequences encoding the vegetative exo-1,3-β-glucanases EXG I and EXG II, previously described.

Only one possible open reading frame long enough to encode a polypeptide of the size estimated for the 1,3-β-glucanase was detected when both DNA strands were examined. This open reading frame is found starting 769 bp from the *Bam*HI site and spans 1,335 nucleotides, with a coding capacity of 445 amino acids. Figure 2 shows the nucleotide sequence of a 2,578-bp region around the open reading frame together with a predicted amino acid sequence for the primary translation product. Nucleotide A of the first in-phase ATG codon within the open reading frame is numbered 1, and it seems likely that this is the translational initiation site since there is no other ATG start codon located prior to the N terminus of the mature protein, as identified by amino-terminal sequence analysis. The proposed AUG start codon, however, is located in a nucleotide context (5′-GUAAAUGGUU-3′) that shows only a 6 of 10 fit with that commonly found in *S. cerevisiae* mRNAs (5′-[A/Y]A[A/U]AAUGUCU-3′) (10), differing from the consensus sequence at positions −4, −3, +4, and +5.

Direct evidence for the expression of this open reading frame came from Northern transfer experiments. Poly(A)-enriched RNA, obtained from the diploid strain AP1 *MAT***a**/*MAT*α maintained for 10 h under sporulation conditions

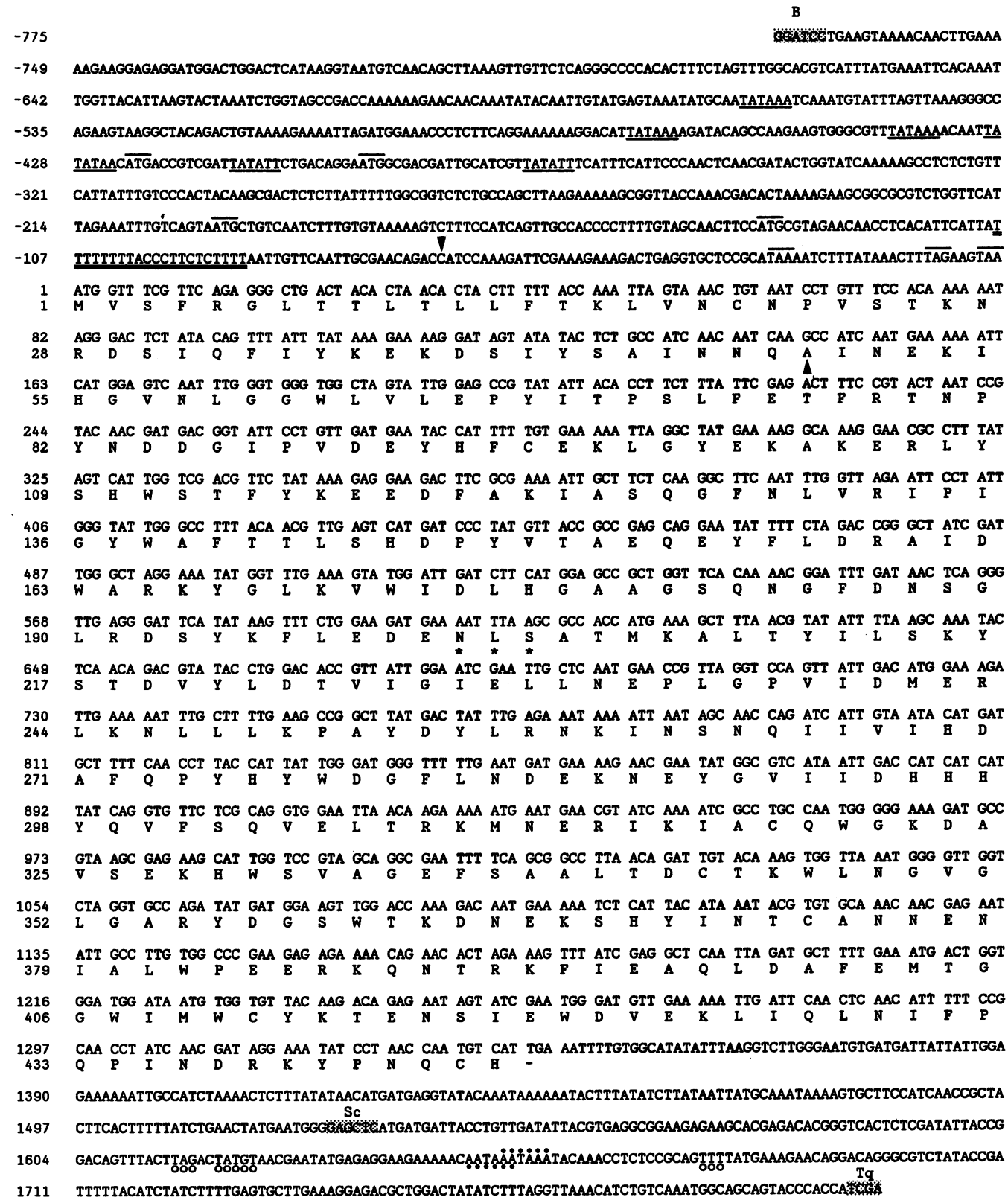

FIG. 2. Nucleotide sequence of the *SSG1* locus and predicted amino acid sequence of the primary translation product. In the 5′-flanking region, consensus TATA elements and TATA-related sequences are underlined by a single line and the pyrimidine-rich tract (C+T) is double underlined. Four upstream ATGs as well as three in-frame stop codons immediately preceding the putative initiation ATG are overlined. The mapped transcription start point is marked by a downward arrowhead. In the 3′-flanking region, sequences matching the consensus transcription termination-polyadenylation signals are indicated by either open circles (for the TAG. . . TATGT. . . TTT element) or solid dots (for the AATAAA element). The first amino acid of the mature protein is marked by an upward arrowhead. A potential site for N glycosylation is marked with asterisks. Restriction sites: B, *Bam*HI; Sc, *Sac*I; Tq, *Taq*I.

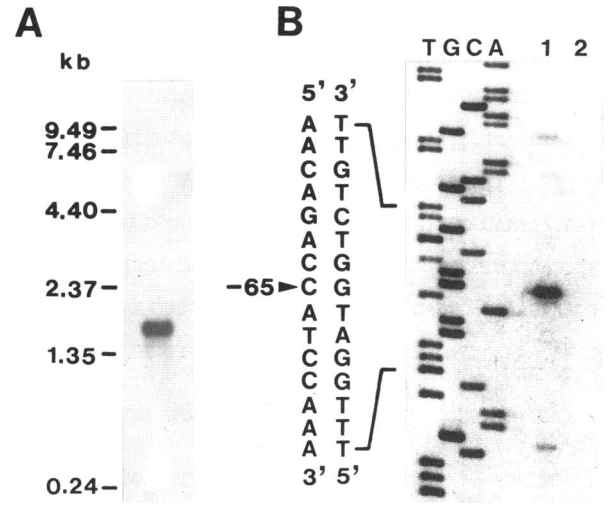

FIG. 3. Characterization of the *SSG1* transcript. (A) Northern analysis at the *SSG1* gene-containing region. Three micrograms of poly(A)-enriched RNA, obtained from sporulating cells of strain AP1 *MAT***a**/*MAT*α, was denatured with glyoxal, resolved by agarose gel electrophoresis, transferred to a nylon membrane, and probed with a labeled *SSG1*-containing fragment. The positions and sizes of RNA markers (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) are indicated on the left. (B) Transcript mapping by primer extension. A synthetic oligonucleotide complementary to the sense strand of the *SSG1* gene between +32 and +9 was labeled at the 5′ end (10^5 cpm, 0.125 pmol) and annealed to 3.5 μg of poly(A)$^+$ RNA from sporulating cells. The primer was then elongated with Rous-associated virus 2 reverse transcriptase, and the extended products (lane 1) were resolved on a sequencing gel alongside a sequencing ladder of the noncoding strand (lanes T, G, C, and A) obtained by using the same oligonucleotide as a primer. Poly(A)$^+$ RNA was substituted by the same amount of yeast tRNA for the reaction shown in lane 2.

(about 2% asci), was fractionated by electrophoresis, transferred to a nylon membrane, and hybridized to the radioactively labeled 1.2-kb *Sal*I-*Sac*I fragment encompassing most of the *SSG1* coding region. Figure 3 shows a single band of hybridization transcript arising from this region, with a size of approximately 1.7 kb, sufficient to accommodate the predicted 445-amino-acid polypeptide. *SSG1* transcription start sites were determined by extending a ^{32}P-labeled synthetic primer with reverse transcriptase following hybridization to poly(A)$^+$ RNA from sporulating cells. A major cDNA product 97 nucleotides long was obtained, indicating that the initiation of transcription preferentially takes place at the cytosine residue 65 bases upstream from the predicted start of translation (Fig. 3). This defines a presumptive 5′-nontranslated leader sequence that is slightly longer than that normally found in *S. cerevisiae* mRNAs (65 nucleotides compared with the usual 20 to 60 nucleotides). Kozak's scanning model for eukaryotic translation postulates that in most cases ribosomes translate from the first AUG encountered downstream from the 5′ end of the mRNA (41). Transcriptional start points should then lie between the ATG of the *SSG1* open reading frame and the closest ATG in the DNA sequence upstream from this point. Upstream from the *SSG1* coding region, there are four ATGs between positions −133 and −422; i.e., the transcripts starting at position −65 fit the Kozak model.

Analysis of the DNA sequence upstream of the *SSG1* gene for transcriptional signals showed that the 5′-flanking region does not display canonical TATA boxes in the interval where these promoter elements are usually located in *S. cerevisiae* (between 40 and 120 bp upstream from the transcription initiation point) (68). Several sequences with a perfect match to the consensus TATAAA sequence can, however, be identified beginning at positions −441, −471, and −565, and three copies of the functional variants TATATT and TATATAA (58) occur at positions −376, −410, and −430. Nevertheless, since these elements are too far upstream from the putative transcriptional start site, it seems unlikely that they would serve as initiation signals, and therefore, transcription of the *SSG1* gene may not depend on a recognizable TATA sequence, as has been observed for other yeast genes, including some involved in the sporulation process, such as *SPO13* (8). A pyrimidine-rich tract (CT block), found in many yeast promoters (20), is located immediately preceding the mRNA start point, spanning nucleotides −107 to −88. The CAAG motif, closely associated (about 10 nucleotides downstream) with the CT block and correlated with a high level of expression in certain yeast genes (20), is not found in the *SSG1* gene, although the related sequence CAAAG is present 26 bp after the CT-rich domain and very close (3 nucleotides downstream) to the mapped transcription start site. Thus far, sequence comparisons with the available 5′-flanking regions of other sporulation-regulated genes did not reveal striking similarities that might represent common regulatory elements responding to a meiosis-specific control.

Downstream of the open reading frame, there are several motifs commonly observed in the 3′ noncoding region of eukaryotic genes and thought to be necessary for either transcription termination, processing of the 3′ end, or addition of poly(A) at the 3′ terminus. Thus, a perfect match to the TAG. . . TATGT. . . TTT tripartite consensus sequence, which has been postulated to act in yeast cells as a transcription termination signal (78), appears between nucleotides 277 and 340 following the stop codon. Within this region, two copies of the hexanucleotide AATAAA that is usually found 10 to 30 nucleotides upstream from the polyadenylation site in higher eukaryotes (59) can be recognized between nucleotides 311 and 320 following the stop codon. Additionally, overlapping these AATAAA motifs, the sequence TAAATAAA matches the consensus TAAATAA (G/A) which is present in many yeast genes approximately 28 to 33 nucleotides upstream from the 3′ end of the mRNA and, for this reason, proposed to be important for yeast transcription termination (5). Taking into account the transcription start at position −65 and the proposed location of the transcription termination signals, an mRNA species of approximately 1.7 kb is expected, in accordance with the estimated size of the *SSG1* transcript detected by Northern blot analysis.

Primary structure of the *SSG1* gene product. Translation of the 1,335-bp open reading frame of the *SSG1* gene reveals that it specifies an acidic protein (calculated pI, 5.39) of 445 amino acids and a predicted molecular weight of 51,790 which contains 38.6% nonpolar, 34.4% polar, 13.5% positively charged, and 13.5% negatively charged residues. This protein fraction carries one potential site for N-linked glycosylation, which in eukaryotic proteins occurs at the tripeptide sequences Asn-X-Thr and Asn-X-Ser, in which X may be any amino acid with the possible exception of aspartate (34); such a sequence corresponds to amino acid positions 201 to 203 (Asn-Leu-Ser), although at this moment we do not know whether this acceptor site bears oligosaccharide chains N glycosidically attached to the asparagine residue.

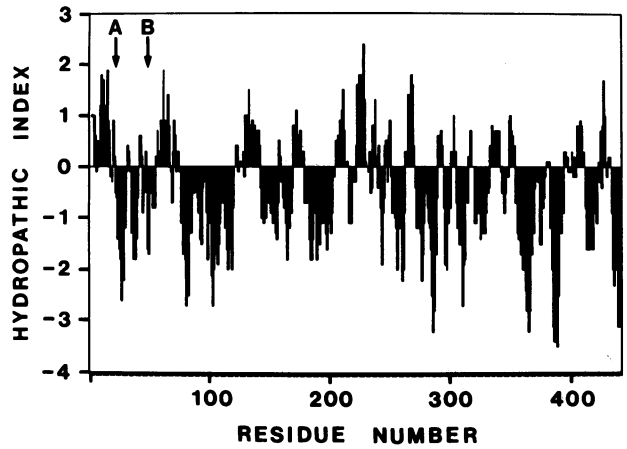

FIG. 4. Hydropathy profile of the complete *SSG1* product. The average hydropathy per amino acid was calculated along the sequence within windows of seven amino acids according to the method of Kyte and Doolittle (44). The first plotted value thus corresponds to amino acid residue 4. Peaks above the midpoint line are hydrophobic regions, and those below are hydrophilic. The extents of the proposed signal peptide (A) and the amino terminus of the mature protein (B) are indicated.

In *SSG1*, 59 of the 61 possible triplets are used and there is no codon bias toward the major isoacceptor tRNA species that characterizes some highly expressed yeast genes. According to the calculations described by Bennetzen and Hall (4), the *SSG1* gene has a codon bias index of 0.045, indicative of a virtually random codon usage and a low level of expression.

Hydropathy analysis of the product inferred from the *SSG1* nucleotide sequence (Fig. 4), based on the calculations of Kyte and Doolittle (44), shows a predominantly hydrophilic polypeptide with a hydrophobic N-terminal region, suggesting that cellular localization of the *SSG1* product involves entrance into the endoplasmic reticulum. The extent of a putative signal sequence at the amino terminus was predicted following the proposal of von Heijne (73), which is based on the frequency of appearance of different groups of amino acids in the vicinity of the signal peptidase cleavage point found in a wide variety of secretory protein precursors. According to this prediction method, the most likely cleavage site would correspond to the peptide bond between Cys-20 and Asn-21, defining a presumptive signal segment of 20 amino acids in length which shares common structural features with known secretory signal sequences: it is a very hydrophobic region (10 amino acids, including the N-terminal methionine), there is a positively charged residue near the N terminus (Arg at position 5), and the last residue immediately preceding the cleavage site is a small side chain amino acid (Cys). Cleavage of this presumptive leader has not been demonstrated, and additionally, the N terminus of the mature protein identified by conventional amino acid sequencing corresponds to residue 49 relative to the initiation codon, indicating that the precursor contains an amino-terminal extended peptide of 48 residues that is cut off during the process of protein maturation. According to this, it would be reasonable to surmise that the sporulation-specific 1,3-β-glucanase is originally synthesized as a precursor protein with complex posttranslational processing involving removal of the N-terminal hydrophobic portion by means of the signal peptidase during translocation to the endoplasmic reticulum, followed by additional proteolytic cleavage to yield a mature polypeptide of 397 amino acids and a calculated molecular size of 46,294 daltons. This size is slightly larger than the 44 kDa estimated on the basis of the migration of the purified enzyme in SDS-polyacrylamide gel electrophoresis. However, although additional C-terminal processing cannot be ruled out, the approximately 2-kDa difference may simply reflect a peculiar electrophoretic mobility of the mature protein.

Comparison to other 1,3-β-glucanase-encoding genes. To date, the nucleotide sequences of only two genes encoding 1,3-β-glucanases in *S. cerevisiae* have been reported; these correspond to the *BGL2* gene (40), coding for a 29-kDa cell-wall-associated glycoprotein with 1,3-β-glucanase activity, and to the *EXG1* gene (72), encoding two extracellular vegetative exo-1,3-β-glucanases (EXG I and EXG II). For other eukaryotic organisms, the nucleotide sequences of 1,3-β-glucanase genes from *Nicotiana tabacum* and *Nicotiana plumbaginifolia* (16, 65) and of a 1,3-1,4-β-glucanase gene of barley aleurone (26) have also been reported.

Comparison of the predicted amino acid sequences of the polypeptides encoded by *BGL2* and *SSG1* showed only limited similarity (11% [19% with conservative substitutions]), just as the *SSG1* product does not present any relevant similarity with the above-mentioned plant β-glucanases. The *EXG1* gene, however, shows a significant degree of similarity to the *SSG1* sequence. At the DNA sequence level, these genes are related over the entire length of the coding region, as shown by diagonal plot-dot matrix analysis of the paired sequences (data not shown), displaying 69% identity under maximal alignment parameters. Consequently, comparison of the amino acid sequences of the predicted primary translation products encoded by *SSG1* and *EXG1* reveals that both proteins are extremely homologous. As shown in Fig. 5, their similarity results from a high percentage of identities and an accumulation of many conservative replacements: in 446 aligned amino acids, 287 residues of the *SSG1* product (64%) are identical to residues in the *EXG1* protein and another 55 residues represent conserved amino acid substitutions, such that there is an overall level of similarity of 76%. Most of the mismatches are found in the hydrophobic N-terminal regions of the predicted polypeptides, which are cleaved off during processing or maturation of the putative precursors. Thus, when comparative analyses are done without these additional peptides at the N termini, the identity rises to 66% (78% similarity with conservative substitutions).

Developmental-stage-specific expression of the *SSG1* gene. Assignment of the *SSG1*-encoded glucanase to the meiotic process was based on the fact that this activity appears in *MAT***a**/*MAT*α diploid cells committed to sporulation but not in vegetatively growing cells or in asporogenous *MAT*α/*MAT*α diploids when subjected to nitrogen starvation (18). There existed, however, the possibility that the enzyme might be synthesized at such small amounts in these cells that it could not be detected or, alternatively, that the enzyme might be produced as a zymogen during vegetative growth and converted into the active form upon sporulation. In order to verify that the glucanase is indeed the product of a gene which remains silent during vegetative growth and whose expression is triggered as a result of the sporulation program (that is, to verify that *SSG1* expression is developmentally regulated), Northern analyses were carried out with a *MAT***a**/*MAT*α strain under vegetative growth as well as with *MAT***a**/*MAT*α and *MAT*α/*MAT*α diploids 10 h after the transfer to sporulation medium. As shown in Fig. 6,

```
EXG1 : MLSLK--TLLCTLLTVSSVLATPVPARDPSSIQFVHEENKKRYYDYDHGSLGEPIRGVNI
SSG1 : MVSFRGLTTL-TLLFTKLVNCNPVSTKNRDSIQFIYKEKDSIYSAINNQAINEKIHGVNL
       *.*..  * * ***    *   **  ..  ****.  *    *   ..  * * *.***.

EXG1 : GGWLLLEPYITPSLFEAFRTNDDNDEGIPVDEYHFCQYLGKDLAKSRLQSHWSTFYQEQD
SSG1 : GGWLVLEPYITPSLFETFRTNPYNDDGIPVDEYHFCEKLGYEKAKERLYSHWSTFYKEED
       *****.************ ****  **.**********. ** . ** ** ******* *.*

EXG1 : FANIASQGFNLVRIPIGYWAFQTLDDDPYVSGLQESYLDQAIGWARNNSLKVWVDLHGAA
SSG1 : FAKIASQGFNLVRIPIGYWAFTTLSHDPYVTAEQEYFLDRAIDWARKYGLKVWIDLHGAA
       ** ******************* **  ****   ** .** ** ***   *****.******

EXG1 : GSQNGFDNSGLRDSYKFLEDSNLAVTTNVLNYILKKYSAEEYLDTVIGIELINEPLGPVL
SSG1 : GSQNGFDNSGLRDSYKFLEDENLSATMKALTYILSKYSTDVYLDTVIGIELLNEPLGPVI
       ******************** **  *   * *** *** . **********.********.

EXG1 : DMDKMKNDYLAPAYEYLRNNIKSDQVIIIHDAFQPYNYWDDFMTENDGYWGVTIDHHHYQ
SSG1 : DMERLKNLLLKPAYDYLRNKINSNQIIVIHDAFQPYHYWDGFLNDEKNEYGVIIDHHHYQ
       **...**  * ***.**** * *.*.*.*********.*** *. .     ** *******

EXG1 : VFASDQLERSIDEHIKVACEWGTGVLNESHWTVCGEFAAALTDCTKWLNSVGFGARYDGS
SSG1 : VFSQVELTRKMNERIKIACQWGKDAVSEKHWSVAGEFSAALTDCTKWLNGVGLGARYDGS
       **   .* * ..*.**.**.**   . * ** * *** ********** **.*******

EXG1 : WVNGDQTSSYIGSCANNDDIAYWSDERKENTRRYVEAQLDAFEMRGGWIIWCYKTESSLE
SSG1 : WTKDNEKSHYINTCANNENIALWPEERKQNTRKFIEAQLDAFEMTGGWIMWCYKTENSIE
       *   .. * **  *****..** * .***.***...********** *****.****** *.*

EXG1 : WDAQRLMFNGLFPQPLTDRKYPNQCGTISN
SSG1 : WDVEKLIQLNIFPQPINDRKYPNQCH----
       ** ..*.   .****. ********
```

FIG. 5. Alignment of the amino acid sequences of the *SSG1* and *EXG1* primary translation products. Identical residues (asterisks) in the two polypeptides and conservative amino acid substitutions (dots) are indicated. The first amino acid in each mature protein is indicated by an arrowhead.

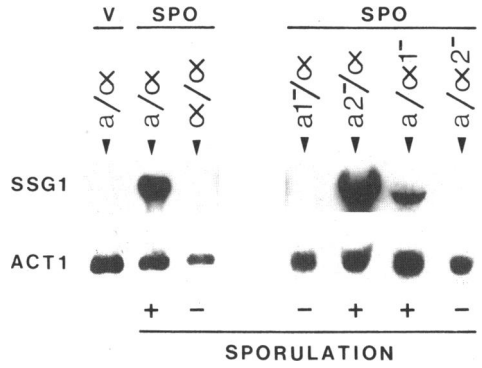

FIG. 6. Developmental-stage-specific expression of the *SSG1* gene. Total RNAs prepared from diploid strains with different configurations at the *MAT* locus (as indicated above each lane), growing vegetatively (V) or at 10 h after transfer to sporulation medium (SPO), were denatured, fractionated by electrophoresis (30 μg per lane) in a 1% agarose gel, and transferred to a nylon membrane. Immobilized RNA was then hybridized to an *SSG1*-containing fragment (1.2-kb *Sal*I-*Sac*I) that had been radioactively labeled by random priming. As a positive control, actin mRNAs were detected in the same blots by reprobing with an *ACT1*-containing fragment (1.7-kb *Bam*HI-*Hin*dIII [53]). The following strains were used: AP1 (*MAT***a**/*MAT*α or *MAT*α/*MAT*α), 23a20 × α131-20 (*mat***a**1/*MAT*α), 23a7-12 × α131-20 (*mat***a**2/*MAT*α), EG123 × 23α113 (*MAT***a**/*mat*α1), and 80 × 23α182 (*MAT***a**/*mat*α2).

SSG1 transcripts accumulate in sporulating *MAT***a**/*MAT*α cells whereas they are not detected in RNA from vegetatively growing cells or in RNA extracted from the asporogenous diploid incubated under identical sporulation conditions, even with longer autoradiographic exposures. To examine whether positive regulation of *SSG1* by the *MAT* locus (*MAT***a**1 and *MAT*α2) occurs at the transcriptional level, total RNA was isolated from diploid cells in which one of the four *MAT* genes was mutated and hybridized to the probe. As shown in the same figure, *SSG1* transcripts are absent from the nonsporulating diploids carrying the *mat***a**1 or *mat*α2 mutations whereas diploid cells carrying the *mat***a**2 or *mat*α1 alleles produce an amount of *SSG1* RNA equivalent to that of wild-type diploid cells. The hybridization pattern obtained with a fragment containing the actin gene (53), expressed in all cell types during vegetative growth and after transfer to sporulation medium, indicated that similar amounts of intact RNAs had been used for the comparisons described above. Taken together, these results confirmed the developmental specificity of expression of the *SSG1* gene, which requires both heterozygosity at the mating-type locus and nutrient deprivation and, like other sporulation-specific events, is dependent on the activation mediated by the **a**1-α2 mating-type product.

The meiotic time course of *SSG1* expression was examined by Northern analysis of *MAT***a**/*MAT*α cells at various stages in the differentiation process. The results revealed a temporal pattern of expression that resembles that of the previously described late class of sporulation-specific transcripts (32, 36). As shown in Fig. 7, *SSG1* transcripts are absent from mRNAs at the time that the cells are shifted to the nitrogen-deficient medium (time zero) and expression of the *SSG1* gene is first detected in cells 6 h after the transfer to sporulation conditions, at the time of meiosis I. Between 6 and 9 h, there is a sharp rise in the amount of transcripts (an approximately 40-fold accumulation), reaching a maximal level between 12 and 15 h, coincident with the moment at which asci can be first detected by light microscopy. From this point onward, there is a progressive but slow decline in *SSG1* transcripts, which, however, still remain abundant in cells that had been in sporulation medium for 27 h, when more than 80% of the population has been converted into asci. To control the integrity of the RNA isolated at early times, the same blot was hybridized to a fragment containing the actin gene; the corresponding transcripts were visible at approximately equal intensities in all the gel lanes. Additionally, a well-characterized sporulation-specific gene, the *SGA1* gene which encodes an intracellular glucoamylase (12, 13, 77), was used to compare its temporal pattern of expression with that observed for *SSG1*. Interestingly, the kinetics of accumulation of both mRNAs are very similar except for a small delay in *SGA1* induction, reflected by the absence of *SGA1* transcripts in 6-h samples, when *SSG1* mRNAs are already detectable.

Translation of *SSG1* transcripts into the encoded product was monitored by assaying 1,3-β-glucanase activity in extracts from *MAT***a**/*MAT*α cells harvested in parallel with those used to prepare mRNAs for Northern analysis. As a control, 1,3-β-glucanase activity was also measured for the isogenic asporogenous *MAT*α/*MAT*α strain, which was subjected to conditions of nutrient deprivation identical to those that trigger the meiotic process in heterozygous *MAT***a**/*MAT*α diploids. On the basis of the results shown in Fig. 7, three clearly defined periods in which the 1,3-β-glucan-degradative activity in sporulating cells parallels the kinetics of *SSG1* transcript accumulation can be distinguished. In the first period, which lasts for approximately 6 h of incubation under sporulation conditions, a basal 1,3-β-glucanase activ-

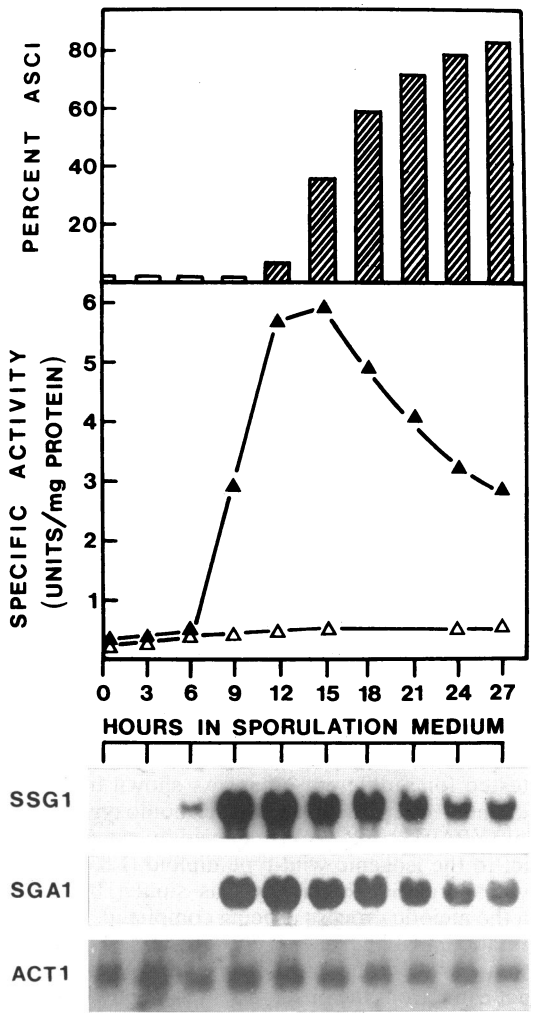

FIG. 7. Meiotic time course of *SSG1* expression. Vegetatively growing cultures of *S. cerevisiae* strains AP1 *MAT***a**/*MAT*α and AP1 *MAT*α/*MAT*α were shifted into sporulation medium (time zero) and incubated at 30°C. At the indicated times after transfer to potassium acetate, cells from 25-ml samples were harvested, broken with glass beads, and assayed for 1,3-β-glucanase activity by measuring the release of reducing sugars from laminarin as the substrate. ▲, strain AP1 **a**/α; △, strain AP1 α/α. Parallel samples (150 ml each) from the AP1 **a**/α culture were processed for mRNA isolation and subjected to Northern analysis (bottom panel). For this purpose, total RNAs (30 μg per lane) were fractionated by electrophoresis through a 1% agarose gel, transferred to a nylon membrane, and then sequentially hybridized to radioactively labeled fragments containing the *SSG1* gene (1.2-kb *Sal*I-*Sac*I fragment), the *SGA1* gene (0.7-kb *Bam*HI-*Sal*I fragment [77]), and the *ACT1* gene (1.7-kb *Bam*HI-*Hin*dIII fragment [53]). Only the portions of the membranes containing transcripts which hybridized with the probes are shown. Appearance of mature asci (top panel) in the AP1 **a**/α culture, was monitored by light microscopy with phase-contrast optics (the data are percentages of cells converted into asci).

ity is present both in the *MAT***a**/*MAT*α strain and in the *MAT*α/*MAT*α strain; this activity can be attributed to the expression of vegetative 1,3-β-glucanases. Coincident with the appearance of *SSG1* transcripts in the *MAT***a**/*MAT*α diploid, there is a second period characterized by a fast rate of synthesis resulting in an approximately 15-fold increase in the amount of 1,3-β-glucanase activity between 6 and 12 h, which reaches a maximum between 12 and 15 h after transfer to sporulation medium. From this point, 1,3-β-glucanase activity declines simultaneously with the reduction in the number of *SSG1* transcripts and concomitantly with the widespread appearance of mature asci. The slight activity in the nonsporulating *MAT*α/*MAT*α diploid remains almost unaltered throughout the incubation, and thus at 27 h, when the activity in the AP1 **a**/α culture has declined to 48% of the value obtained at 15 h, the activity in the α/α culture is still fivefold lower than that in **a**/α cells.

Disruption of the *SSG1* gene. The confinement of the *SSG1* gene expression to the meiotic process suggested that the encoded 1,3-β-glucanase might serve functions essential for ascospore wall development. To test this assumption, mutational analyses were performed by the single-step gene replacement procedure (62). First, a plasmid that contained the sequences upstream and downstream of the *SSG1* gene but that had the entire translated region of the gene deleted (from nucleotide −769 to 187 nucleotides beyond the stop codon) and replaced with a 1.2-kb DNA fragment containing the *URA3* gene was constructed. This plasmid was treated with *Xho*I to separate the yeast sequence from the vector sequence, and the resulting 3.1-kb linear DNA fragment (containing the disrupted gene) was then used to transform a *MAT***a** *ura3* strain (W3031A) and a *MAT*α *ura3* strain (α131-20) to Ura$^+$ (Fig. 8). *MAT***a** *ssg1*::*URA3* (YPA92) and *MAT*α *ssg1*::*URA3* (YPA93) transformants were then mated to obtain a homozygous mutant diploid, with which Southern blot analyses were carried out to verify that integration of the substituted allele occurred at the genomic locus corresponding to the cloned gene. The results confirmed that both chromosomal copies of the *SSG1* gene had been replaced by the *URA3* gene (data not shown). Thus, when the 2.3-kb *Bam*HI-*Sac*I *SSG1*-containing fragment was used as a probe, wild-type diploid yeast DNA cut with *Xho*I displayed the expected 4.2-kb band, while no hybridizing signal was visible in a parallel blot with DNA isolated from the diploid strain carrying the presumed *URA3* substitutions. Furthermore, probing with the 1.2-kb *Hin*dIII *URA3*-containing fragment detected in the homozygous mutant diploid strain an additional 3.1-kb band, whose size correlated with the size of the *Xho*I plasmid-borne *URA3*-disrupted fragment, that was not present in wild-type diploid DNA, which showed only a single hybridizing band corresponding to the resident *URA3* gene copy.

As expected, 1,3-β-glucanase activity was strongly reduced in homozygous *ssg1*/*ssg1* mutants, which after 14 h of exposure to sporulation conditions showed a 1,3-β-glucan-degradative capacity about fivefold lower (0.907 U/mg of protein) than that exhibited by the corresponding isogenic wild-type strain (4.440 U/mg of protein) (Fig. 8). This result confirmed that *SSG1* is indeed the gene responsible for the increase in 1,3-β-glucanase levels observed during sporulation. However, if one takes into account the approximately 12-fold increase in activity at about the same time between a sporulating (AP1 **a**/α) and an asporogeneous (AP1 α/α) isogenic diploids, one must conclude that 1,3-β-glucanases other than that encoded by the *SSG1* gene might be subject to changes in their levels of expression during the meiotic process.

The homozygous *ssg1*/*ssg1* mutant strain was examined for different parameters in order to evaluate the effect of *SSG1* deletion on the ability of these cells to complete the meiotic program. Microscopic examination of cells subjected to sporulation conditions revealed that *SSG1* is not essential for sporulation, since after incubation periods

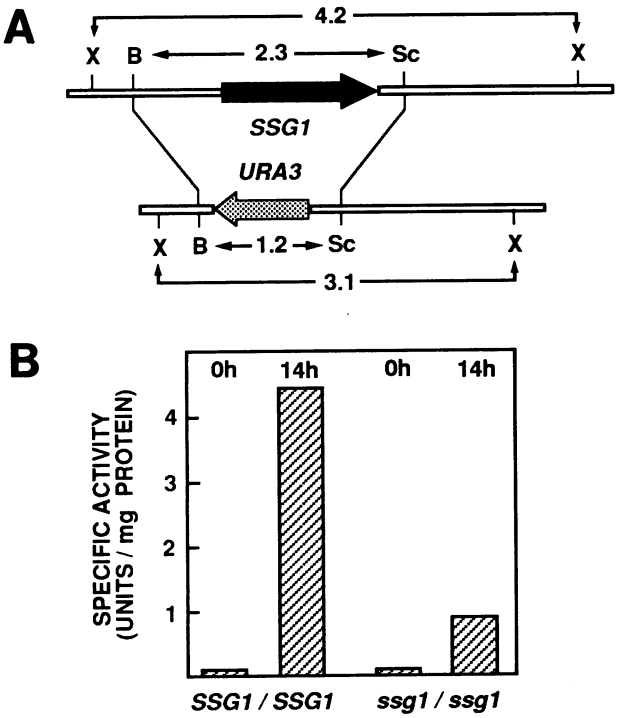

FIG. 8. Deletion substitution in the *SSG1* region. (A) Restriction map of the wild-type *SSG1* locus and the locus at which the entire *SSG1* gene had been replaced by the *URA3* gene. To achieve this replacement, a 1.2-kb *Hin*dIII fragment containing the *URA3* gene was blunt ended with Klenow DNA polymerase and ligated into the *Sma*I site at the polylinker of vector pGEM-3Z (Promega Corporation). From this plasmid, the *URA3* gene was recovered with *Bam*HI and *Sac*I and used to replace the 2.3-kb *Bam*HI (B)-*Sac*I (Sc) *SSG1*-containing region. The 3.1-kb *Xho*I (X) fragment from the resulting construction was then used to transform *ura3* cells to Ura$^+$ cells, in which the chromosomal *SSG1* gene was expected to be replaced by the disrupted allele. Substitution was confirmed by Southern analysis (not shown). (B) Levels of 1,3-β-glucanase activity in wild-type and mutant diploids. Vegetatively growing cultures of *SSG1/SSG1* and *ssg1/ssg1* diploids were shifted to sporulation medium and incubated at 30°C. At the time of the transfer (time zero) and after 14 h of incubation, cells from 25-ml samples were harvested, broken with glass beads, and assayed for 1,3-β-glucanase activity by measuring the release of reducing sugars from laminarin.

longer than 40 h in nitrogen-deficient medium, the homozygous mutants form asci to about the same extent as the heterozygous and homozygous wild-type controls. Nor did the null *ssg1::URA3* allele have any drastic effect on spore viability or germination. Dissection of 18 complete tetrads from the homozygous *SSG1*-disrupted strain estimated an overall viability of 73.6%; by comparison, 83.3% of spores from the wild-type diploid and 79.2% from the heterozygous strain were viable. Finally, in a search for a more subtle defect, we tested the thermotolerance of the mutant ascospores by determining the plating efficiency of 37-h sporulated cultures after exposure to 50°C for various periods. No significant differences between mutant and wild-type cells were found, pointing to a similar capacity of both types of ascospores to survive heat stress.

An analysis of the time course of ascus formation, however, revealed differences between the mutant and wild-type strains that may be considered meaningful. As shown in Table 1, there is a delay in the appearance of asci in the

TABLE 1. Sporulation abilities of *SSG1* wild-type and mutant strains

Cross[a]	Genotype (*MAT***a**/*MAT*α)[b]	% Sporulation after the following period (h) in medium[c]:			
		12	16	20	37
W3031A × α131-20	*SSG1/SSG1*	6	45.5	66.5	90
YPA92 × YPA93	*ssg1/ssg1*	0	28	46	82
YPA92 × α131-20	*ssg1/SSG1*	4	44	68	90
L839 × α131-20	*SSG1/SSG1*	5	41	75	96
YPA94 × YPA93	*ssg1/ssg1*	0	18.5	43.5	90
YPA94 × α131-20	*ssg1/SSG1*	3	36.5	68	96

[a] YPA92 (*MAT***a** *ssg1::URA3*), YPA93 (*MAT*α *ssg1::URA3*), and YPA94 (*MAT***a** *ssg1::URA3*) are transformants of W3031A, α131-20, and L839, respectively.

[b] The first three diploids are isogenic except at *SSG1*; the last three diploids are also isogenic except at *SSG1*.

[c] Diploids were sporulated on liquid sporulation medium following pregrowth in YEPA medium. Cells were cultured and sporulated simultaneously.

SSG1-disrupted strain such that, for example, after 16 h in sporulation medium 45% of wild-type diploid cells have been converted into asci, only 28% of the homozygous mutant population have sporulated. These differences are reduced at longer incubation periods, reaching almost similar percentages at 37 h, when ascus formation is nearly completed. Similar results were obtained when the ability to sporulate was tested for a different cross. As shown in Table 1, the development of mature asci in the homozygous *ssg1/ssg1* mutant (YPA94 × YPA93) is delayed by several hours with respect to the isogenic wild-type diploid (L839 × α131-20), and yet sporulation efficiency was similar by the time at which the meiotic process is being completed. In both cases, confirmation that the phenotype of the *MAT***a** *ssg1/MAT*α *ssg1* mutant was due to the absence of the *SSG1* gene product came from the observation, in parallel experiments, that the kinetics and efficiency of appearance of asci in the corresponding *ssg1::URA3/SSG1* heterozygotes (YPA92 × α131-20 and YPA94 × α131-20) were similar to those observed for the isogenic wild-type diploids.

In order to determine whether the observed retardation in spore formation was a consequence of a delay in earlier events in the meiotic program, 4′,6-diamidino-2-phenylindole staining was used to compare the kinetics of the nuclear divisions in the different strains. In all cases, binucleate cells first appeared between 5 and 6 h after the transfer to sporulation conditions and reached maximum levels at approximately 7 h; similarly, tetranucleate cells appeared in all cultures by 6 to 7 h (data not shown). Thus, meiotic divisions followed with normal kinetics in the disrupted strains, and we therefore concluded that the *SSG1*-encoded 1,3-β-glucanase is not essential for meiosis and spore formation in yeast cells, although it does contribute to ascus development.

The lack of a more drastic defect and the fact that after prolonged periods in the sporulation medium the *ssg1/ssg1* diploid recovers, reaching ascus-formation percentages similar to those of the wild-type control, could be an indication that another product with an overlapping function might be able to substitute for the *SSG1*-encoded 1,3-β-glucanase. Although functionally redundant proteins do not necessarily have to be encoded by homologous genes, the fact that vegetative 1,3-β-glucanases persist during the meiotic process and the strong similarity exhibited by the *SSG1*-encoded 1,3-β-glucanase with those encoded by the *EXG1* and

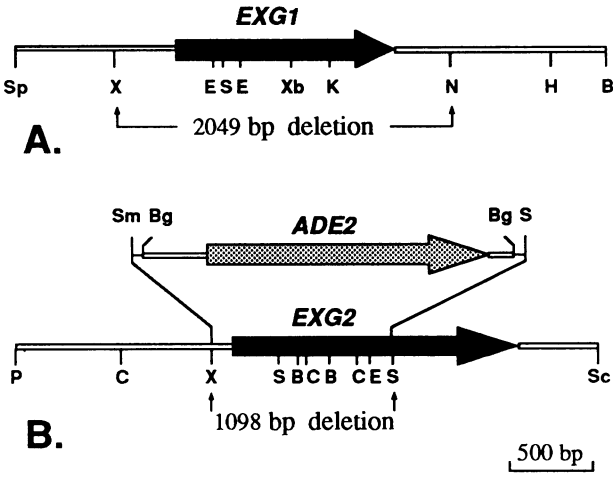

FIG. 9. Disruption of the *EXG1* and *EXG2* genes. (A) Construction of the deletion allele *exg1-Δ1* was accomplished by gene transplacement (6, 74). First, a 3.5-kb *Sph*I-*Bam*HI fragment containing the *EXG1* gene and flanking sequences was inserted between the corresponding sites on the *URA3*-bearing YIp5 vector. From the resulting plasmid, pRM1, the 2.0-kb *Xho*I-*Nae*I fragment encompassing the *EXG1* coding region was removed, the *Xho*I end was repaired by incubation with the Klenow fragment of DNA polymerase I, and the blunt-ended DNA was religated to generate plasmid pCV22, which was used for integrative transformation. Excision of the plasmid from Ura$^+$ transformants was then selected by growth of cells on 5-fluoro-orotic acid, and finally, gene-replaced strains were examined by Southern analysis to confirm transplacement of the wild-type *EXG1* gene with the intended mutation (data not shown). (B) The deletion allele *exg2::ADE2* was obtained by the one-step gene replacement method (62). First, a 2.3-kb *Bgl*II-*Bgl*II fragment containing the *S. cerevisiae ADE2* gene was isolated from the shuttle vector pASZ11 (67) and cloned into the *Bam*HI site at the polylinker of plasmid pUC119 (48) to create plasmid pPS42. From this plasmid, the *ADE2* gene was recovered by digesting at the *Sma*I and *Sal*I flanking sites and then used to replace the 1.1-kb *Xho*I-*Sal*I region in the *EXG2*-bearing plasmid pPS45 that had previously been digested with *Xho*I, flush ended by treatment with DNA polymerase I, and finally, digested with *Sal*I. This mutant allele was used to replace the wild-type chromosomal copy of *EXG2* through transformation of *ade2* receptor strains to adenine independence by using the 4.6-kb *Pst*I-*Sac*I linear fragment containing the *ADE2* gene flanked by 5′ and 3′ untranslated *EXG2* region DNA. Subsequent Southern analysis confirmed that Ade$^+$ transformants carried the *exg2::ADE2* deletion (data not shown). Restriction sites: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; N, *Nae*I; P, *Pst*I; S, *Sal*I; Sc, *Sac*I; Sm, *Sma*I; Sp, *Sph*I; X, *Xho*I; Xb, *Xba*I.

EXG2 genes would support the notion of the presence of two or more gene products that could serve complementary functions such that the cell would be able to compensate for the absence of one of them.

To test this possibility, we constructed a set of isogenic diploids lacking several functions simultaneously (doubly and triply disrupted strains) for evaluating the effect of a diminished 1,3-β-glucanase activity on the sporulation efficiency of the cells. Disruption of the vegetative 1,3-β-glucanase-encoding gene *EXG1* was accomplished in the different haploid parental strains by deleting, through gene transplacement (6, 74), a 2.0-kb fragment spanning the entire coding region and neighboring 5′ and 3′ untranslated sequences, thus creating the *exg1-Δ1* allele (Fig. 9). Mutations at the *EXG2* locus were generated by replacing a 1.1-kb fragment, from position −110 to position +988 within the open reading frame, by the *ADE2* gene, thus deleting the sequence coding for the 330 N-terminal amino acids (about two-thirds of the protein) of the *EXG2*-encoded 1,3-β-glucanase (Fig. 9). As in the case of *SSG1* disruptions, gel transfer hybridization analyses of genomic DNA were carried out to verify that the corresponding transformants bore the intended mutations (data not shown).

TABLE 2. Sporulation efficiencies of strains homozygous for 1,3-β-glucanase gene disruptions[a]

Cross (*MAT***a** × *MATα*)	Disrupted gene(s)[b]	% Sporulation after the following period (h) in medium[c]:			
		12	16	20	36
W3031A × α131-20	None	11	41	70	86
YPA95 × YPA96	*EXG1*	9	41	69	89
YPA97 × YPA98	*EXG2*	9.5	42	63	82
YPA92 × YPA93	*SSG1*	3	28	49	75
YPA99 × YPA100	*EXG1 EXG2*	7	42	71	80
YPA101 × YPA102	*EXG1 SSG1*	1	27	51	75
YPA103 × YPA104	*EXG2 SSG1*	3	29	49	75
YPA105 × YPA106	*EXG1 EXG2 SSG1*	2	24	47	67

[a] Haploid strains were constructed as follows. Strains harboring single 1,3-β-glucanase-encoding gene mutations were obtained directly from parental strains W3031A (YPA95, YPA97, and YPA92) and α131-20 (YPA96, YPA98, and YPA93) by introducing mutant alleles at the corresponding loci, *EXG1* (*exg1-Δ1*), *EXG2* (*exg2::ADE2*), and *SSG1* (*ssg1::URA3*). The *exg1 exg2* double mutants YPA99 and YPA100 were obtained through disruption of the *EXG2* gene (*exg2::ADE2*) in the *exg1-Δ1*-bearing strains YPA95 and YPA96; *exg1 ssg1* and *exg2 ssg1* double mutants were obtained from *exg1-Δ1* strains (YPA95 and YPA96) or from *exg2::ADE2* strains (YPA97 and YPA98) after the *SSG1* gene was replaced by the *URA3* marker (*ssg1::URA3*), yielding strains YPA101, YPA102, YPA103, and YPA104. The same substitution was carried out in the *exg1-Δ1 exg2::ADE2* double mutants YPA99 and YPA100 to generate strains YPA105 and YPA106, bearing mutant alleles at the three loci.

[b] All diploids were isogenic except at their *EXG1*, *EXG2*, and *SSG1* loci.

[c] Diploids were sporulated on liquid sporulation medium following pregrowth in YEPA medium. Cells were cultured and sporulated simultaneously.

Homozygous mutant diploids were obtained by crossing the appropriate haploid parental strains, and they were subjected to sporulation conditions to evaluate the effects of mutant alleles on the ability to form asci. The results, depicted in Table 2, clearly indicated that the subtle defect in *SSG1* singly disrupted diploids was not due to a functional complementation exerted by the *EXG1*- or *EXG2*-encoded 1,3-β-glucanases, since double *ssg1/ssg1 exg1/exg1* and *ssg1/ssg1 exg2/exg2* mutants, and even the triple *ssg1/ssg1 exg1/exg1 exg2/exg2* mutant, showed ascus formation kinetics almost identical to that observed for *SSG1* singly disrupted diploids. This apparent lack of a direct involvement of the *EXG1*- and *EXG2*-encoded products in the sporulation process is corroborated by the fact that *exg1/exg1* and *exg2/exg2* diploids, and even the double *exg1/exg1 exg2/exg2* mutant, are able to form asci with the same efficiency and in the same temporal pattern as the wild-type control. In support of this assumption, although indirectly, is the low contribution of the *EXG1*- and *EXG2*-encoded products to the 1,3-β-glucan-degradative capacity of sporulating cells: enzymatic measurement of the 1,3-β-glucanase content in cell extracts from the triple disruptant after 14 h in sporulation medium showed that it still retained about 82% of the 1,3-β-glucanase activity detected on extracts from the *SSG1* singly disrupted diploid maintained for the same period under sporulation conditions.

DISCUSSION

Progress through the meiotic pathway in the yeast *S. cerevisiae* is marked by dramatic changes in gene expression. These changes fall into two categories: those that are associated with the starvation conditions required for sporulation and those that are concomitant with the development of asci. Some of these correspond to products whose function is crucial to the sporulation process, but most of them, although in some way important to the process, do not seem to play any essential role, as assessed by null mutations that render levels of sporulation indistinguishable from that for wild-type controls.

The evidence presented in this article substantiates previously published observations regarding the induction of the glucan-degradative capacity upon sporulation in *S. cerevisiae*, with the molecular cloning and characterization of a gene encoding a 1,3-β-glucanase associated with the meiotic program. In this way, *SSG1* now becomes one of the few genes described that specifically accompany the sporulation process and for which some knowledge about the nucleotide structure and biochemical activity of the encoded protein is available.

Thus far, the study of carbohydrate metabolism in *S. cerevisiae* has provided the best documented examples of sporulation-specific enzyme activities. In this context, it is worth emphasizing the close similarity between the genetic system governing 1,3-β-glucanase synthesis and that responsible for glucoamylase production. As has been demonstrated, some *S. cerevisiae* strains (var. *diastaticus*) have the ability to digest starch due to the existence of any of three unlinked homologous genes, *STA1*, *STA2*, and *STA3*, encoding the extracellular glycosylated glucoamylase isozymes I, II, and III (71). All three *STA* genes contain a region common to the nonsecreted glucoamylase-encoding *SGA1* gene which corresponds to the catalytic domain of the protein, and on this basis, it has been proposed that *STA* genes originated as the result of gene duplications of *SGA1* followed by recombinational events to provide different 5′-flanking regions as well as the structural information to target the corresponding polypeptides to the secretory pathway (76, 77). In view of the strong homology between the *EXG1*- and *SSG1*-encoded products and taking into account the different properties exhibited by them regarding their fates and timing of production, a similar situation could be envisaged for the 1,3-β-glucanase system in this yeast. In support of this notion of a family of closely related genes is the finding that the protein deduced from the nucleotide sequence of a different vegetative exo-1,3-β-glucanase gene (*EXG2*), cloned in parallel with *EXG1* by partial complementation of *exg1* mutants, shows a significant degree of similarity to the *EXG1* gene product (14, 19, 51). It is then tempting to postulate a common evolutionary origin of the exo-1,3-β-glucanase genes with the acquisition of distinct structural and regulatory features which would allow them to respond to specific signals and to perform different roles in the yeast life cycle.

The enzymatic activity of the *SSG1*-encoded product together with its timing of production in the ordered sequence of events which constitutes the meiotic process in *S. cerevisiae* is suggestive of a direct participation of this protein in morphogenetic processes related to ascospore wall synthesis. As is known, the initiating event in the development of the spore envelope appears to be the coalescence of small vesicles to generate a flattened sac on the cytoplasmic side of the outer spindle plaque; this double-membrane structure, the prospore wall, expands around the nuclear lobe until it encloses the haploid nucleus as well as a portion of the cytoplasm. With the nascent spore thus delimited, the spore wall then forms by deposition of mannoproteins and glucans between the two membranes, and its formation is followed by the appearance of a glucosamine-containing outer spore coat (2, 3, 47). An analysis of the in vitro coding capacity of RNA from sporulating cells to correlate the appearance of specific messages with the major morphogenetic events of sporulation led Kurtz and Lindquist (42, 43) to propose that genes which are presumably involved in spore wall biogenesis are coordinately induced about 8 h after the transfer of cells to nitrogen-deficient medium, giving rise to a set of transcripts whose localization is restricted to the ascus cytoplasm. Interestingly, translation of these messages seems to be membrane associated, and the corresponding products (eight polypeptides migrating as species of 68, 65, 50, 38, 31, 25, 20, and 17 kDa) are most likely structural proteins or proteins involved in cell wall assembly which have to be targeted to the double-membrane structure surrounding the nascent spore. The relationship between this set of transcripts and those arising from the late class of sporulation-specific genes has been demonstrated by the fact that *SPS4*, a gene included in this group, encodes the 38-kDa polypeptide identified by in vitro translation of 8-h mRNAs (28). As shown here, the same program of message induction is observed for *SSG1* and for the glucoamylase-encoding *SGA1* gene, a well-known prototype of the late class; hence, we assume that both genes are members of the same group. Although we do not have direct evidence, it is possible that the *SSG1* gene characterized in this study could specify the 8-h sporulation-specific transcript described by Kurtz and Lindquist as encoding the 50-kDa polypeptide. The existence of an amino-terminal hydrophobic sequence extending to a potential cleavage site fulfills the structural requirement for incorporation into a membranous system that allows its translocation into the spore wall. It is worthwhile to point out that, interestingly, this amino-terminal sequence is the most divergent region between the primary translation products encoded by *SSG1* and *EXG1*. Thus, this leader sequence might perform a decisive role in defining their targeting to distinct subcellular locations, with the *EXG1*-encoded 1,3-β-glucanases being mainly secreted to the periplasmic space and then to the surrounding medium whereas the *SSG1*-product would remain as an intracellular protein, presumably related to the nascent ascospore envelope.

The most interesting question would be to determine the exact physiological function of this exo-1,3-β-glucanase in the sporulation process. Because sporulation in *S. cerevisiae* can occur on potassium acetate alone, it seems apparent that extensive recycling of the parent cell wall components is required to provide precursors for the synthesis of spore macromolecules, and since the appearance of the sporulation-specific exo-1,3-β-glucanase occurs largely before the majority of ascospores have actually formed, it may well have a metabolic function directed toward the mobilization and organization of vegetative cell wall 1,3-β-glucan into ascospore walls. Precedents for such a glucanase function have been reported previously for *Aspergillus nidulans* (79), in which after glucose depletion a sharp increase in 1,3-α-glucanase production is related to the reutilization of the mycelial cell wall 1,3-α-glucan, apparently essential for cleistothecium formation. Thin-section electron microscopy of asci in *S. cerevisiae*, however, does not suggest that the parent wall itself is extensively reutilized (27), and the

recycling is probably restricted to the cell contents. Consistent with this are the well-documented fact that glycogen serves as a storage carbohydrate in the meiotic process and the fact that massive synthesis as a response to starvation conditions early on in sporulation is followed by a rapid breakdown, coincident with the appearance of asci (13, 33, 38); it is then possible that some of the glucose residues could be redistributed from glycogen to spore wall polysaccharides. A more direct involvement of the *SSG1*-encoded product in the construction of the ascospore cell wall should then be considered. However, although it is conceivable to envisage a participation of exo-1,3-β-glucanases in situations in which it seems imperative to soften the preexisting wall, such as in budding, in order to allow emergence of the new growth, it is difficult to imagine what an exo-hydrolytic activity would be required for in a process consisting mainly of the deposition of polymeric components to build a new envelope around the meiotic products. As an alternative function, recent results that have assigned a glucosyltransferase activity to an exo-1,3-β-glucanase from *Candida albicans* seem to be of interest. This finding has allowed authors to speculate that, in the region of new wall growth, glucanases may function as transferases that elongate the 1,3-β-glucan being extruded by the glucan synthetase (70). Although we have not checked for this activity in the *SSG1*-encoded 1,3-β-glucanase, there is no doubt that such biosynthetic properties may be of functional significance in spore wall biogenesis.

Whatever its physiological function, it is clear that *SSG1* is not essential for successful completion of sporulation, and homozygous *ssg1/ssg1* diploids undergo meiosis and give rise to four apparently normal, viable ascospores. This is not surprising if one takes into account that previous mutational analyses of several other genes that display a late sporulation-specific expression pattern in most cases failed to reveal drastic effects on spore formation. Thus, with the exception of the *SPS1* gene, whose disruption has been shown to give rise to an asporogenous phenotype (56), nonessential functions have been attributed to the products encoded by several late genes such as *SGA1* (75), *SPS2* (56), *SPS4* (28), *LGN2* (29), and *SPR2*, *SPR6*, and *SPR9* (37, 39). Of course, this does not rule out the possibility that the *SSG1*-encoded 1,3-β-glucanase might serve some important, but not indispensable, function in the formation of ascospores, and this suggestion is supported by the slight but significant delay in the appearance of mature asci occurring in the homozygous mutant diploid compared with the wild-type or heterozygous strain. It seems logical to speculate that the absence of a product presumably involved at some point of the synthesis or assembly of the 1,3-β-glucan polymer would lead to an impairment in the deposition of the different constituents required for the formation of an envelope around the meiotic products.

On the basis of the significant degree of similarity between the *SSG1*-encoded product and the 1,3-β-glucanases encoded by the *EXG1* and *EXG2* genes, the possibility that the lack of a phenotype severely defective in spore formation in *SSG1*-disrupted strains might be the result of overlapping activities by the vegetative 1,3-β-glucanases was really attractive. Functional redundancy has also been invoked to explain why mature spores from homozygous *SPS100*-disrupted diploids are as resistant to ether as are wild-type controls, despite there being a significant delay in the onset of ether resistance in developing mutant asci, and also to account for the failure to readily detect a mutant phenotype for other sporulation-specific genes such as *SPS2*, *SPS4*, and *SPS101* (28, 46, 55). The sporulation efficiencies of doubly and triply disrupted strains clearly showed that this was not the case, although one cannot totally rule out the possibility of a functional complementation. Thus, the fact that the triple mutant still retains most of the glucan-degradative capacity exhibited by the singly *SSG1*-disrupted mutant indicates, on one hand, the small contribution of the *EXG1*- and *EXG2*-encoded products to the total 1,3-β-glucanase content during sporulation and, even more interestingly, the presence during this process of other 1,3-β-glucan-hydrolyzing enzymes. At the present moment, we are unable to elucidate which enzymatic forms might be responsible for these levels of 1,3-β-glucanase activity. On one hand, it should be taken into account that, apart from the *EXG1*- and *EXG2*-encoded 1,3-β-glucanases, other minor exo- and endo-1,3-β-glucanases have been described as occurring during vegetative growth, and their synthesis could be maintained or even activated during the meiotic program. Alternatively, it is not possible to dismiss the possibility of the existence of some enzymatic form with exo- or endo-1,3-β-glucanase activity whose induction, like that of the *SSG1*-encoded product, is specifically coupled to sporulation events.

A detailed analysis of this residual 1,3-β-glucan-degradative capacity of triply disrupted strains will allow us to discern between these two possibilities and to investigate the molecular cloning of other 1,3-β-glucanase-encoding genes, whose characterization will afford us further insight into this complex glucan-hydrolyzing system in *S. cerevisiae*. In addition, a detailed ultrastructural study of the ascospore cell wall as well as determination of its 1,3-β-glucan content (by cell wall fractionation) in *SSG1*-disrupted strains should be carried out to confirm the direct participation of the *SSG1*-encoded 1,3-β-glucanase in the synthesis and assembly of the spore envelope.

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