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A 15-Base-Pair Element Activates the *SPS4* Gene Midway through Sporulation in *Saccharomyces cerevisiae*

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Sporulation of the yeast *Saccharomyces cerevisiae* represents a simple developmental process in which the events of meiosis and spore wall formation are accompanied by the sequential activation of temporally distinct classes of genes. In this study, we have examined expression of the *SPS4* gene, which belongs to a group of genes that is activated midway through sporulation. We mapped the upstream boundary of the regulatory region of *SPS4* by monitoring the effect of sequential deletions of 5′-flanking sequence on expression of plasmid-borne versions of *SPS4* introduced into a *MAT*a/*MAT*α Δ*sps4*/Δ*sps4* strain. This analysis indicated that the 5′ boundary of the regulatory region was within 50 bp of the putative TATA box of the gene. By testing various oligonucleotides that spanned this boundary and the downstream sequence for their ability to activate expression of a heterologous promoter, we found that a 15-bp sequence sufficed to act as a sporulation-specific upstream activation sequence. This 15-bp fragment, designated UAS^{SPS4}, activated expression of a *CYC1-lacZ* reporter gene midway through sporulation and was equally active in both orientations. Extending the UAS fragment to include the adjacent 14-bp enhanced its activity 10-fold. We show that expression of *SPS4* is regulated in a manner distinct from that of early meiotic genes: mutation of *UME6* did not lead to vegetative expression of *SPS4*, and sporulation-specific expression was delayed by mutation of *IME2*. In vivo and in vitro assays suggested that a factor present in vegetative cells binds to the UAS^{SPS4} element. We speculate that during sporulation this factor is modified to serve as an activator of the *SPS4* gene or, alternatively, that it recruits an activator to the promoter.

Sporulation of the yeast *Saccharomyces cerevisiae* is a process of cellular differentiation that begins when *MAT***a**/*MAT*α diploid cells are starved for nitrogen in the presence of a nonfermentable carbon source. Starved cells exit the cell cycle at G_1 and initiate meiotic DNA replication. This is followed by chromosome pairing, a high level of genetic recombination, and the two meiotic divisions in which first homologous chromosomes and then sister chromatids segregate. Deposition of spore walls around the haploid meiotic products completes the process, generating four spores enclosed within an ascus (reviewed in references 12, 14, 22, and 23). The sporulation program is accompanied by the sequential expression of distinct groups of sporulation-specific genes, classified as early, middle, mid-late, or late on the basis of their time of expression (reviewed in reference 38). Sporulation in *S. cerevisiae* therefore provides a simple model system for a study of the regulatory mechanisms providing temporal control of gene expression during development.

The genetic signals controlling entry into the sporulation pathway have been well studied. The *MAT***a***1* and *MAT*α*2* gene products regulate expression of *RME1* (11, 27, 40) and *IME4* (50), which in turn control expression of *IME1*, a key positive regulator of entry into meiosis (27). Ime1p has recently been shown to contain a transcriptional activation domain (35, 53). Fine-tuning of the level and activity of Ime1p is achieved by an array of genes which include *MCK1* (42); *RIM1*, *RIM8*, *RIM9*, and *RIM13* (57); *RIM11* (2); and *SME3* (28). Transduction of the starvation signal is also complex. Although a reduction in the activity of cyclic AMP-dependent kinase activity in response to nutrient deprivation enhances expression and activity of Ime1p and promotes sporulation (reviewed in references 5 and 34; 36, 51), nutritional regulation also acts at points downstream of *IME1* (reviewed in reference 25).

Ime1p activates expression of early sporulation genes. These are genes which are first expressed at the beginning of meiotic prophase and encode proteins involved in meiotic recombination and chromosome segregation (reviewed in reference 38). *IME1* is also required for expression of *IME2* (54, 63), which encodes a protein kinase (63). Expression of *IME2* from a heterologous promoter allows activation of many early sporulation genes in the absence of *IME1*. *IME2* is also essential for expression of middle sporulation genes (39). In a simple scenario, *IME2* activates a global regulator of expression of early genes and the product of one or more of these genes then serves to activate middle sporulation genes.

The promoter elements of the early meiotic genes *SPO13* (6), *HOP1* (60), and *IME2* (1) have been partially characterized. Each of these genes contains an URS1 site which represses transcription of these genes in vegetative cells and contributes to their activation during meiosis. The URS1 element was first identified as a site mediating repression of *CAR1* (58) and has since been found in the promoter regions of numerous genes involved in activities such as nitrogen metabolism, electron transfer, carbon metabolism, inositol metabolism, and meiosis (reference 33 and references therein). In addition to the URS1 site, a site referred to as UAS_H has been shown to contribute to meiotic activation of *HOP1* (60). Similarly, an activation element referred to as a T_4C site has been found to act in conjunction with an URS1 site to direct meiotic activation of *IME2* (1). Bowdish and Mitchell (1) suggest that modification of an URS1 complex by Ime1p leads to meiotic activation of *IME2*. Sequence inspection has shown that an URS1 site and a UAS_H or a T_4C site are present in the

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upstream region of nearly all early meiotic genes (reviewed in reference 38). This leads to the notion that an URS1 site in conjunction with differing UAS sites coordinately controls expression of early meiotic genes. Recently, the *UME6*/*CAR80* gene product, a protein with a six cysteine-containing zinc finger DNA-binding motif, has been shown to interact with the URS1 site of *SPO13* (56), whereas Buf1p and Buf2p, which are encoded by *RPA1* and *RPA2* (33), have been found to bind to $URS1^{CAR1}$ (32). The role of these proteins in mediating the regulatory effects of URS1 remains to be determined.

Expression of the late sporulation gene *SGA1* also has been shown to depend on two sequence elements (30). A UAS element responds to nutritional control, and a negative element responds to both mating-type control and nutritional control (30).

In the study described here, we have identified the promoter element of *SPS4*, a sporulation-specific gene which is expressed as the meiotic divisions are nearing completion. This gene, which was identified in a differential hybridization screen (44), encodes an abundant, but nonessential, sporulation-specific transcript (17). We find that a 15-bp sequence from the 5′-flanking region of *SPS4* suffices to activate gene expression midway through sporulation.

MATERIALS AND METHODS

Yeast strains. All strains of *S. cerevisiae* used in this study were derived from the isogenic strains W3031A (*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*) and W3031B (*MAT*α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*) (constructed by R. Rothstein) or their derivatives W3031A-H (*MAT***a** *ade2-1 leu2-3,112 trp1-1 ura3-1 can1-100*) and W3031B-T (*MAT*α *ade2-1 his3-11,15 leu2-3 ura3-1 can1-100*) (31) with the exception of strain 20B-12 (α *trp pep4-3*) used for preparation of protein extracts. LP112, provided by S. Lindquist, represents the diploid obtained by mating W3031A and W3031B (45). With the exception of LP112 and *MAT***a**/*MAT***a** strains, all diploids were obtained by mating of haploids followed by prototrophic selection of diploids taking advantage of chromosomal markers or markers introduced on plasmids for this purpose. A *MAT***a**/*MAT***a** version of LP112 was created with pGAL-HO (26) (provided by P. Sadowski) as described by Connolly et al. (10) to induce mating-type switching. The Δ*sps4* allele was constructed by replacement of the 5′ region of *SPS4* (from nucleotides [nt] −159 to +362) with a DNA fragment containing the *URA3* gene, as described previously (17). Appropriate integrative transformation in the haploid strains W3031A-T and W3031B-H was confirmed by Southern blot analysis of genomic DNA from Ura^+ transformants. A *MAT***a** Δ*sps4* strain and a *MAT*α Δ*sps4* strain were then mated to give the diploid strain YTG1 (*MAT***a**/*MAT*α Δ*sps4*/Δ*sps4*). YSH1 (*MAT***a**/*MAT***a** Δ*sps4*/Δ*sps4*) was created from YTG1 by using pJH283, a derivative of pGAL-HO (constructed by F. Heffron and provided by P. Sadowski), to induce mating-type switching.

The yeast strains YKE1, YKE2, and YSH2 were constructed by strategies similar to that described for YTG1. YKE1 (*MAT***a**/*MAT*α *ime1*::*TRP1*/*ime1*::*TRP1*) was generated with pSH113B (provided by A. Mitchell) as described elsewhere (54). The haploid *MAT*α *ime1*::*TRP1* strain was transformed with pRS316 (52) to provide *URA3* as a selectable marker to facilitate selection of diploids. YKE2 (*MAT***a**/*MAT*α *ime2*::*LEU2*/*ime2*::*LEU2*) was constructed with pAM412-2, a derivative of pAM412 (provided by A. Mitchell), as described elsewhere (39). YSH2 (*MAT***a**/*MAT*α *ume6*::*LEU2*/*ume6*::*LEU2*) was constructed with pPF5914 (56) (provided by R. E. Esposito) that had been digested with *Hin*dIII. As previously noted (1, 56), this strain grew very slowly on minimal medium. All yeast transformations were carried out by the lithium acetate procedure of Gietz et al. (18). Southern blot analyses were carried out as described previously (44).

Media and culture conditions. Rich medium (YEPD) contained 2% Bacto Peptone, 1% yeast extract, 2% glucose, and 40 mg of adenine sulfate per ml. Minimal medium (SD) contained 2% glucose, 0.67% yeast nitrogen base without amino acids, and the required auxotrophic supplements. Presporulation medium (YEPA) contained 1% potassium acetate (BDH Chemicals Inc.), 1% Bacto Peptone, 0.5% Bacto Yeast Extract, and 40 mg of adenine sulfate per ml. Sporulation medium contained 1% potassium acetate plus the required auxotrophic supplements. All yeast cultures were grown at 30°C.

For sporulation of yeast strains containing plasmids, a single colony was taken from an SD plate to inoculate SD selective for the plasmid. If the strain did not contain a plasmid, then the colony was taken from a YEPD plate. The cultures were grown to late log phase and used to inoculate YEPA at a density of 1.3×10^6 cells per ml. When the YEPA culture reached a density of 1.5×10^7 to 2.0×10^7 cells per ml, the cells were harvested by centrifugation, washed twice with 1% potassium acetate, and resuspended in sporulation medium at a density of 1.5×10^7 cells per ml. At appropriate times, aliquots of cells were harvested by centrifugation, and the pellets were stored at −20°C for subsequent analysis. The time of transfer of cells to sporulation medium is referred to as 0 h. The efficiency of ascus formation was 35 to 60% as assessed by light microscopy.

Construction of *SPS4* genes with deletions in the 5′-flanking region. Plasmids that contained the *SPS4* gene with various amounts of 5′-flanking sequence were named p4LEX, with X denoting the endpoint of the deletion relative to the transcriptional start site (+1). To generate the plasmids p4LE812 and p4LE159, the intermediate plasmid pHX was first constructed by cloning a *Hin*dIII-*Xho*I fragment of *SPS4*, which extends from nt +866 into the 3′-flanking sequence of the gene (17), into the corresponding sites of pRS315 (52). The *Hin*dIII-*Hin*dIII fragment of *SPS4* that extends from nt −812 to +866 was then cloned into the *Hin*dIII site of pHX to generate p4LE812. The *Mlu*I-*Hin*dIII fragment of *SPS4* that extends from nt −159 to +866 was blunted at the *Mlu*I end with the Klenow form of DNA polymerase and cloned between the *Sma*I and *Hin*dIII sites of pHX to generate p4LE159. The construction of p4LE89 is described elsewhere (13).

Unidirectional deletions of the 5′-flanking region of *SPS4* were generated with p4LE159 and an Erase-a-Base kit (Promega). Plasmid DNA that had been digested with *Sst*I and *Bam*HI was treated with exonuclease III at 4°C for various amounts of time, treated with S1 nuclease, ligated, and transformed into *Escherichia coli* TG1 cells. The deletion endpoints were determined by dideoxy sequence analysis (49) using single-stranded DNA as the template and the −20 universal primer (United States Biochemicals).

Use of an actin-globin gene to test fragments for UAS activity. The multicopy plasmid pAB0 (41) (provided by Alan Wildeman) contains the 5′ region of the *ACT1* gene of *S. cerevisiae*, including its UAS, TATA box, and start sites of transcription, fused at the ATG to a portion of the coding region of the rabbit β-globin gene. The plasmid contains *LEU2* as a selectable marker. pAB1-4 is a derivative of pAB0 in which the *ACT1* sequence from nt −473 to −319 (relative to the ATG codon), including the *ACT1* UAS, has been replaced with a unique *Bam*HI site (41). *SPS4*-derived sequences were inserted into this *Bam*HI site and tested for UAS activity. pAB56 was constructed by cloning a 56-bp *Mlu*I-*Ase*I fragment of *SPS4* (nt −158 to −103; see Fig. 3) into the *Bam*HI site of pAB1-4 after the restriction endonuclease-generated ends had been filled in with the Klenow form of DNA polymerase. pAB19 was constructed by cloning a 19-bp fragment of *SPS4* (nt −140 to −122; see Fig. 3) formed by annealing complementary oligonucleotides containing 5′-GATC overhangs into the *Bam*HI site of pAB1-4. pAB37U and pAB38L were constructed by digestion of pAB19 and pAB56 with *Bss*HII and *Hin*dIII, which cut at unique sites in the *SPS4*-containing insert and in the vector sequence, respectively. The 9.5-kb fragment from pAB56 was ligated with the 500-bp fragment from pAB19 to generate pAB37U; the 500-bp fragment from pAB56 was ligated with the 9.5-kb fragment from pAB19 to generate pAB38L. This regenerated pAB1-4 with the inserts 37U (nt −158 to −122 of *SPS4*) and 38L (nt −140 to −103 of *SPS4*) (see Fig. 3) at the *Bam*HI site. All constructs were verified by dideoxy sequence analysis (49) using double-stranded DNA as the template and a 19-base oligonucleotide corresponding to part of the *ACT1* sequence as the primer.

Use of a *CYC1-lacZ* gene to test fragments for UAS activity. The multicopy *CYC1-lacZ*-containing plasmids pLGΔ312(Bgl) and pLGΔ312SΔSS(Bgl) were constructed by A. Mitchell and provided by B. Andrews. pLGΔ312(Bgl) is a derivative of pLGΔ312 (21) that contains a *Bgl*II linker inserted at the *Xho*I site (nt −178) present between the *CYC1* UAS sites and the TATA box. pLGΔ312SΔSS(Bgl) is a derivative of pLGΔ312S (20) in which the *Sma*I-*Sal*I fragment containing the *CYC1* UAS sites has been replaced with a *Bgl*II linker. A 56-bp *Mlu*I-*Ase*I fragment from *SPS4* (nt −158 to −103) was cloned into the unique *Bgl*II site of pLGΔ312SΔSS(Bgl) after the restriction endonuclease-generated ends had been filled in with the Klenow form of DNA polymerase. Fragments 25U, 25M, 24L, 15M, 29, and 21U with 5′-GATC overhangs were obtained by annealing complementary oligonucleotides (see Fig. 3) and were cloned into the unique *Bgl*II site of pLGΔ312(Bgl) or pLGΔ312SΔSS(Bgl). Four tandem copies of fragment 29 that had been inserted at the *Bgl*II site of pLGΔ312(Bgl) were excised by digestion with *Sal*I. The *Sal*I fragment was treated with the Klenow form of DNA polymerase and then inserted at the *Sma*I site upstream of the *CYC1* UAS in pLGΔ312(Bgl). All constructs were verified by dideoxy sequence analysis using double-stranded DNA as the template and a 20-base oligonucleotide corresponding to the *CYC1* sequence as the primer, except for the insert at the *Sma*I site, which was sequenced using an oligonucleotide corresponding to part of the *URA3* sequence.

Fragments with mutations. pLGΔ312SΔSS(Bgl) containing fragment 15M with a mutation of the C residue at nt −127 to a T residue [15M (C→T)] or with a deletion of an A residue from the A_5 tract at nt −123 to −119 [15M (ΔA)] were identified fortuitously by sequence analysis of plasmids constructed with fragment 15M. Fragment 15M-4, which differs from fragment 15M at four positions, was obtained by annealing complementary oligonucleotides with 5′-GATC overhangs. The sequence of the upper oligonucleotide was 5′-gatCG<u>A</u>G<u>T</u>CA CAAAA<u>TT</u>G; mutant bases are underlined, and the portion of the 5′ overhang that differs from the *SPS4* sequence is in lowercase.

RNA isolation and Northern analysis. RNA was prepared from yeast and analyzed by Northern blot as described previously (44). Each lane of a 1.5% agarose–formaldehyde gel contained 10 μg of total RNA. RNA was transferred to a nylon membrane and hybridized with gene-specific probes that had been

labeled with [α-^{32}P]dCTP by the random priming method of Feinberg and Vogelstein (15). The probes used to detect various transcripts were as follows: *SPS1*, a 550-bp *Cla*I-*Eco*RV fragment from pSPS1-URA3 (16); *DIT1*, a 1.8-kb *Hin*dIII-*Hin*dIII fragment from pPB-13 (4); *SPO13*, an 870-bp *Eco*RI-*Xba*I fragment from p(SPO13)BglII which was created from p(SPO13)15 (62) by replacing the *URA3*-containing *Hin*dIII fragment with a *Bgl*II linker; *PYK1*, a 950-bp *Hpa*I-*Hpa*I (7) fragment from pPYK101 (provided by B. McNeil); *SPS4*, a 521-bp *Mlu*I-*Cla*I fragment from p4LE812; *HOP1*, a 3.0-kb *Eco*RI-*Eco*RV fragment from pNH59-2 (24); *IME2*, a 2.5-kb *Bgl*II-*Bgl*II fragment from pMM101-2 (39); and *IME1*, a 2.5-kb *Nco*I-*Bam*HI fragment from pAC55-2 (54).

Primer extension assay. A 25-base oligonucleotide complementary to a portion of the globin transcript of the actin-globin gene present in pAB1-4 was labeled at its 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase, as described elsewhere (48), and then purified with a Biospin-6 column (Bio-Rad). A 10-μl reaction mixture containing 60 μg of total RNA, 1 pmol of primer (10^4 to 10^5 Cerenkov counts per minute), 50 mM Tris-HCl (pH 8.0), 40 mM NaCl, and 2 mM EDTA was heated at 65°C for 3 min and then incubated at 58°C for 1 h. The reaction mixture was then adjusted to contain 0.6 mM (each) dGTP, dATP, dTTP, and dCTP; 12.5 mM dithiothreitol, 3.7 mM $MgCl_2$, 0.4 U of the RNase inhibitor RNAguard (Pharmacia), and 4 U of avian myeloblastosis virus reverse transcriptase were added, giving a final volume of 15 μl. After incubation for 1 h at 42°C, the reaction mixtures were extracted with phenol-chloroform and the nucleic acids were recovered by ethanol precipitation with oyster glycogen as a carrier. The cDNA-RNA hybrids were denatured at 85°C, and the extended primers were analyzed on a 4.8% polyacrylamide–8 M urea sequencing gel beside the products of dideoxy sequencing reactions performed using the globin oligonucleotide as the primer and pAB1-4 as the template.

β-Galactosidase assay. Frozen cell pellets derived from 2 ml of cells grown in YEPD or 10 ml of cells in sporulation medium or YEPA were resuspended in 200 μl of Z buffer (37), and the cells were broken by vortexing with 500-μm-diameter glass beads for six 15-s pulses (19). Z buffer (0.8 ml) was added, and the samples were adjusted to 0.005% sodium dodecyl sulfate and 2% chloroform. Aliquots of the extracts were then added to Z buffer to give a final volume of 0.5 ml. The samples were prewarmed at 28°C for 2 min before addition of 0.1 ml of an *O*-nitrophenyl-β-D-galactopyranoside (ONPG) solution (4 mg of ONPG per ml of 0.1 M $NaPO_4$ [pH 7.0]). After incubation at 28°C, the reactions were stopped by addition of 0.25 ml of 1 M Na_2CO_3. The samples were centrifuged briefly, and the optical density at 420 nm of the supernatant was recorded. β-Galactosidase activity is given as nanomoles of ONPG cleaved per minute per milligram of protein at 28°C. The protein concentration of the extract was determined by the Bradford assay. The β-galactosidase activity measured in cells containing pLGΔ312SΔSS(Bgl) (<1 U) was subtracted from all values. All reported values are the average activities obtained from two to four independent transformants, each measured at two concentrations of protein extract.

Gel retardation assay. The protein present in the high-speed supernatant of a crude extract prepared from strain 20B-12 growing in YEPD was concentrated by ammonium sulfate precipitation and dialyzed as described previously (59). Gel retardation assays were performed as described by Challice and Segall (8) using as a probe fragment 29 (see Fig. 3) whose 5′ overhangs had been labeled by filling in with the Klenow form of DNA polymerase in the presence of [α-^{32}P]dCTP. A 20-μl gel retardation reaction mixture contained ~10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.9), 75 mM KCl, 11 mM $MgCl_2$, 50 μM $ZnSO_4$, 10% glycerol, 0.65 mM dithiothreitol, 0.25 mM EDTA, 2 μg of poly(dI · dC)-poly(dI · dC), 0.04 pmol of radioactively labeled fragment 29, 1.5 μl of crude extract, and the indicated competitor DNAs. The crude extract was the last component added to the reaction. After an 8-min incubation, the samples were applied to an 8% polyacrylamide gel running at 60 V. The gel buffer and running buffer were 45 mM Tris-borate (pH 8.3)–1 mM EDTA. Gels were prerun for 20 min at 120 V and then run at 120 V at room temperature.

General methods. Custom-made oligonucleotides were synthesized by Vetrogen Corp. Sequencing reactions were performed with a Sequenase kit (United States Biochemical). All plasmids were harbored in *E. coli* DH5α unless specified otherwise.

RESULTS

The 5′ boundary of the regulatory region of the *SPS4* gene maps downstream of −135. As a first step in identifying a potential regulatory element(s) promoting sporulation-specific expression of the *SPS4* gene, we mapped the 5′ boundary of the regulatory region of the gene. For these experiments, we monitored the effects of sequential deletions of 5′-flanking sequence on expression of *SPS4* using plasmid-borne versions of the gene introduced into a *MAT***a**/*MAT*α Δ*sps4*/Δ*sps4* strain. Chromosome-derived *SPS4* transcripts are not present in this strain (Fig. 1A, second lane of each panel) because the 5′-flanking and initial coding regions of both chromosomal copies of the *SPS4* gene have been deleted. We initially used conveniently positioned restriction sites to introduce versions of the *SPS4* gene with 5′ endpoints at nt −812, −159, and −89 into the centromere-containing plasmid pRS315. These plasmids were introduced into the *MAT***a**/*MAT*α Δ*sps4*/Δ*sps4* strain, and expression of the plasmid-borne SPS4 gene was monitored by Northern blot analysis of RNA prepared from cells growing vegetatively and after transfer to sporulation medium. This analysis indicated that *SPS4* genes with deletion endpoints at nt −812 and −159 were expressed in a sporulation-specific manner (Fig. 1A, fourth and fifth lanes of each panel). The level of expression of the plasmid-borne genes was approximately the same as that of the chromosomal gene of a *MAT***a**/*MAT*α *SPS4*/*SPS4* strain (Fig. 1A, compare first, fourth, and fifth lanes). The *SPS4* gene containing a deletion endpoint at nt −89 was not activated during sporulation (Fig. 1A, third lane of each panel). These results suggested that the 5′ boundary of a sporulation-specific UAS was located between nt −159 and −89 of the *SPS4* gene.

To delimit the 5′ boundary of the putative sporulation-specific regulatory element more precisely, we used exonuclease III to generate a series of 5′-flanking deletions in the *SPS4* gene with endpoints positioned approximately every 10 to 15 bp between nt −159 and −89. As in the initial analysis described above, plasmids containing these *SPS4* genes were introduced into the *MAT***a**/*MAT*α Δ*sps4*/Δ*sps4* strain and *SPS4* expression was monitored by Northern blot analysis. The pattern of expression of genes containing deletion endpoints at nt −159, −151, −141, and −135 essentially corresponded to that of the chromosomal *SPS4* gene of a *MAT***a**/*MAT*α strain (Fig. 1B, compare first and third through sixth lanes of each panel). The genes were expressed in a sporulation-specific manner and were activated prior to 10 h of sporulation, with maximal transcript accumulation occurring at 15 h. We noted that transcripts from the plasmid-borne *SPS4* genes with deletion endpoints at nt −159 and −141 (Fig. 1B, third and fifth lanes) reproducibly accumulated to a slightly higher level than did transcripts from the chromosomal *SPS4* gene (Fig. 1B, first lane) and the plasmid-borne *SPS4* genes with deletion endpoints at nt −151 and −135 (Fig. 1B, fourth and sixth lanes). It is possible that sequences in this region of the *SPS4* gene serve to modulate the rate of transcription from the promoter. Extending the deletion of the 5′-flanking sequence of *SPS4* to nt −117 abolished expression of the gene (Fig. 1B, seventh lane). As a control, the blots were reprobed with *SPS1*, a sporulation-specific gene that is expressed at about the same time as is *SPS4*. As expected, *SPS1* transcripts could be detected in all the strains 10 h after transfer to sporulation medium (Fig. 1B). Reprobing the blots with *PYK1* confirmed that similar amounts of RNA were present in all lanes (Fig. 1B). In summary, this analysis indicated that the 5′ boundary of a putative regulatory element required for expression of the *SPS4* gene lies between nt −135 and −117.

The chromosomal *SPS4* gene is not activated in *MAT***a**/*MAT***a** cells or *MAT*α/*MAT*α cells transferred to sporulation medium (17), since the homozygous *MAT* cells are unable to enter the sporulation pathway in response to nutrient deprivation. We therefore ascertained that expression of the plasmid-borne versions of the *SPS4* gene was also subject to mating-type control. Plasmids containing the *SPS4* gene with deletion endpoints at nt −812, −159, −135, and −117 were introduced into a *MAT***a**/*MAT***a** Δ*sps4*/Δ*sps4* strain. *SPS4*-derived transcripts could not be detected in RNA isolated from the resultant strains either shortly after transfer to sporulation medium or after extended incubation in sporulation medium (Fig. 1C). We therefore concluded that the sequence upstream of nt

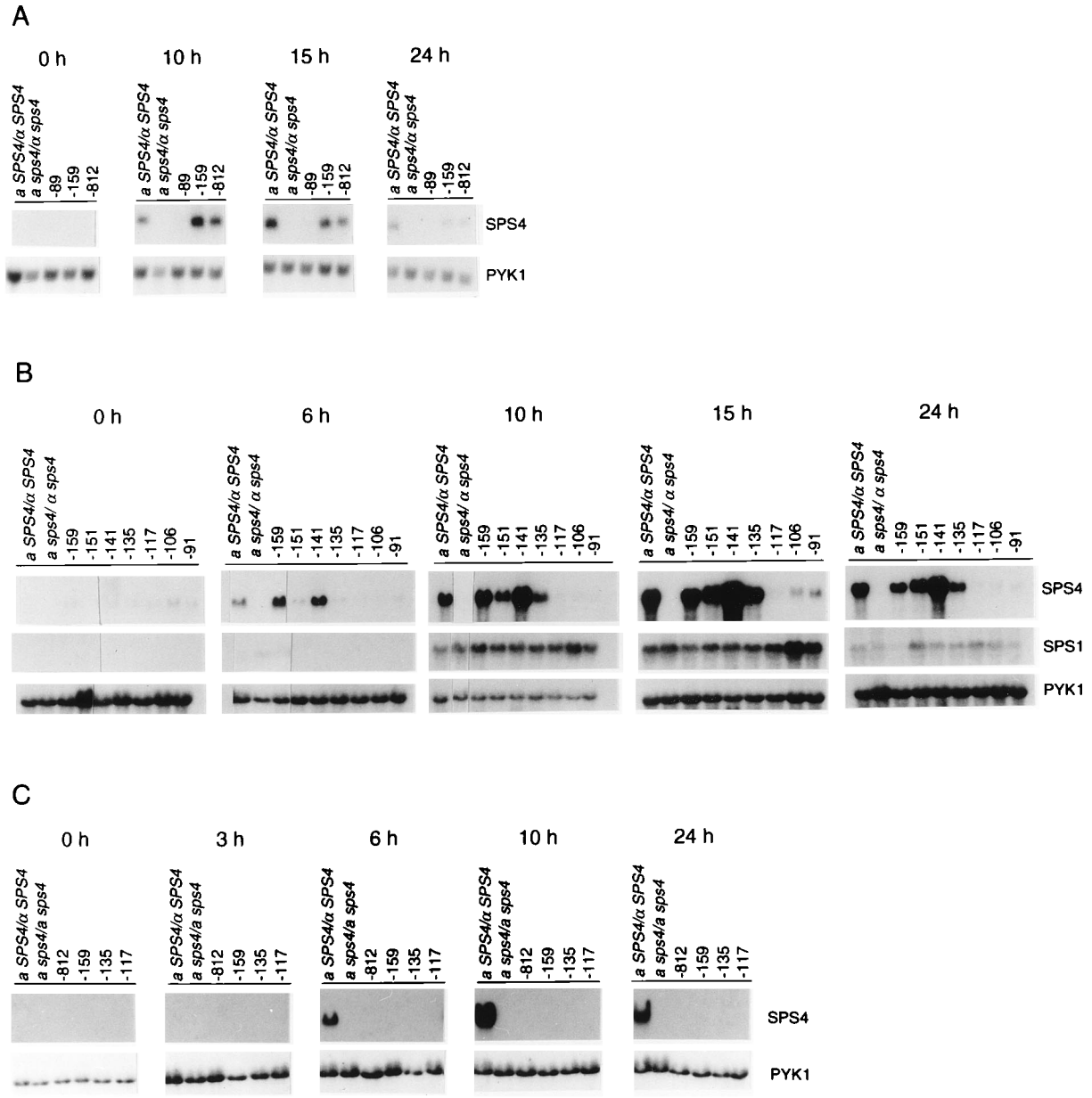


FIG. 1. The 5′ boundary of the regulatory region of the *SPS4* gene is located between nt −135 and −117. Northern blot analysis was performed with RNA isolated from vegetatively growing cells (0 h) and from cells harvested at various times after transfer to sporulation medium, as designated above the panels. The filters were sequentially hybridized with the radioactively labeled probes indicated on the right of the panels. (A) The sequence upstream of nt −159 is dispensable for sporulation-specific expression of *SPS4*. Analysis of RNA isolated from LP112 (*MAT***a**/*MAT*α *SPS4*/*SPS4*) (first lane of each panel) YLE1 (*MAT***a**/*MAT*α Δ*sps4*/Δ*sps4*) (second lane of each panel), and YLE1 transformed with pRS315-derived plasmids containing the *SPS4* gene with deletions of the 5′-flanking sequence (deletion endpoints [89, 159, and 812 bp upstream of the start site of transcription] are indicated above the lanes). (B) The 5′ boundary of the regulatory region of *SPS4* is located between nt −135 and −117. Northern blot analysis was performed as described for panel A. (C) The sequence upstream of nt −135 of the *SPS4* gene is not required for mating-type control of gene expression. Analysis of RNA isolated from LP112 (*MAT***a**/*MAT*α *SPS4*/*SPS4*) (first lane of each panel), YSH1 (*MAT***a**/*MAT***a** Δ*sps4*/Δ*sps4*) (second lane of each panel), and YSH1 transformed with pRS315-derived plasmids containing the *SPS4* gene with the indicated deletion endpoints.

−135 of the *SPS4* gene is not essential for mating-type control or nutritional control of its expression.

A 38-bp DNA fragment from the 5′ region of the *SPS4* gene promotes sporulation-specific expression from a heterologous promoter. The results of the deletion analysis indicated that the 5′ boundary of the regulatory region of the *SPS4* gene was within 50 bp of the putative TATA box of the gene. We therefore tested whether a DNA fragment spanning the 5′ boundary of the regulatory region and extending towards the TATA box, which is centered at nt −81, would act as a sporulation-specific UAS on a heterologous promoter. As a reporter gene, we initially used an actin-globin fusion gene lacking the *ACT1* UAS, which we refer to as a UAS-less reporter gene. The fusion gene contains the TATA box and transcription initiation sites of the yeast *ACT1* gene fused to coding sequence of the rabbit β-globin gene (41). We cloned a 56-bp restriction endonuclease-generated fragment of the *SPS4* gene (nt −158 to −103) upstream of this reporter gene. Plasmid-borne versions

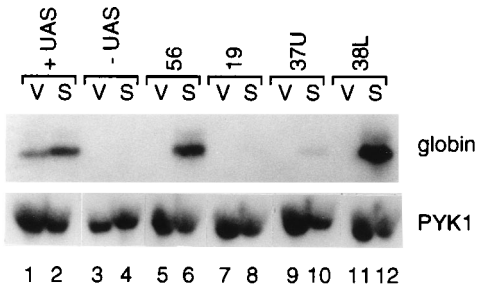
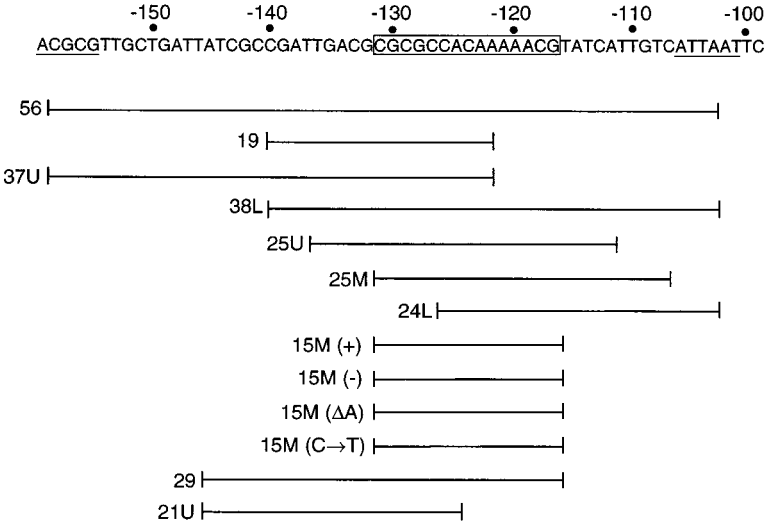


FIG. 2. A 38-bp fragment isolated from the 5′-flanking region of *SPS4* promotes sporulation-specific expression from a heterologous promoter. LP112 cells contained the following plasmids: pAB0, which contains an actin-globin reporter gene containing the yeast *ACT1* regulatory region, including its UAS, fused to a portion of the rabbit β-globin coding region (see Materials and Methods) (lanes 1 and 2); pAB1-4, which contains a UAS-less version of the actin-globin reporter gene (lanes 3 and 4); and versions of pAB1-4 containing the *SPS4*-derived inserts 56, 19, 37U, and 38L (lanes 5 to 12) (see the text and Fig. 3 for a description of these inserts). RNA was isolated from cells growing vegetatively (lanes V) and at 10 h after transfer to sporulation medium (lanes S). (Top) RNA was analyzed by primer extension using a globin-specific oligonucleotide as the primer (see Materials and Methods). Only the portion of the autoradiogram depicting the extended products is shown. The major start site that we detected corresponds to site C3 as reported elsewhere (41). (Bottom) RNA was analyzed by Northern blotting. The filter was probed with *PYK1*.

of the actin-globin gene were introduced into a *MAT***a**/*MAT*α strain, and RNA isolated from these strains during vegetative growth and at 10 h after transfer to sporulation medium was analyzed by primer extension using a primer specific for the globin transcript (see Materials and Methods). Globin transcripts were readily detected in RNA prepared from both vegetative and sporulating cells of a control strain that contained the reporter gene under the control of the *ACT1* UAS (Fig. 2, upper panel, lanes 1 and 2). Fusion transcripts were not detected in a strain containing the UAS-less reporter gene (Fig. 2, lanes 3 and 4). Insertion of the 56-bp *SPS4*-derived fragment upstream of the UAS-less reporter gene led to sporulation-specific expression of the gene (Fig. 2, upper panel, lanes 5 and 6). Northern blot analysis of the RNAs, probing for *PYK1* transcripts, confirmed that the primer extension reactions contained similar amounts of RNA (Fig. 2, lower panel).

We then tested a 19-bp fragment that extended from nt −140 to −122 of the *SPS4* gene and contained a GC-rich partially palindromic region for UAS activity (see Fig. 3, insert 19). This fragment did not activate the reporter gene (Fig. 2, upper panel, lanes 7 and 8). Since this fragment spanned the 5′ boundary of the regulatory region, we anticipated that the addition of downstream sequence would generate a fragment with UAS activity. Indeed, we found that the *SPS4*-derived sequence from nt −140 to −103 promoted sporulation-specific expression of the reporter gene (Fig. 2, upper panel, lanes 11 and 12; Fig. 3, insert 38L). In contrast, the *SPS4*-derived sequence from nt −158 to −122 promoted a barely detectable level of expression (Fig. 2, upper panel, lanes 9 and 10; Fig. 3, insert 37U). This analysis indicated that a 38-bp DNA fragment containing the sequence just upstream of the putative TATA box of the *SPS4* gene is sufficient to promote sporulation-specific transcription from a heterologous promoter. This

A



B

reporter					
ACT-globin			CYC-lacZ		
*MAT***a**/*MAT*α			*MAT***a**/*MAT*α		*MAT***a**/*MAT***a**
VEG			VEG	SPO	SPO
−	+		1.5	100	0.2
−	−		1.1	0.0	ND
−	−		ND	ND	ND
−	+		ND	ND	ND
			1.0	16.3	0.5
			0.1	7.0	0.4
			0.1	0.1	ND
			0.7	11.2	0.8
			1.5	16.8	ND
			0.9	1.7	0.3
			1.1	2.0	0.4
			2.4	147	1.1
			1.6	0.4	ND

FIG. 3. Delimitation of a 15-bp sequence from the 5′-flanking region of *SPS4* that promotes sporulation-specific expression from a heterologous promoter. (A) The sequence of the 5′-flanking region of the *SPS4* gene from nt −159 to −99 is given (17). +1 is the start site of transcription; the putative TATA box (not shown) is centered at −81. An *Mlu*I site at −159 and an *Ase*I site at −106 are underlined. The 15-bp regulatory element identified in this study is boxed. The horizontal bars below the sequence denote fragments from this region that were tested for UAS activity, with their names given on the left. 15M(−) indicates that fragment 15M was in the opposite orientation in the reporter gene as in the *SPS4* gene; 15M(ΔA) and 15M(C→T) refer to mutations in 15M as described in the text. (B) Ability of these fragments to activate expression from heterologous promoters. The first two columns (designated ACT-globin) denote the ability of the fragments to promote expression of a UAS-less yeast actin-rabbit β-globin reporter gene and summarize the primer extension analysis presented in Fig. 2 (+, extended products readily detected; −, no significant amount of extended products). The last three columns (designated CYC-lacZ) denote the ability of the fragments to promote expression of a UAS-less *CYC1-lacZ* reporter gene (see Materials and Methods). The activities of these fragments were determined by measurements of β-galactosidase activity and are expressed as percentages of the activity of fragment 56 (top line), which was 23.7 U (see Materials and Methods). As indicated, plasmids containing the reporter genes were present in a *MAT***a**/*MAT*α strain (LP112) or a *MAT***a**/*MAT***a** strain (LP112 *MAT***a**/*MAT***a**). Expression was assessed in vegetatively growing cells (VEG) and in cells at 15 h after transfer to sporulation medium (SPO). ND, not determined.

experiment also showed that transcription promoted by the *SPS4* regulatory sequence started at the same site as did transcription promoted by the *ACT1* UAS (Fig. 2, compare lanes 6, 12, and 1).

A 15-bp element is sufficient for sporulation-specific UAS activity. To further delimit the sporulation-specific UAS element, we tested a series of overlapping *SPS4*-derived DNA fragments spanning the region from nt −145 to −103 for their ability to promote sporulation-specific expression of a UAS-less *CYC1-lacZ* reporter gene. Use of this reporter gene allowed us to readily compare the UAS activities of the various DNA fragments. First, we cloned the 56-bp fragment that extended from nt −158 to −103 of the *SPS4* gene upstream of the UAS-less *CYC1-lacZ* gene present in pLGΔ312SΔSS(Bgl) (see Materials and Methods). The resultant plasmid was introduced into a *MAT***a**/*MAT*α strain, and β-galactosidase activity was measured in extracts of vegetatively growing cells and cells harvested at 15 h after transfer to sporulation medium. As expected, the 56-bp fragment promoted sporulation-specific expression of *CYC1-lacZ* (Fig. 3A, insert 56; Fig. 3B, third and fourth columns). Of the smaller inserts tested, we found that fragment 25U (nt −136 to −112 of the *SPS4* gene) and fragment 25M (nt −131 to −107 of the *SPS4* gene) promoted sporulation-specific expression of *CYC1-lacZ*, albeit less efficiently than did fragment 56 (Fig. 3, inserts 25U and 25M). Fragment 24L (nt −126 to −103), on the other hand, lacked UAS activity (Fig. 3, insert 24L). This suggested that the sequence between nt −131 and −126 was essential for the activity of the regulatory element and that the sequence downstream of nt −112 was not. Consistent with these observations, we found that fragment 15M (nt −131 to −117) supported sporulation-specific expression of *CYC1-lacZ*. The fragment was active in either orientation [Fig. 3, inserts 15M(+) and 15M(−)]. Deletion of an A from the A_5 tract between nt −123 and −119 or mutation of the C residue at nt −127 to a T residue led to a large reduction in the level of expression promoted by 15M [Fig. 3, inserts 15M(ΔA) and 15M(C→T)].

Although fragment 15M supported sporulation-specific gene expression, we note that this fragment was 10-fold less efficient than was the 56-bp fragment in activating the reporter gene. A 29-bp fragment extending from nt −145 to −116, however, was as active as was the 56-bp fragment (Fig. 3, insert 29). The sequence from nt −145 to −131, which enhanced the activity of fragment 15M, did not by itself have any significant UAS activity (Fig. 3, inserts 37U and 21U; Fig. 2, lanes 9 and 10). We therefore concluded that the 15-bp sequence extending from nt −131 to −117 of the *SPS4* gene contains a regulatory element(s) that suffices to activate sporulation-specific gene expression and that the sequence between nt −145 and −131, upstream of this minimal regulatory region, serves to enhance the level of activation. None of the *SPS4*-derived UAS-containing inserts were able to promote significant expression of *CYC1-lacZ* in *MAT***a**/*MAT***a** cells incubated in sporulation medium (Fig. 3B, last column) indicating that the regulatory element(s) is subject to mating-type control.

We next monitored the time at which the *SPS4*-derived regulatory element became active during sporulation. β-Galactosidase activity was measured in extracts of cells harvested at various times after transfer to sporulation medium. Expression from the *CYC1-lacZ* reporter gene containing the more active 56-bp fragment was first detected at 10 h of sporulation (Fig. 4). Expression from the *CYC1-lacZ* reporter gene containing the less efficient 15M fragment was first detected at 12 h of sporulation (Fig. 4). Maximal levels of β-galactosidase activity were measured at 15 h of sporulation. The *SPS4*-derived regulatory element(s) therefore retains appropriate temporal control of gene expression, activating the reporter gene at about the same time that transcripts begin to accumulate from the chromosomal *SPS4* gene (Fig. 1B).

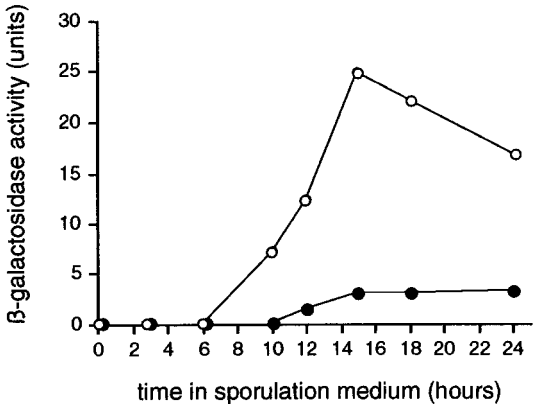


FIG. 4. *SPS4*-derived fragments activate a *CYC1-lacZ* reporter gene midway through sporulation. Aliquots of *MAT***a**/*MAT*α cells (LP112) containing fragment 56 (open circles) or fragment 15M (closed circles) (Fig. 3) inserted upstream of the UAS-less *CYC1-lacZ* reporter gene of pLGΔ312SΔSS(Bgl) were harvested at various times after transfer to sporulation medium, and β-galactosidase activity was determined (see Materials and Methods).

Expression of *SPS4* is *IME1* and *IME2* dependent. Entry into sporulation is controlled by *IME1*, a key regulatory gene which is responsible for expression of most, if not all, sporulation genes (reviewed in reference 38). Expression of *IME1* leads to activation of early meiotic genes such as *SPO13*, *HOP1*, and *IME2* (39, 54, 55, 60). Activation of *IME2* is required for cells to progress through the sporulation program and appears to be necessary for down regulation of *IME1* RNA levels and for activation of middle sporulation genes (39). We determined the effect of mutation of *IME1* and *IME2* on expression of *SPS4* during sporulation. RNA was isolated from wild-type cells, *ime1*/*ime1* cells, and *ime2*/*ime2* cells at various times after transfer to sporulation medium, and expression of three early meiotic genes (*IME2*, *HOP1*, and *SPO13*), the middle sporulation genes *SPS1* and *SPS4*, and the mid-late sporulation gene *DIT1* was monitored by Northern analysis. The analysis of RNA from wild-type cells revealed the expected sequential expression pattern of these temporally distinct classes of genes (Fig. 5, left panel). We also found a peak of expression of *IME1*, *IME2*, *HOP1*, *SPO13*, *SPS1*, and *SPS4* at 36 h after transfer of cells to sporulation medium. As expected, none of the meiotic genes tested, including *SPS4*, were expressed in the *ime1*/*ime1* strain transferred to sporulation medium (Fig. 5, middle panel). Consistent with previous observations (39), expression of *IME1* was prolonged and expression of *SPO13* and *HOP1* was both delayed and prolonged in the *ime2*/*ime2* strain (Fig. 5, compare left and right panels). As observed for the middle sporulation genes *SPS1* and *SPS2* (39), we found that expression of *SPS4* could be detected only after very prolonged incubation of the *ime2*/*ime2* strain in sporulation medium (Fig. 5, right panel). This was also the case for the mid-late gene *DIT1* (Fig. 5, right panel). Thus, expression of *SPS4*, like that of *SPS1* and *SPS2*, is much more sensitive to the absence of Ime2p than is expression of early meiotic genes.

***UME6* is not required for vegetative repression of *SPS4*.** Most early meiotic genes contain an URS1 element (reviewed in reference 38), which was initially identified as a regulatory element involved in repression of *CAR1* under noninducing conditions (58). The URS1 element has been shown to contribute to both mitotic repression and meiotic activation of

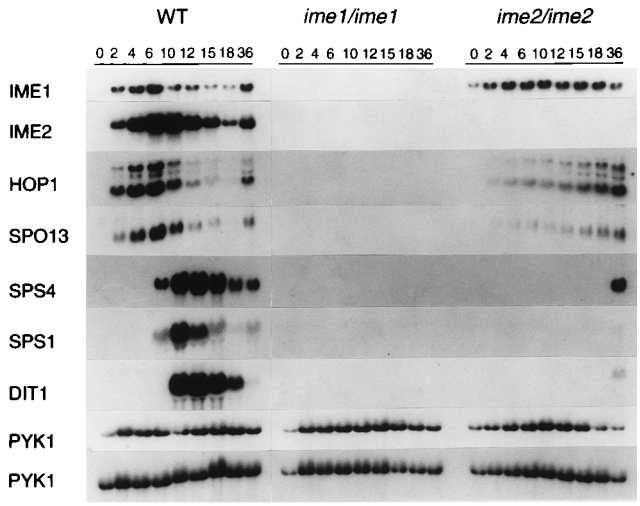


FIG. 5. Analysis of gene expression in wild-type, *ime1*/*ime1*, and *ime2*/*ime2* strains on transfer to sporulation medium. RNA was purified from wild-type (WT) (LP112), *ime1*/*ime1* (YKE1), and *ime2*/*ime2* (YKE2) strains during vegetative growth (0 h) and at various times after transfer to sporulation medium, as indicated (in hours) above the panels. Northern blots of these RNAs were hybridized with radioactively labeled probes containing the genes indicated on the left (see Materials and Methods). One filter was sequentially hybridized with probes for *SPS4*, *DIT1*, *IME2*, *PYK1* (upper of the two PYK1 rows), and *IME1*. A duplicate filter was sequentially hybridized with probes for *PYK1* (lower of the two PYK1 rows), *SPS1*, *SPO13*, and *HOP1*. Two *HOP1*-hybridizing transcripts are seen (24).

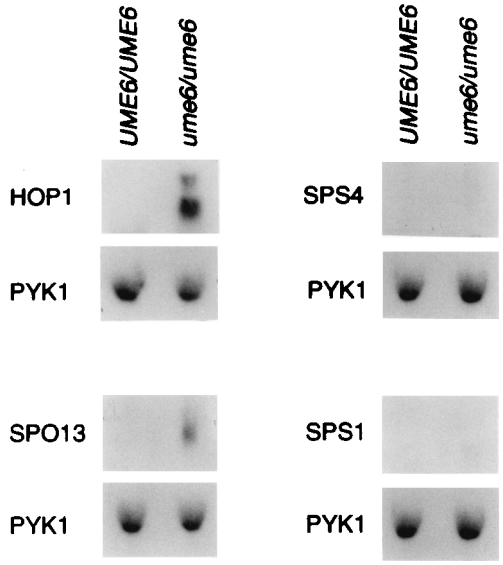


FIG. 6. Mutation of *UME6* does not lead to vegetative expression of *SPS4*. Northern blots were prepared with RNA purified from LP112 (*MAT***a**/*MAT*α *UME6*/*UME6*) and from YSH2 (*MAT***a**/*MAT*α *ume6*/*ume6*) strains growing vegetatively in YEPD. The filters were hybridized with radioactively labeled probes for *HOP1*, *SPS1*, *SPO13*, and *SPS4* and then rehybridized with a probe for *PYK1*.

SPO13 (6), *IME2* (1), and *HOP1* (60). It is thought that Ume6p/Car80p mediates at least some of the regulatory effects of URS1 (43, 56). Mutation of *UME6* leads to high levels of expression of the early meiotic genes *SPO11*, *SPO13*, and *SPO16* in mitotic cells, whereas the middle sporulation genes *SPO12* and *SPS2* remain inactive (56). The observation that *SPS4* lacks an apparent URS1 site suggested that *UME6* would not be involved in mitotic repression of this gene. Indeed, we were able to readily detect *HOP1* and *SPO13* transcripts in Northern blot analysis of RNA from a *ume6*/*ume6* strain growing vegetatively, but we were unable to detect *SPS4* transcripts or *SPS1* transcripts in these cells (Fig. 6). We concluded that *SPS4* is not subject to *UME6*-dependent repression in mitotic cells. This genetic analysis of the effect of mutation of *UME6* and *IME2* on expression of *SPS4* clearly indicated that regulation of *SPS4* is distinct from the regulation of early meiotic genes.

Evidence that a protein present in vegetative cells binds to the *SPS4* regulatory element. To test the possibility that a protein is bound to the *SPS4* regulatory element in vegetative cells, we cloned fragment 29 (nt −145 to −116 of the *SPS4* gene; Fig. 3) into the *Bgl*II site of pLGΔ312(Bgl) (Fig. 7). This places the *SPS4* sequence between the *CYC1* UAS and the TATA box of the *CYC1-lacZ* reporter gene. Previous studies with this vector have shown that insertions of short sequences between the *CYC1* UAS and TATA box have little or no effect on expression of the reporter gene whereas the binding of a protein to the inserted sequence interferes with activation of transcription (3). Although insertion of one copy of fragment 29 had only a marginal effect on expression of the reporter gene, the presence of three copies reduced expression 10-fold and the presence of four copies reduced expression 200-fold (Fig. 7, second through fourth lines). This repressive effect was, at least in part, independent of the orientation of the insert (Fig. 7, third line). This experiment suggested that a protein, or protein complex, binds to the *SPS4* regulatory element in vegetative cells. The bound protein could interfere with activation indirectly by steric hindrance (e.g., see reference 3) or directly by repression (e.g., see references 29 and 61). To distinguish between these possibilities, we inserted four copies of fragment 29 at the *Sma*I site upstream of the *CYC1* UAS in the *CYC1-lacZ* reporter gene. At this site, interference with transcrip-

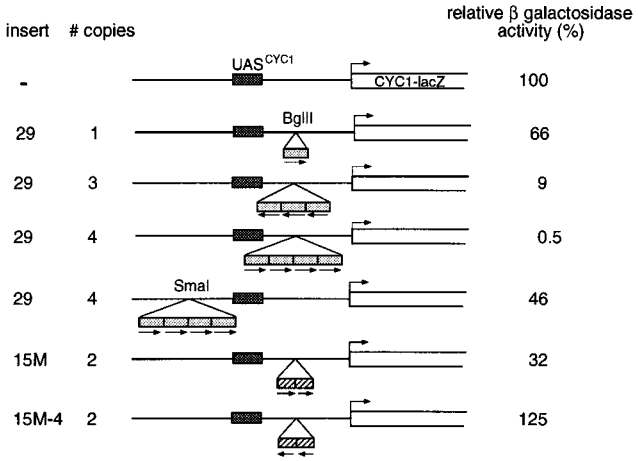


FIG. 7. In vivo evidence that a protein binds to the *SPS4* regulatory region in vegetatively growing cells. The *CYC1-lacZ* reporter gene of pLGΔ312(Bgl) is shown on the top line. The plasmid contains a unique *Bgl*II site between the *CYC1* UAS and TATA box and a unique *Sma*I site upstream of the *CYC1* UAS. One, three, and four copies of *SPS4*-derived fragment 29 (second to fourth lines, respectively), two copies of fragment 15M (sixth line) and two copies of fragment 15M-4 (seventh line) were cloned into the *Bgl*II site. Four copies of fragment 29 were cloned into the *Sma*I site (fifth line). The sequences of fragments 29 and 15M are given in Fig. 3. Fragment 15M-4 is a mutant version of fragment 15M (see Materials and Methods). The orientation of the inserts relative to the *SPS4* gene is denoted by the arrows. The right column gives the relative β-galactosidase activity measured in LP112 cells grown vegetatively in YEPD and containing the indicated plasmid-borne *CYC1-lacZ* gene.

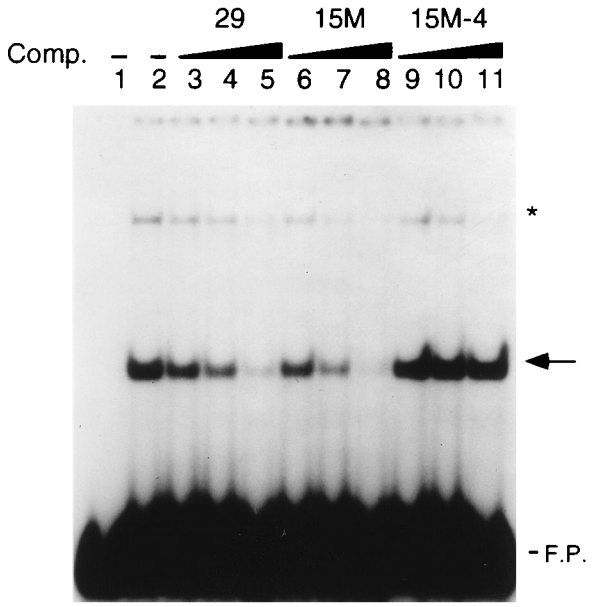


FIG. 8. Specific binding of protein present in a crude extract of vegetative cells to the *SPS4* regulatory region. An aliquot (1.5 μl) of a crude extract prepared from vegetatively growing cells was incubated with radioactively labeled fragment 29 (Fig. 3). After an 8-min incubation at 25°C, the reaction mixture was loaded onto a nondenaturing 8% polyacrylamide gel to separate protein-DNA complexes from free DNA. The reaction of lane 1 contained no protein. The reactions of lanes 3 to 5 contained fragment 29 as the competitor DNA; the reactions of lanes 6 to 8 contained fragment 15M as the competitor DNA; the reaction of lanes 9 to 11 contained fragment 15M-4, a version of 15M with four base pair changes (see Materials and Methods), as the competitor DNA. The reactions of lane 3, 6, and 9 contained a 10-fold molar excess of nonlabeled DNA; the reactions of lanes 4, 7, and 10 contained a 25-fold molar excess of nonlabeled DNA; and the reactions of lanes 5, 8, and 11 contained a 100-fold molar excess of nonlabeled DNA. The asterisk denotes an apparent nonspecific protein-DNA complex. F.P., free probe.

tional activation by steric hindrance would be minimal whereas interference by direct repression would most probably be maintained. We found that insertion of the *SPS4*-derived sequence at the *Sma*I site had only a minor effect on expression of the reporter gene in vegetative cells (Fig. 7, fifth line). This suggested that the putative protein that binds to the *SPS4*-derived sequence is not a direct repressor of gene expression. The interference observed when the *SPS4*-derived sequence is present downstream of the *CYC1* UAS might result from an inactive form of a transcriptional activator bound to UAS^{SPS4} in vegetative cells.

We also inserted two copies of fragment 15M (nt −131 to −117 of the *SPS4* gene; Fig. 3) between the UAS and TATA box of the *CYC1-lacZ* reporter gene. This smaller UAS^{SPS4}-containing fragment, which is less efficient at promoting sporulation-specific expression than is fragment 29, led to a threefold reduction in β-galactosidase expression in vegetatively growing cells (Fig. 7, sixth line). As a control, the presence of two copies of a mutated version of fragment 15M did not reduce expression of *CYC1-lacZ* (Fig. 7, seventh line).

The results of the experiment described above prompted us to test crude extracts of vegetative cells for a factor that binds to the *SPS4* regulatory element. Figure 8 presents a gel retardation experiment which reveals the formation of a protein-DNA complex when a crude extract of cells is incubated with radioactively labeled fragment 29 (lane 2). Formation of this complex (denoted by the arrow in Fig. 8) was inhibited by the presence of excess nonlabeled fragment 29 (Fig. 8, lanes 3 to 5) or fragment 15M (lanes 6 to 8) but not by the presence of an excess of fragment 15M-4, a version of 15M containing four base pair changes (lanes 9 to 11). This indicates that this protein-DNA complex reflects a specific interaction of a protein present in the vegetative extract with the *SPS4* sequence represented by fragment 15M. In summary, both an in vivo assay and an in vitro assay provide preliminary evidence for the presence in vegetative cells of a factor that binds to the *SPS4* regulatory element. The role of this factor in regulating expression of the *SPS4* gene remains to be determined.

DISCUSSION

The *SPS4* gene of *S. cerevisiae* is expressed midway through the sporulation program, with maximal transcript accumulation occurring as the meiotic divisions are nearing completion. In this study, we identified a 15-bp element from the 5′-flanking region of *SPS4* that directs sporulation-specific expression. This element, designated UAS^{SPS4}, is subject to mating-type and nutritional control and activates a heterologous promoter following the same temporal pattern of expression as the chromosomal *SPS4* gene. Consistent with the notion that activation of *SPS4* midway through sporulation occurs in a manner distinct from that of activation of early meiotic genes, we found that *SPS4* was not expressed in vegetative cells of a *MAT***a**/*MAT*α *ume6*/*ume6* strain. Mutation of *UME6* has been shown to lead to vegetative expression of a number of early meiotic genes (56). We also found that activation of *SPS4* during sporulation was more sensitive to mutation of the regulatory gene *IME2* than was activation of early meiotic genes. This is consistent with the observation that early meiotic genes can be activated by both an *IME1*-dependent and an *IME2*-dependent pathway but that later genes require the *IME2*-dependent pathway for their activation (reviewed in reference 38).

Regulatory elements of sporulation genes. The minimal regulatory element of *SPS4* that suffices to promote sporulation-specific gene expression appears to be a relatively simple site. In contrast, distinct regulatory sites that act together to achieve sporulation-specific expression have been identified in studies of other sporulation genes. One of these sites, URS1, was initially identified as being involved in repression of *CAR1* (58) and was subsequently found to contribute to the regulation of a diverse set of genes, including early meiotic genes. An URS1 element that is present near the 5′ boundary of the regulatory region of *SPO13* was the first such site found to contribute to the regulation of a meiotic gene (6). The element was shown to serve a dual role, acting to prevent mitotic expression and to enhance the level of meiotic induction of *SPO13* (6). The near universal presence of URS1 in the 5′ region of early meiotic genes, first noted by Buckingham et al. (6), has been further documented (reviewed in reference 38). Candidate proteins for mediating the regulatory role of URS1 include Ume6p/Car80p (1, 43, 56) and the heteromeric Bufp-Rpap complex (32, 33).

The URS1 site, referred to as $URS1_H$, present in a 200-bp region that promotes meiosis-specific expression of *HOP1* was also shown to be essential for mitotic repression and full meiotic induction of this gene (60). A second element within the regulatory sequence, UAS_H, functions as an independent activator site and contributes to full meiotic expression (60). Sequences with similarity to UAS_H have been identified in several early meiotic genes (60). The observation that a 100-bp fragment containing the UAS_H and $URS1_H$ sites fails to promote meiosis-specific expression indicates that additional *HOP1* regulatory sites remain to be identified (60). Bowdish and Mitchell (1) characterized a 50-bp region upstream of

IME2 that responds to *IME1*-dependent regulation. The regulatory region includes an activation element, referred to as T_4C, and an URS1 element. Again, the URS1 site serves both positive and negative functions. Sequence inspection reveals the presence of T_4C in the 5′ region of several early meiotic genes (1). We find no good matches to the URS1, UAS_H, or T_4C site in the *SPS4* regulatory region.

The *SGA1* gene, a late sporulation gene encoding a glucoamylase, is regulated by an activation element that responds to nutritional conditions and a negative element that is subject to both nutritional and mating-type control (30). Kihara et al. (30) noted that the *SPS4* sequence from nt −191 to −172 (+1 being the transcriptional start site) was similar to a sequence within the negative regulatory region of *SGA1*. Our present study indicates that if this sequence contributes to regulation of *SPS4*, it is not an essential site because deletions through this region do not lead to a loss in mating-type or nutritional control of *SPS4* expression (Fig. 1).

The *SPS4* activation element. The 15-bp sporulation-specific UAS element of *SPS4* maps from nt −131 to −117. This places the regulatory element in close proximity to the putative TATA box of *SPS4*, which is centered at nt −81. The early meiotic genes *HOP1* and *SPO11* also have presumptive regulatory elements close to the transcriptional start site (6, 60; discussed in reference 38). UAS^{SPS4} contains a G+C-rich sequence adjacent to an ACA_5 sequence. Both regions appear to be essential for activation because mutation of the C residue at nt −127 in the G+C-rich sequence or deletion of an A residue from the A_5 tract led to a large reduction in the level of expression. Whereas early meiotic genes and *SGA1* are subject to negative control in vegetative cells, our data provide no evidence for negative control of *SPS4.*

We found that the sequence immediately upstream of the 15-bp UAS^{SPS4} element enhanced activation 10-fold. Unlike the UAS_H site of *HOP1* (60) and the T_4C element of *IME2* (1), which also serve to enhance a low level of sporulation-specific gene expression, the sequence of *SPS4* upstream of the 15-bp regulatory element had no significant UAS activity on its own. Inspection of this upstream sequence revealed the presence of a CGCC motif that is also present in the 15-bp element. The significance of this repeating motif is not clear.

Similarity between the *SPS4* UAS and *SPR2* UAS. We anticipated that the coordinate expression of at least some middle *SPS* genes would reflect activation through a common regulatory site. Given that the UAS^{SPS4} element can activate sporulation-specific expression in an orientation-independent manner, we scanned the 5′ region of sporulation genes in both orientations for sequences with similarity to the *SPS4* regulatory element. This search revealed similarities with the *SPR2* regulatory region (9, 47). Figure 9 shows a 30-bp fragment of *SPR2* that was shown to activate sporulation-specific expression of *CYC1-lacZ* in an orientation-independent manner (46, 47). A 10-of-10-bp match is found between nt −261 to −252 of *SPR2* and nt −119 to −128 of *SPS4*. A 14-of-16-bp match is found between nt −251 to −236 of *SPR2* and nt −115 to −130 of *SPS4*. We also noted a 15-of-17-bp match between nt −119 to −135 of *SPS4* and nt −529 to −509 of *DIT1*, a mid-late sporulation-specific gene (4) (Fig. 9). Future work will indicate whether this identity reflects a common mechanism of activation.

Mechanism of regulation at the *SPS4* UAS. We presume that a transcriptional activator that is functional only during sporulation is recruited to the 15-bp UAS^{SPS4} element. The adjacent upstream sequence, which enhances the level of expression, may serve as a binding site for a factor which stimulates formation of an activation complex at UAS^{SPS4}. Alternatively, a protein bound to the upstream sequence might act to augment the level of sporulation-specific expression promoted by the activation complex at UAS^{SPS4}.

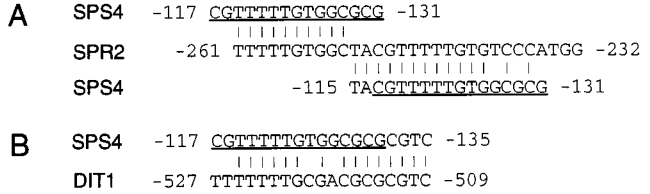


FIG. 9. Comparison of the *SPS4* UAS with *SPR2* and *DIT1* sequences. (A) The top line presents the sequence of the lower strand of *SPS4* from nt −117 to −131 (+1 is the transcriptional start site). The middle line presents the sequence of the top strand of *SPR2* (46, 47) from nt −261 to −232 (+1 is the translational start site). The third line presents the sequence of the lower strand of *SPS4* from nt −115 to −131. (B) The top line presents the sequence of the lower strand of *SPS4* from nt −117 to −135. The bottom line presents the sequence of the upper strand of *DIT1* (4) from nt −527 to −509 (+1 is the transcriptional start site). The *SPS4* sequence represented in fragment 15M is underlined, and identities between sequences are denoted by vertical lines.

In vitro and in vivo assays suggest that a protein(s) that binds to the 15-bp regulatory element is present in vegetative cells. Evidence for a protein bound to UAS^{SPS4} in vivo was provided by the observation that tandem copies of a wild-type UAS^{SPS4} element, but not a mutated element, inserted between the endogenous UAS and TATA box of a *CYC1-lacZ* fusion gene led to a decrease in vegetative expression of β-galactosidase. We found that four copies of the larger UAS^{SPS4}-containing fragment 29 led to a 200-fold decrease in expression of the *CYC1-lacZ* reporter gene when inserted downstream of the *CYC1* UAS but had only a minor effect on expression when inserted upstream of the *CYC1* UAS. This suggested that the protein bound to the *SPS4*-derived fragment in vegetative cells interfered with expression by steric hindrance rather than by active repression. A gel retardation assay also indicated that a factor present in a crude extract of vegetative cells interacts specifically with fragment 29; this interaction was inhibited by a large excess of fragment 15M but not a mutated version of this fragment. We have not yet tested extracts of sporulating cells for DNA-binding proteins that recognize the regulatory element. We speculate that the protein bound to the *SPS4* regulatory site in vegetative cells is an inactive form or component of a transcriptional activator. The protein might be modified during sporulation to reveal an activator function, or it might recruit a sporulation-specific activator to the *SPS4* gene. Future studies will identify the regulatory protein(s) that acts on the UAS^{SPS4} element during sporulation and reveal how expression of the gene is restricted to the middle portion of the sporulation program.

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