

Transcriptional Regulation of the *SMK1* Mitogen-Activated Protein Kinase Gene during Meiotic Development in *Saccharomyces cerevisiae*

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Meiotic development (sporulation) in *Saccharomyces cerevisiae* is characterized by an ordered pattern of gene expression, with sporulation-specific genes classified as early, middle, mid-late, or late depending on when they are expressed. *SMK1* encodes a mitogen-activated protein kinase required for spore morphogenesis that is expressed as a middle sporulation-specific gene. Here, we identify the *cis*-acting DNA elements that regulate *SMK1* transcription and characterize the phenotypes of mutants with altered expression patterns. The *SMK1* promoter contains an upstream activating sequence (UAS^S) that specifically interacts with the transcriptional activator Abf1p. The Abf1p-binding sites from the early *HOP1* and the middle *SMK1* promoters are functionally interchangeable, demonstrating that these elements do not play a direct role in their differential transcriptional timing. Timing of *SMK1* expression is determined by another *cis*-acting DNA sequence termed MSE (for middle sporulation element). The MSE is required not only for activation of *SMK1* transcription during middle sporulation but also for its repression during vegetative growth and early meiosis. In addition, the *SMK1* MSE can repress vegetative expression in the context of the *HOP1* promoter and convert *HOP1* from an early to a middle gene. *SMK1* function is not contingent on its tight transcriptional regulation as a middle sporulation-specific gene. However, promoter mutants with different quantitative defects in *SMK1* transcript levels during middle sporulation show distinct sporulation phenotypes.

The life cycle of the yeast *Saccharomyces cerevisiae* comprises a series of interconnected growth states and developmental programs that are under both genetic and environmental control. Meiotic development (sporulation) is induced when a diploid cell is starved for essential nutrients and a fermentable carbon source (21). Following induction, the cell withdraws from the mitotic cycle at G₁ and enters meiotic prophase, during which a single round of DNA replication, synaptonemal complex formation, and recombination occur. Meiotic prophase is followed by the meiosis I reductional and meiosis II equational divisions. Spore wall morphogenesis initiates with the outgrowth of a double membranous structure (the prospore wall) from the outer plaques of each meiosis II spindle pole body which envelops each haploid meiotic product. Two layers that appear similar to the vegetative cell wall and two protective spore-specific layers are subsequently assembled from within and around the prospore wall. The end product of sporulation is the differentiated ascus which contains four dormant haploid spores. The tightly regulated sequence of cell cycle and morphogenetic events that occur during sporulation provides a model system for study of the mechanisms that regulate and coordinate development.

During sporulation, specific sets of genes that can be classified as early, middle, mid-late, or late are sequentially expressed. Early genes are expressed at the onset of sporulation and are involved in events including formation of the synaptonemal complex and the meiotic divisions. The transcriptional

regulation of the early genes has been extensively studied, and regulatory *cis*-acting DNA sequences and proteins that bind to many of these sites have been identified (21, 26). One element commonly found in early promoters is recognized by the Abf1p DNA-binding protein, a general transcriptional activator (16). URS1, a second *cis*-acting element found in most and perhaps all early promoters, is recognized by the Ume6p DNA-binding protein (26, 29, 35, 37). During vegetative growth, Ume6p interacts with a complex of proteins which includes Sin3p and the Rpd3p histone deacetylase, to repress transcription (19). During early sporulation, changes in the Ume6p-complex occur that lead to transient derepression as well as transcriptional activation (4, 35). Gene products required for the developmentally regulated conversion from a repression to an activation complex include the Rim11p protein kinase, as well as Ime1p, which has transcriptional activation properties and may interact directly with Ume6p (3, 30).

In comparison to the early meiotic genes, less is known about transcriptional regulation of the later temporal classes of sporulation-specific genes. The *cis*-acting promoter sequences responsible for middle-transcriptional regulation have been identified in the *SPS4* and *SPR3* genes (17, 27, 28). The *SPR3* promoter contains a consensus Abf1p binding site which is required for transcriptional activation. An additional site found in both *SPS4* and *SPR3*, as well as in several other middle promoters, is the MSE (middle sporulation element), which has been shown to be required for the activation of middle gene expression and to be capable of activating heterologous promoters during middle sporulation (17, 28). Recently, Ndt80p has been shown to specifically interact with MSE DNA *in vitro* and to activate middle sporulation-specific gene expression *in vivo* (7). *NDT80* is expressed predominantly

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TABLE 1. Yeast strains

Strain ^a	Genotype	Source or reference
LN150	<i>MATa/MATα leu2-hisG/leu2-hisG trp1-hisG/trp1-hisG lys2/lys2 his4-N/his4-G ura3/ura3 ho::LYS2/ho::LYS2</i>	L. Neigeborn
LN153	<i>MATa/MATα leu2-hisG/leu2-hisG trp1-hisG/trp1-hisG lys2/lys2 ura3/ura3 ho::LYS2/ho::LYS2</i>	L. Neigeborn
MDPY10	<i>MATa/MATα leu2-hisG/leu2-hisG trp1-hisG/trp1-hisG lys2/lys2 his4-N/his4-G ura3/ura3 ho::LYS2/ho::LYS2 smk1::LEU2/smk1::LEU2</i>	42
YV16	<i>MATa/MATα trp1/TRP1 ura3-52/ura3-x can1/CAN1 cyh2/CYH2 ade2-R8/ade2-1</i>	39
LN1273	<i>MATa/MATα leu2-hisG/leu2-hisG trp1-hisG/trp1-hisG lys2/lys2 gal80::LEU2/gal80::LEU2 IME1-14-TRP1/IME4 ura3/ura3 ho::LYS2/ho::LYS2</i>	L. Neigeborn
MDPY1003	<i>MATa leu2-hisG trp1-hisG lys2 his4-N ura3 ho::LYS2 smk1::LEU2</i>	This study
MDPY1004	<i>MATα leu2-hisG trp1-hisG lys2 his4-N ura3 ho::LYS2 smk1::LEU2</i>	This study
MDPY34	MDPY10 + <i>URA3/pRS406/ura3</i>	This study
MDPY35	MDPY10 + <i>URA3::pMDP149/ura3</i>	This study
MDPY36	MDPY10 + <i>URA3::pMDP152/ura3</i>	This study
MDPY48	MDPY10 + <i>URA3::pRS406/URA3::pMDP199</i>	This study
MDPY51	MDPY10 + <i>URA3::pRS406/URA3::pMDP183</i>	This study
MDPY44	MDPY10 + <i>URA3::pRS406/URA3::pMDP185</i>	This study
MDPY46	MDPY10 + <i>URA3::pRS406/URA3::pMDP187</i>	This study

^a All strains are SK1 background (1), with the exception of YV16.

as a middle gene, and it has been proposed that completion of a meiotic checkpoint is required for its full activity (7, 17a, 43). Mid-late and late genes appear to require distinct *cis*-acting elements. A negative regulatory element (NRE^{DIT}) that requires the Ssn6p and Tup1p corepressor complex regulates the divergently transcribed mid-late *DIT1* and *DIT2* genes (14). NRE^{DIT} derepression during sporulation requires complex interactions with at least two additional *cis*-acting elements. Thus, the different temporal classes of sporulation-specific promoters require multiple transcriptional activators that are expressed at different times during sporulation, as well as distinct transcriptional-repression pathways.

SMK1 encodes a middle sporulation-specific mitogen-activated protein (MAP) kinase homolog that is required for spore wall morphogenesis. In an *smk1* null homozygous mutant, multiple abnormal spore wall assembly patterns are observed within a single ascus, with spore wall layers inverted or missing (20). Different *smk1* missense mutants stall at distinct stages in spore morphogenesis. Furthermore, modest increases in dosage of certain *smk1* alleles can restore wild-type spore wall morphogenesis, and different spore resistance phenotypes require distinct *smk1* allelic thresholds (41, 42). These results show that *SMK1* plays a central role in coordinating multiple events that are required for spore formation and suggest that different morphogenetic events can have distinct MAP kinase threshold requirements.

The transcriptional regulation of *SMK1* raises a number of interesting questions that are now amenable to experimental investigation. What are the *cis*-acting promoter elements that regulate *SMK1* expression? What is the functional significance of the tight control of *SMK1* transcription during spore development? Can promoter mutants cause distinct phenotypes similar to those seen with the amino acid substitution mutants? Analysis of *SMK1* transcriptional regulation should also provide insights on how the expression of different temporal classes of genes is regulated during development.

MATERIALS AND METHODS

Yeast strains and culture conditions. The genotypes and sources of yeast strains used in this study are shown in Table 1. Vegetative cultures were propagated in either YEPD (1% yeast extract, 2% peptone, 2% glucose), YEPA (1% yeast extract, 2% peptone, 2% potassium acetate), SD (0.67% Difco yeast nitrogen base without amino acids, 2% glucose) or SA (0.67% yeast nitrogen base without amino acids, 1% potassium acetate, 1% phthalic acid [pH 5.5]) supplemented with nutrients essential for auxotrophic strains at the levels specified by

Sherman et al. (31). For synchronous sporulation of strains containing plasmids, cells were pregrown in SA-uracil, and for strains lacking plasmids, cells were pregrown in YEPD. Logarithmically growing cultures were used to inoculate YEPA, the cultures were expanded overnight at 30°C to a density of 1×10^7 cells/ml (4 to 5 generations), and cells were collected by centrifugation, washed once in 2% potassium acetate, and resuspended at 4×10^7 cells/ml in SM (2% potassium acetate, 10 µg of adenine/ml, 5 µg of histidine/ml, 30 µg of leucine/ml, 7.5 µg of lysine/ml, 10 µg of tryptophan/ml, 5 µg of uracil/ml) prewarmed to 30°C. Under these conditions, the strains used in this study completed spore formation within 12 h. For testing the effects of *ume6*, *sin3*, and *tpd3* mutations on *SMK1* expression, the FT5-derived strains described by Kadosh and Struhl were used (19). The effects of *ssn6* and *tup1* mutations were tested by using the strains described by Friesen et al. (14).

Plasmids. Plasmids, markers, and sources are detailed in Table 2. Mutations in the *SMK1* promoter were introduced by using PCR-based strategies and confirmed by dideoxynucleotide sequence analysis. The *SMK1-lacZ* reporter plasmid pMDP83 was constructed by replacing the *BglII-SalI* fragment of pLAK42 (codons 183 to 388 of *SMK1* and the 3' noncoding region) with a 3,072-bp *BglII-SalI* fragment containing *lacZ* generated by PCR using pMC1871 as a template. The 5' primer changed the first and last nucleotides of the *BamHI* site found in pMC1871 to A and T, respectively, to generate the *BglII* site, and the 3' primer included the *SalI* site in pMC1871. Thus, pMDP83 contains 219 bp of the *SMK1* promoter and 546 bp of the *SMK1* coding region fused in frame to *lacZ*. *SMK1* promoter deletion constructs pMDP86, -89, -126, -95, and -92 were generated by replacing the *KpnI-BglII* fragment of pMDP83 with *KpnI-BglII* fragments generated by PCR that lacked the indicated base pairs. The pseudo-URS^{1S} mutation removed bp -84 to -90, and the mse^S mutation changed the sequence TTTG at positions -79 to -76 to CCCA. Both of these mutations were introduced by using an *XhoI* site that was introduced into the *SMK1* promoter by a T→C A→G double substitution at positions -68 and -65, respectively (see Fig. 1). In control experiments the *XhoI* site reduced the level of expression twofold but had no effect on vegetative repression or the timing or pattern of *SMK1* expression. To test for mutant *smk1* promoter phenotypes, an integrating *SMK1* plasmid was constructed by subcloning the *KpnI-XhoI* fragment of *SMK1* (containing the entire *SMK1* gene and its promoter) into these sites in pRS406 to generate pMDP199. Promoter mutations were introduced into pMDP199 by replacing the *KpnI/BglII* fragment of pMDP199 with the *KpnI/BglII* fragments from the *lacZ* expression plasmids. The *SMK1* promoter in pLAK42 was replaced with a 200-bp PCR fragment of the *HOP1* promoter (measured from the ATG) to generate pJS1 by using the unique *BstXI* site located at codon 13 of the *SMK1* gene and the unique *KpnI* site at the plasmid/insert junction.

To construct *HOP1-lacZ* reporter promoters with the wild-type and mutant *SMK1* Abf1p binding sites, synthetic oligonucleotides containing the top and bottom strands of each site were annealed and ligated into the *BglII* site of plasmid pAV130 as described previously (16). pAV130 contains a *HOP1-lacZ* fusion gene in which there is an 8-bp substitution in the Abf1p-binding site of the *HOP1* promoter (39). pCC83 was derived from pAV130 by inserting a 19-mer double-stranded oligonucleotide containing the wild-type *HOP1* Abf1p-binding site into the *BglII* site 10 bp upstream of the disrupted site (16). pJX33 was derived from pAV130 by inserting the wild-type *SMK1* Abf1p-binding site 5'-g atcTATCGCGCGCGACGA-3' (top-strand oligonucleotide with lowercase bases used for cloning into the restriction site) in the same manner.

To construct *HOP1-lacZ* reporter promoters with the *SMK1* pseudo-URS^{1S} and MSE^S sites, synthetic oligonucleotides containing the top and bottom

TABLE 2. Plasmids

Plasmid	Markers	Source or reference
pRS316	<i>CEN URA3</i>	33
pLAK40	pRS316 + <i>SMK1</i>	20
YEp352	2 μ m <i>URA3</i>	18
pMC1871	<i>lacZ</i>	6
pLAK42	YEp352 + <i>SMK1</i>	20
pJS1	YEp352 + <i>HOP1/SMK1</i>	This study
pMDP83	YEp352 + <i>SMK1/lacZ</i> (-219)	This study
pMDP86	YEp352 + <i>SMK1/lacZ</i> (-181)	This study
pMDP89	YEp352 + <i>SMK1/lacZ</i> (-139)	This study
pMDP126	YEp352 + <i>SMK1/lacZ</i> (-124)	This study
pMDP95	YEp352 + <i>SMK1/lacZ</i> (-96)	This study
pMDP92	YEp352 + <i>SMK1/lacZ</i> (-71)	This study
pMDP119	pMDP89 + pseudo-urs1 ^S	This study
pMDP174	pMDP89 + mse ^S	This study
pMDP176	pMDP89 + pseudo-urs1 ^S mse ^S	This study
pRS406	<i>URA3</i>	33
pMDP149	pRS406 + <i>SMK1</i> (-124)	This study
pMDP152	pMDP149 + C118A	This study
pMDP199	pRS406 + <i>SMK1</i> (-139)	This study
pMDP183	pMDP199 + pseudo-urs1 ^S	This study
pMDP185	pMDP199 + mse ^S	This study
pMDP187	pMDP199 + pseudo-urs1 ^S + mse ^S	This study
pMDP113	pMDP89 + UAS ^H	This study
pAV124	2 μ m <i>URA3 HOP1/lacZ</i> - URS1 ^H	39
pCC51	pAV124 + URS1 ^H	15
pJX42	pAV124 + pseudo-URS ^S	This study
pJX43	pAV124 + MSE ^S	This study
pAV130	2 μ m <i>URA3 HOP1/lacZ</i> - UAS ^H	39
pCC83	pAV130 + UAS ^H	16
pJX33	pAV130 + UAS ^H	This study
pAV73	2 μ m <i>URA3 CYC1/lacZ</i>	39
pJX49	pAV73 + MSE ^S	This study

strands of each site were annealed and ligated into the *XhoI* site of plasmid pAV124, as described previously (15). pAV124 contains a 207-bp region of the *HOP1* promoter and the region coding for the first 115 residues of the protein fused in frame to the *lacZ* gene. Five base pairs within the *HOP1* URS1^H site in the promoter have been mutated to create an *XhoI* site into which the pseudo-URS1^S 5'-tcgacTAGAATTCGGCGCCACTAATc-3' or MSE^S 5'-tcgacCCACTAATTTGTGACACTTgc-3' (top strands) was cloned by using duplex oligonucleotides. pCC51 contains a wild-type *HOP1* URS1^H site that was inserted into the *XhoI* site of pAV124 and was used as a control for normal *HOP1* expression (15). The ability of MSE^S to repress the *CYC1* promoter was tested by subcloning the MSE^S oligonucleotides described above into the *XhoI* site of pAV73 (39) to generate pJX49.

Assays for spore wall assembly. For light microscopy, cells were fixed in ethanol and stained with the DNA-specific dye 4',6-diamidino-2-phenylindole (DAPI) (31). Samples were viewed and photographed as a wet mount under phase-contrast oil immersion optics by using a Nikon Optiphot equipped for epifluorescence. The procedure for the fluorescence assay has been described previously (42). Spore viability after heat shock (40 min at 55°C) or treatment with glucanase (1 h at 26°C) was determined as described by Briza et al. (5). Sensitivity of cells to ether exposure (3 min with constant gentle rocking) was assayed according to the method of Dawes and Hardie (8).

EMSA. For electrophoretic mobility shift assays (EMSA), oligonucleotides were end labeled with [γ -³²P]ATP by using polynucleotide kinase and were purified by Nensorb columns (NEN). The oligonucleotides were made double-stranded by mixing with a threefold excess of the matching strand, incubating at 90°C for 20 min, and slowly cooling to 25°C overnight. Binding reactions were carried out in a solution containing 10 mM Tris-HCl (pH 7.5), 40 mM NaCl, 4 mM MgCl₂, 6% (wt/vol) glycerol, 10 μ g of sonicated salmon sperm DNA/ml, and ³²P-labeled oligonucleotide (10,000 cpm) in a total volume of 20 μ l at room temperature for 20 min. Crude extracts and partially purified Abf1p were prepared as previously described (16). Protein dilutions were made in a solution containing 20 mM Tris-HCl (pH 8), 50 mM NaCl, 1 mM EDTA, 1 mg of bovine serum albumin (BSA)/ml, 5 mM β -mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Samples were analyzed on a 6% polyacrylamide gel (run in 0.5 \times Tris-borate-EDTA [TBE] buffer for 60 min at 250 V). Gels were dried after electrophoresis, exposed to a phosphor screen, scanned, and quantitated on a Model 425E Molecular Dynamics PhosphorImager.

Miscellaneous methods. Preparation of total RNA and Northern blot hybridization analysis were carried out as previously described (20). DNA probes from the coding regions of the indicated genes were isolated from preparative agarose gels, radiolabeled with ³²P by random priming (2), and used in hybridization analysis at 10⁶ dpm/ml. The DNA hybridization probe used for *SMK1* consisted of the 0.8-kb *SylI* fragment of its coding region. This probe does not detect any transcripts that may be produced at the *smk1::LEU2* locus, since it is internal to the boundaries of this deletion/insertion mutation (20). The DNA probe used for *SPO12* was the 0.4-kb *EcoRI-BamHI* fragment of its coding region, and that for *HOP1* was the *BamHI-SacI* restriction fragment of its coding region. The PC4/2 control probe for RNA loading has been described previously (22, 38). For β -galactosidase assays, cell pellets were frozen at -80°C and subsequently resuspended with an equal volume of glass beads and breaking buffer (100 mM Tris-HCl, 1 mM dithiothreitol, 20% glycerol, 1 mM PMSF). Cell lysis was achieved by vortexing for seven 1-min intervals separated by cooling on ice. Samples were centrifuged for 10 min at 13,000 \times g and stored at -80°C. Protein concentrations were determined by the method of Bradford with BSA as a standard, and β -galactosidase was assayed by using *o*-nitrophenyl- β -D-galactopyranoside (ONPG). Oligonucleotides were synthesized on an Applied Biosystems 392-5 DNA synthesizer, and when appropriate, purified by C₁₈ reverse-phase high-performance liquid chromatography (HPLC).

RESULTS

The *SMK1* promoter contains distinct activating and repressing transcriptional control elements. We have previously shown that *SMK1* is expressed as a middle sporulation-specific gene (20). To assay the *SMK1* promoter we constructed pMDP83, which contains 219 bp of the promoter and 546 bp of the coding region fused in frame to *lacZ* (Table 2). The SK1 strain background (LNY150; Table 1) was used for these studies because it can be induced to undergo sporulation in a relatively rapid and synchronous manner. pMDP83 diploid transformants were sporulated, cells were harvested at 2-h intervals, and β -galactosidase activities were assayed. Figure 1A compares the *SMK1* mRNA expression profile determined by Northern blot hybridization analysis (20) to the *lacZ* expression profile. The levels of β -galactosidase and endogenous *SMK1* mRNA were low in vegetative cells and remained low until around the time of completion of meiosis II (6 h). Both β -galactosidase and *SMK1* mRNA levels peaked around the time when the major steps of spore wall morphogenesis were occurring (8 h). β -Galactosidase levels remained high and decayed relatively slowly thereafter. In contrast, *SMK1* mRNA levels decayed rapidly. These results demonstrate that 219 bp of the *SMK1* promoter are sufficient for the regulated expression of *SMK1*. In addition, these results show that *SMK1-lacZ* fusions can be used to assay the onset and magnitude of *SMK1* transcription.

In order to further define the *cis*-acting sequences that regulate *SMK1* transcription, a series of deletion constructs was generated from pMDP83, and the expression patterns were measured in vegetative cells and cells that had been sporulated for 10 h. As can be seen in Fig. 1B, successive deletions of the *SMK1* promoter from -219 through -181, -139, and -124 had only modest (less than twofold) effects on the level of expression seen in both vegetative and sporulation cultures. In contrast, removal of an additional 28 bp (from -124 to -96) reduced the level of sporulation-specific expression over 200-fold while leaving the low level of vegetative expression unaffected. Thus the region between -124 and -96 is required for sporulation-specific expression. We refer to this upstream activating site in the *SMK1* promoter as UAS^S. Removal of an additional 25 bp resulted in a 50-fold increase in vegetative expression. Thus, the region between -96 and -71 is required for repression of *SMK1* in vegetative cells. Expression of the derepressed -71 deletion construct in vegetative cells appears to require the remaining *SMK1* promoter sequences, since substitution mutations in this interval reduced promoter activity (data not shown). However, the -71 construct does not

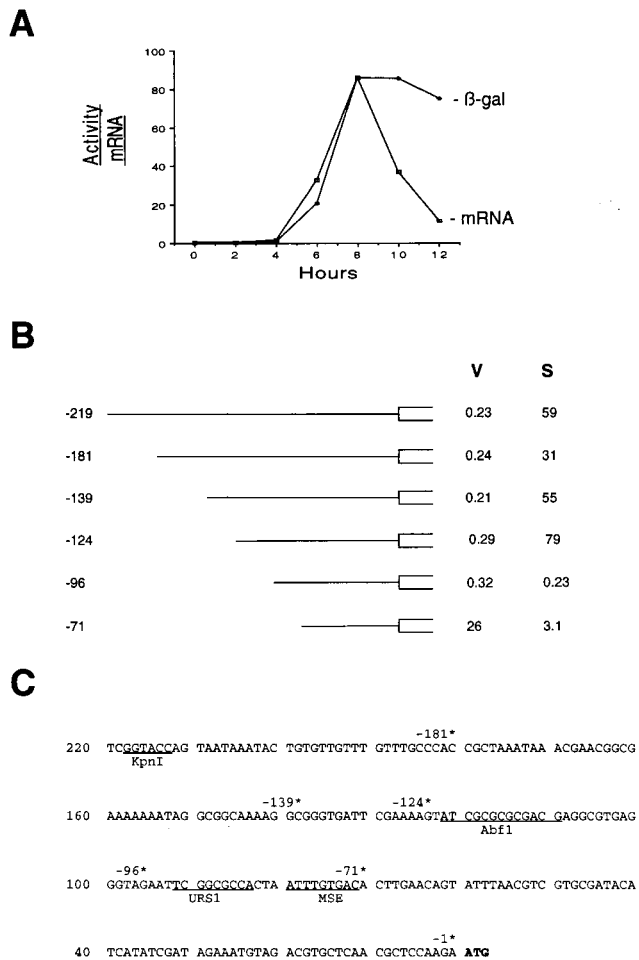


FIG. 1. Analysis of the *SMK1* promoter using *SMK1-lacZ* expression plasmids. (A) Comparison of relative *SMK1* mRNA levels and *SMK1-lacZ* enzyme activities during sporulation. β -Galactosidase activity levels were measured at 2-h intervals postinduction by using strain LNY150 transformed with pMDP83. Activity is expressed as units of β -galactosidase per milligram of total protein. *SMK1* mRNA levels were quantitated by Northern blot hybridization (20). (B) Deletion analysis of the *SMK1* promoter. The indicated deletions were generated from the -219 promoter construct pMDP83 (see Table 2). β -Galactosidase activities were determined in vegetative cells (V) and 10 h postinduction (S) and are averages from at least two separate determinations. In each case the deletion point indicates the number of base pairs between the initiator ATG and the C that is the most 3' base of the *KpnI* restriction endonuclease site of the parental plasmid. (C) Nucleotide sequence of the *SMK1* promoter. Deletion endpoints diagrammed in panel B are indicated (*), and the initiator ATG of *SMK1* is boldfaced. Consensus elements referred to in the text and the *KpnI* restriction enzyme site used in the construction of pMDP83 are underlined.

appear to direct any transcription during sporulation, because the low level of β -galactosidase in sporulated transformants can be accounted for by the stability of vegetative pools of the enzyme (see Fig. 1A, and compare Fig. 4 and 5).

Abf1p interacts with UAS^S. The Abf1p DNA-binding protein is required for the regulated expression of many diverse genes. It appears to play an important role in sporulation-specific gene expression, and Abf1p-binding sites are found in a high percentage of sporulation-specific promoters (16, 27, 28). High-affinity Abf1p binding in vitro has previously been shown to require the 13-bp sequence RTCRYBNNNACG (where Y is pyrimidine, R is purine, B is G, C, or T, and N is any base) (10, 12, 13). Within the 28-bp region that is required for UAS^S activity, an element that conforms to this consensus

is found starting at position -122 (Fig. 1C). We tested whether Abf1p would bind to this site in vitro. As a positive control, binding to the characterized Abf1p-binding site, which is required for transcriptional activation of the early sporulation *HOP1* promoter (UAS^H), was also assayed. As shown in Fig. 2A, Abf1p specifically bound to a 13-bp oligonucleotide duplex from UAS^S. Furthermore, substitution of the CG base pair corresponding to position -118 with an AT base pair (referred to below as the -118A substitution) eliminated detectable Abf1p binding, consistent with previously described binding requirements. In contrast, substitution of the GC base pair at position -115, which is found in the degenerate core of the Abf1p-binding site, with an AT base pair had no effect on binding. Although Abf1p bound to the UAS^S site, it bound with lower affinity than to the UAS^H site. We were therefore interested to determine if the *SMK1* Abf1p site would be functional in the context of the *HOP1* promoter and vice versa. It has previously been shown that removal of UAS^H from the *HOP1* promoter reduces the early sporulation-specific expression peak (Fig. 2B) (39). Introduction of the *SMK1* Abf1p-binding site into this mutant *HOP1* promoter restored activity. Furthermore, the early sporulation timing of the *HOP1* promoter containing the *SMK1* Abf1p site was indistinguishable from the timing of the *HOP1* promoter reconstituted with the *HOP1* Abf1p site. Similarly, replacement of the Abf1p-binding site in the *SMK1* promoter with the Abf1p-binding site from *HOP1* had no detectable effect on the timing or level of *SMK1* expression (Fig. 2B). These results show that the Abf1p sites from these promoters are functionally interchangeable. Thus, both biochemical and functional assays indicate that Abf1p can activate the *SMK1* promoter. These results also suggest that different Abf1p-binding sites do not play a direct role in setting the timing of these temporally distinct classes of promoters.

We were interested in identifying mutations that reduce *SMK1* promoter activity to different extents in order to test the functional consequences of reducing *SMK1* expression by defined thresholds. Toward this goal, a series of substitution mutations were introduced in UAS^S and their effects on the expression of *SMK1-lacZ* were assayed. As shown in Fig. 3A, substitutions that were predicted to reduce Abf1p binding strongly, such as -118A and -117A (10, 13, 16), strongly reduced the level of expression. Also, as expected, mutations in bp -116, -115, -114, and -113, which correspond to the degenerate core of the site, caused only modest transcriptional effects. Surprisingly however, the double -110A -111A substitution, which changes two positions that are strongly conserved among Abf1p-binding sites, reduced the level of *SMK1-lacZ* expression only modestly. The effects of substitutions in positions that are adjacent to the consensus Abf1p-binding site (-108A, -107A, -106A, and -105A) were also tested. Some of these mutations strongly reduced *SMK1* promoter activity. The most severe of these mutations (-106A) also strongly reduced the activity of the *SMK1* promoter containing the Abf1p-binding site from *HOP1*. Surprisingly, we found that the transcriptional defect of the -106A substitution was partially suppressed by mutations in the center of the site (see the -115A -106A, -114A -106A, and -113A -106A double substitutions).

Mutational analysis of Abf1p-binding sites in the promoters of other genes has shown that in general, substitutions that eliminate UAS activity in vivo also eliminate Abf1p binding in vitro. We therefore tested several of the UAS^S point mutations for their effects on Abf1p-binding affinity (Fig. 3A). Based on the predicted consensus requirements of RTCRYBNNNACG for Abf1p recognition, all of the point mutations that were predicted to reduce Abf1p binding did so (10, 13, 16).

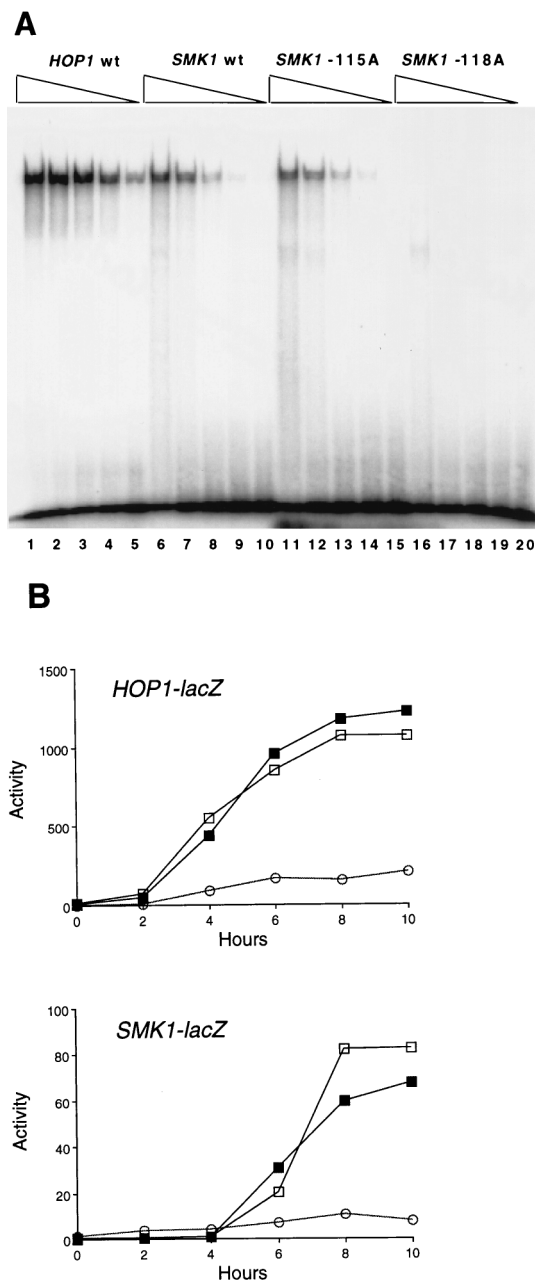


FIG. 2. Comparison of UAS^S and UAS^H sites. (A) EMSA of Abf1p binding. Partially purified Abf1p was serially diluted fivefold and used in binding reactions to radiolabeled 13-bp oligonucleotide duplexes containing the *HOP1* Abf1p-binding site (lanes 1 to 5), the wild-type *SMK1* Abf1p-binding site (lanes 6 to 10), and the mutant *SMK1* Abf1p sites containing the -115A (lanes 11 to 15) and -118A (lanes 16 to 20) substitutions. (B) Analysis of *HOP1* and *SMK1* promoters containing heterologous Abf1p-binding sites. The *HOP1* promoter (upper panel) lacking its Abf1p-binding element (open circles), reconstituted with the *HOP1* binding site (open squares), or reconstituted with the *SMK1* Abf1 site (closed squares) was tested by using the *HOP1-lacZ* plasmid pAV130, pCC83, or pJX33, respectively. The *SMK1* promoter (lower panel) lacking a functional Abf1p-binding site (-118A mutant; open circles), containing its normal Abf1p-binding site (open squares), or reconstituted with the Abf1p-binding site from *HOP1* (closed squares) was tested for expression of β -galactosidase by using the *SMK1-lacZ* plasmid pMDP126-118A, pMDP126, or pMDP113, respectively, in yeast strain LNY150 as described in Materials and Methods. The experiment was performed independently three times with similar results.

Interestingly, the -110A -111A substitution, which had a relatively modest effect on transcriptional activation in vivo, caused a large reduction in DNA-binding affinity. A similar difference in DNA-binding affinity and transcriptional activation has been observed in the mutational analysis of the Abf1p-binding site in the *HOP1* promoter (16). The -105A, -106A, and -107A substitutions, which are outside the Abf1p-binding consensus, had no effect on Abf1p-binding affinity in vitro. Our results suggest that the Abf1p-UAS^S interaction is complex and that recognition by the protein may be influenced by additional factors in vivo (see Discussion).

SMK1 promoter sequences from *SMK1-lacZ* plasmids whose expression was reduced by varying extents were used to reconstitute the *SMK1* gene in an integrating plasmid vector. The resulting *SMK1* promoter mutants were integrated into the chromosome at the *ura3* locus, and *SMK1* mRNA levels were determined by Northern blot hybridization. The expression of the middle sporulation-specific *SPO12* gene was determined in the same samples as a normalization control. Quantitation of the hybridization signals showed that mutations that reduced the level of *SMK1-lacZ* expression also reduced expression of *SMK1* in the chromosomal context. This series of strains proved useful for assessing the functional significance of the level of *SMK1* expression (see below).

The MSE is required for vegetative repression as well as sporulation-specific activation of the *SMK1* promoter. Within the -96-to--71 deletion interval that derepressed vegetative *SMK1* expression, there are two sequence elements of interest. The first element is similar to the previously described MSE. The MSEs of the *SPS4* and *SPR3* middle genes have been shown to activate middle expression in heterologous promoter constructs (17, 27). A consensus MSE sequence (GNCRC AAAA/T) (28) is found at positions -72 to -80 of the *SMK1* promoter, and we refer to it hereafter as MSE^S. The second DNA element of interest is similar to the consensus URS1 element (TCGGCGGCT) and is found at positions -84 to -92 of the *SMK1* promoter (Fig. 1). URS1 sites have been shown to interact with Ume6p, and in the context of early sporulation promoters, they function to repress expression during vegetative growth (29, 37, 39). URS1 sites have also been shown to function as meiosis-specific activator elements (3, 30). The transcriptional regulatory properties of the URS1 consensus in *SMK1* differs in certain respects from the URS1s that have been characterized in early sporulation-specific genes (see below). We therefore refer to this element in *SMK1* as pseudo-URS1^S (or Ψ -URS1^S) hereafter.

To address the significance of MSE^S and pseudo-URS1^S in the *SMK1* promoter, mutations were constructed in these sites in the context of the *SMK1-lacZ* reporter gene. Promoter mutants and wild-type controls were synchronously sporulated, and β -galactosidase activities were determined at 2-h intervals. An almost complete removal of pseudo-URS1^S by deletion of bp -84 to -90 (pMDP119) had no effect on the timing of expression (compare Fig. 4A and B). In contrast, a 4-bp substitution in MSE^S (bp -73 to -76, which make up the core of MSE similarity; pMDP174) significantly derepressed the promoter in vegetative cells, and high levels of β -galactosidase were detected at all time points tested (Fig. 4C). In the double pseudo-urs1^S mse^S mutant promoter (pMDP176), the level of β -galactosidase in vegetative cells was indistinguishable from that in the single mse^S mutant. However the double pseudo-urs1^S mse^S mutant showed lower β -galactosidase activities than the single mse^S mutant during late sporulation (compare Fig. 4C and D). These data show that MSE^S is a strong transcriptional repressor site in vegetative cells and that it is also required for the sporulation-specific peak of *SMK1* expression.

A

Abf1 con	R T C R Y B N N N N A C G											lacZ-V	lacZ-S	Binding										
	-122	-120	-118	-116	-114	-112	-110	-108	-106															
WT	G	T	A	T	C	G	C	G	C	G	C	G	A	C	G	A	G	G	C	G	T	0.3	100	100
-110A, -111A	_____ A A _____											0.8	34	<1										
-118A	_____ A _____											0.4	1.0	<1										
-117A	_____ A _____											0.4	3.8	10										
-116A	_____ A _____											0.2	54											
-115A	_____ A _____											0.6	39	100										
-114A	_____ A _____											0.9	152											
-113A	_____ A _____											0.6	34	40										
-108A	_____ A _____											0.2	91	200										
-107A	_____ A _____											0.3	11	100										
-106A	_____ A _____											0.3	2.0	100										
-105A	_____ A _____											0.3	25											
-115A, -106A	_____ A _____ A _____											0.1	8.2											
-114A, -106A	_____ A _____ A _____											0.1	35											
-113A, -106A	_____ A _____ A _____ A _____											0.2	10											
HOP1-Abf1	_____ A - T T C A C _____											0.3	55	2500										
HOP1-Abf1, -106A	_____ A - T T C A C _____ A _____											0.3	0.9	2500										

FIG. 3. Mutational analysis of UAS^S. (A) Effects of UAS^S mutations on expression of *SMK1* and binding to Abf1p. Strains with the indicated mutations in the -124 promoter *SMK1-lacZ* plasmid pMDP126 were assayed for β -galactosidase activity in vegetative (V) and sporulated cultures at 10 h postinduction (S). β -Galactosidase activities are averages from at least two separate comparisons to the wild-type plasmid (pMDP126). Both vegetative and sporulation values are shown as percentages of the wild-type sporulation value (79 ± 8 U/mg of total protein). Relative complex formation between oligonucleotide duplexes containing the indicated mutation and partially purified Abf1p (Binding) was quantitated from a single titration curve performed as shown in Fig. 2. The mutations are referred to by the numbers at the left. Sequence requirements for Abf1p binding (Abf1 con) are shown above for comparison. The *HOP1*-Abf1 mutation contains a 6-bp substitution to generate the *HOP1* Abf1p-binding site, which reads 5'-ATCACTCACACG-3'. (B) Hybridization analysis of UAS^S promoter mutants. Indicated promoter mutations in the context of the wild-type *SMK1* gene were integrated at the *ura3* locus, and RNA was prepared from vegetative (V) or sporulated (S) cultures 10 h postinduction. Hybridization analysis was performed on total RNA by Northern blot analysis using an *SMK1*-specific probe. The same filter was subsequently hybridized with the middle sporulation *SPO12*-specific probe as a normalization control. The *SMK1*-specific hybridization signal in sporulating samples (normalized for *SPO12* hybridization) was reduced to 51, 48, 23, and 17% of the wild-type signal in the -116A, -115A, -115A -106A, -117A, and -118A mutant strains, respectively.

B

These data also suggest that pseudo-URS1^S can function in a positive fashion during sporulation, but this is only apparent in the *mse*^S mutant promoter background.

To directly assay the effects of the promoter mutations on mRNA levels, the mutant promoters were used to replace the promoter in *SMK1* on an integrating plasmid vector (to generate pMDP199, pMDP183, pMDP185, and pMDP187 [Table 2]). Wild-type and mutant promoters were integrated at the *ura3* locus in a *smk1::LEU2* strain, and diploid transformants were synchronously sporulated. Cultures were harvested at 2-h intervals, and RNA was prepared and analyzed by Northern blot hybridization with a *SMK1* probe specific for the integrated promoter mutant (see Materials and Methods). Northern hybridization analysis was also carried out by using representative early, middle, and constitutively expressed gene sequences as controls. As can be seen in Fig. 5, the pseudo-urs1 mutation modestly increased the level of *SMK1* expression (two- to threefold) at all time points, but it had no detectable effect on the overall timing of expression, consistent with the effect of this mutation in the *lacZ* expression assay system. In the *mse*^S mutant samples, *SMK1* mRNA levels were derepressed in vegetative samples and the middle peak of expression was undetectable. This result directly confirms that MSE^S is required for transcriptional repression in vegetative cells as well as for the peak of sporulation-specific expression. Interestingly, a new peak of expression that is significantly earlier than that seen in the wild-type control is observed in the *mse*^S mutant. This early peak is absent in the *mse*^S pseudo-urs1^S double mutant. These results show that in an *mse*^S mutant promoter, pseudo-URS1^S can function positively during early meiotic development, consistent with the ability of URS1 elements to activate the expression of a variety of early sporu-

lation promoters (4). Furthermore, the level of vegetative derepression in the double pseudo-urs1^S *mse*^S mutant is indistinguishable from that seen in the single *mse*^S mutant. This result indicates that pseudo-URS1^S is unable to repress the vegetative expression of *SMK1*.

To further characterize the activities of the MSE^S and pseudo-URS1^S elements, these sites were used to replace the Ume6p-binding site (URS1^H) in the early sporulation *HOP1* promoter. It has previously been shown that a mutation in the URS1^H site allows expression of *HOP1* in vegetative cells and also leads to low-level constitutive expression throughout sporulation (39). Wild-type regulation can be restored by inserting the URS1^H site back into this mutant promoter (Fig. 6) and (15). Pseudo-URS1^S inserted into the mutant promoter repressed *HOP1* expression to an extent similar to that seen in the URS1^H-containing recombinant but only weakly activated *HOP1* expression during sporulation. The URS1^H in the *HOP1* promoter was also replaced with a 22-bp fragment containing MSE^S. Strikingly, MSE^S fully repressed vegetative expression of the *HOP1* promoter and converted this gene from an early to a tightly controlled middle sporulation-specific gene whose expression pattern is indistinguishable from that with the wild-type *SMK1* promoter. Thus, MSE^S can set the developmental timing of middle gene activation and also repress expression in vegetative and early meiotic cells.

The ability of MSE^S to repress vegetative expression was also tested by using the *CYCI* promoter, which normally is expressed in vegetative cells and which, unlike the *SMK1* and *HOP1* promoters, does not contain an Abf1p-binding consensus element. For these experiments the MSE^S was inserted into the *CYCI-lacZ* fusion plasmid pAV73 to generate pJX49. The levels of β -galactosidase in pAV73 and pJX49 transformants

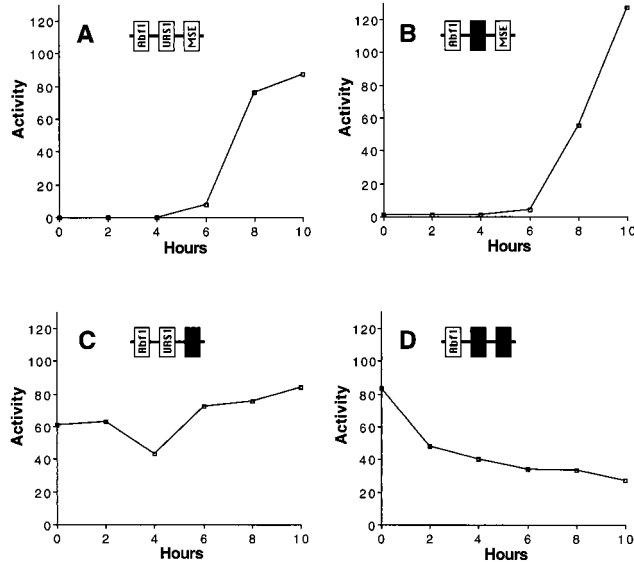


FIG. 4. Expression of β -galactosidase by mse^S and pseudo- $urs1^S$ *SMK1* promoter plasmids during sporulation. Yeast strain LNY150 transformed with a plasmid expressing β -galactosidase under the control of the wild-type (A), pseudo- $urs1^S$ (B), mse^S (C), or pseudo- $urs1^S mse^S$ (D) -139 promoter (pMDP89, pMDP119, pMDP174, or pMDP176, respectively) was synchronously sporulated, and β -galactosidase levels (expressed as units per milligram of total protein) were determined at 2-h intervals. The experiment was performed at least twice for each promoter with similar results.

were 102 ± 5.7 and 5.6 ± 0.7 U, respectively. Thus, MSE^S can repress the expression of heterologous promoters that are not normally expressed during sporulation, as well as those that are sporulation specific.

The MSE^S and $URS1$ elements appear to function in a similar fashion in several key respects: both can repress expression during vegetative growth, and both activate expression during sporulation (but during different temporal windows).

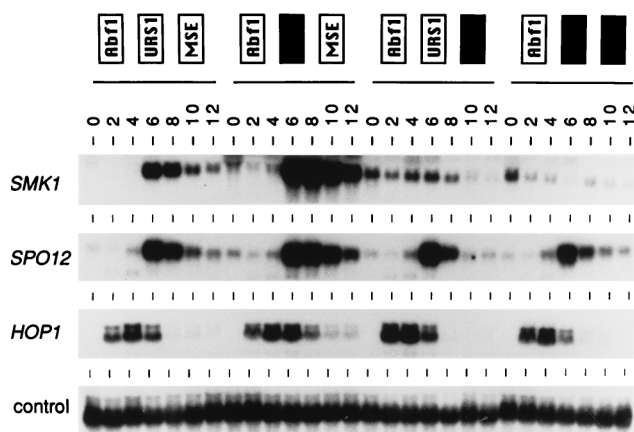


FIG. 5. Expression of *SMK1* mRNA by mse^S and $urs1^S$ promoter mutants during sporulation. Diploid yeast containing a single integrated copy of wild-type *SMK1* coding information under the control of the indicated mutant promoter was transferred to sporulation medium, and total RNA was prepared from cells harvested at the indicated times and assayed by Northern blot hybridization. The indicated *smk1* promoter mutants were generated using the following integrating plasmids, from left to right: wild-type (pMDP199), pseudo- $urs1^S$ (pMDP183), mse^S (pMDP185), and pseudo- $urs1^S mse^S$ (pMDP187) (see Table 2). The same filter was probed with sequences specific for the *SPO12* middle gene, the *HOP1* early gene, and the *pC4/2* constitutive expression control.

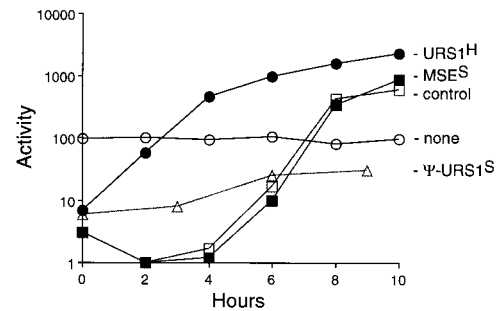








FIG. 6. Reconstitution of the *HOP1* promoter with MSE^S and $URS1^S$. Yeast cells of strain LNY273 transformed with *HOP1* promoter-*lacZ* plasmids containing the indicated *cis*-regulatory elements were synchronously sporulated, and β -galactosidase levels were measured. pAV124 contains a 5-bp substitution that destroys the naturally occurring $URS1^{H1}$ and introduces an *XhoI* site (open circles); pCC51 contains $URS1^{H1}$ (closed circles); pJX42 contains pseudo- $URS1^S$ (open triangles); and pJX43 contains MSE^S (closed squares) in the *XhoI* site of pAV124. The control is pMDP89 (open squares), which is the *SMK1* promoter-*lacZ* plasmid. Data are averages from three determinations.

$URS1$ -dependent repression requires the *UME6*, *SIN3*, and *RPD3* gene products, which encode a DNA-binding protein, a hypothesized coadapter, and a histone deacetylase, respectively (19, 29, 37, 40). *DIT1* and *DIT2* are mid-late sporulation-specific genes that are regulated by the *SSN6* and *TUP1* transcriptional corepressor complex (14). These observations prompted us to determine whether the *UME6*, *SIN3*, *RPD3*, *SSN6*, or *TUP1* gene product is required for the vegetative repression of the *SMK1* promoter. The wild-type *SMK1* promoter/*lacZ* plasmid pMDP89 was transformed into mutant and wild-type control strains, and the level of β -galactosidase expression was determined in vegetative cultures (see Materials and Methods). For *ume6*, *sin3*, *rdp3*, *ssn6*, and *tup1* mutants, the β -galactosidase levels were 1.2-, 1.5-, 1.3-, 0.4-, and 3.0-fold that seen in the corresponding congenic control strain (values are averages of two independent experiments carried out in triplicate). These small differences could easily be due to indirect effects. Similar results were obtained with the pseudo- $urs1^S$ promoter mutant (plasmid pMDP-119). These data show that MSE repression functions through a pathway distinct from the *SIN3/RPD3* and *SSN6/TUP1* pathways.

***SMK1* function during spore morphogenesis is not contingent on its expression as a sporulation-specific middle gene.** To assess the functional significance of the tight transcriptional regulation of the *SMK1* MAP kinase, we examined the phenotypes of the collection of integrated *SMK1* promoter mutants that directed the expression of wild-type Smk1p. *smk1* promoter mutants were sporulated for 36 h, and the end stage products were assayed for the completion of meiosis by DAPI staining, for spore wall morphogenesis by phase-contrast microscopy, and for acquisition of resistance to glusulase, heat shock, and ether (Table 3). The $-118A$ promoter mutant that expressed *SMK1* mRNA as a middle gene, but to only 17% of the level seen in the wild-type promoter control (Fig. 3B), formed spores with near-wild-type efficiency, as judged by phase-contrast microscopy. Based on these results, and taking into account modest differences between the expression of *SMK1* from its normal chromosomal locus and from the *ura3::SMK1* locus (data not shown) and the fact that the *smk1* promoter alleles in these experiments were present in only a single copy, we estimate that spore wall formation can occur when the level of *SMK1* mRNA is reduced to 10% of the level seen in wild-type *SMK1* homozygotes. However, while spore wall formation did occur in these backgrounds, the resistance

TABLE 3. Sporulation phenotypes of *smk1* promoter mutants^a

PROMOTER	SPORES	GLUSULASE	ETHER	HEAT SHOCK
 (wild-type)	9.2×10^{-1}	7.0×10^{-1}	7.1×10^{-1}	3.4×10^{-1}
 (ψ -urs1)	9.3×10^{-1}	1.1×10^0	1.6×10^0	7×10^{-1}
 (-118A)	9.5×10^{-1}	7.0×10^{-1}	9.2×10^{-1}	3×10^{-4}
 (<i>mse</i> ^S)	6.7×10^{-1}	1.5×10^{-1}	3.5×10^{-2}	2.3×10^{-5}
 (ψ -urs1 <i>mse</i> ^S)	1.0×10^{-2}	4.6×10^{-3}	2.5×10^{-3}	1.8×10^{-5}
 (<i>smk1</i> - Δ)	$< 1.0 \times 10^{-3}$	3.0×10^{-3}	6×10^{-4}	8×10^{-6}

^a Diploid *smk1*- Δ strains containing a single copy of the indicated *smk1* promoter constructs were sporulated, and the fraction of cells that formed spores, as assayed by phase-contrast microscopy ($n > 200$), and fractional resistance to glusulase, ether, or heat shock treatment were determined as described in Materials and Methods. Completion of meiosis was greater than 84% in all samples, as monitored by fluorescence microscopy of DAPI-stained samples. The *smk1* promoter mutants were generated by using the following integrating plasmids, from top to bottom: wild-type (pMDP149), pseudo-urs1^S (pMDP183), -118A substitution (pMDP152), *mse*^S (pMDP185), and pseudo-urs1^S *mse*^S (pMDP187). These strains are identical to those assayed by Northern blot analysis, for which results are shown in Fig. 3B and 5. Values are averages from at least two separate experiments.

phenotypes were not like the wild-type phenotypes. In other experiments, we have shown that *SMK1* is required to complete multiple steps in spore morphogenesis (20, 41, 42). In addition, we have isolated a series of *smk1* missense hypomorphic alleles that fail to complete distinct steps in sporulation (41). Some of these mutants are defective only in their resistance to heat shock, some are defective in heat shock and ether resistance, and others are defective in all of the resistance phenotypes tested. The -118A *SMK1* promoter mutant was resistant to glusulase and ether treatments. However, this mutant is over 10^3 -fold more sensitive to heat shock than the control strain (Table 3). The -117A mutant strain, which expresses levels of *SMK1* mRNA only slightly higher than those of the -118A mutant (Fig. 3B), is indistinguishable from the wild type for all resistance phenotypes (data not shown). These results demonstrate that it is possible to reduce the magnitude of *SMK1* expression by roughly 90% and still generate wild-type spores, but when expression is reduced below this level, there are threshold requirements for the acquisition of different resistance phenotypes. These data suggest that acquisition of heat shock resistance requires higher levels of *SMK1* expression than either spore morphogenesis or the acquisition of glusulase or ether resistance.

The phenotypes of mutants that express *SMK1* at inappropriate times were also characterized. In this respect it is worth noting that the derepressed *smk1-mse* promoter mutant grew at wild-type rates as a haploid or diploid. We have also expressed *SMK1* using the strong *GAL1,10* promoter and similarly found that this strain grows well in galactose or glucose media (data not shown). Thus, vegetative expression of *SMK1* mRNA does not appear to be deleterious to vegetative growth. The derepressed *smk1-mse* mutant undergoes meiosis and gen-

erates spore walls with near-wild-type efficiency. Thus, vegetative expression and early misexpression of *SMK1* do not adversely affect the execution of these events. While the glusulase resistance phenotype is only modestly reduced in the *smk1-mse* mutant (roughly fivefold compared to that in the wild type), the ether resistance phenotype is reduced by more than 2 orders of magnitude and heat shock resistance is reduced by more than over 4 orders of magnitude. These phenotypes are all recessive (strains are completely resistant when heterozygous over wild type). This indicates that these sensitivity phenotypes are not the consequence of Smk1p misexpression but of reduced Smk1p levels. The double *smk1-mse pseudo-urs1* promoter mutant, which accumulates the lowest level of *SMK1* mRNA during middle sporulation (Fig. 5), failed to form spores as assayed by phase-contrast microscopy and was fully sensitive to glusulase, heat shock, and ether.

The observation that the *smk1-mse* mutant forms spore walls while the *smk1-mse pseudo-urs1* mutant does not, with the only difference between these promoters being the peak of early expression, suggests that *SMK1* expressed as an early gene can complement certain aspects of spore wall morphogenesis. To determine whether *SMK1* expressed as an early gene could fully complement all of the sporulation phenotypes, the *SMK1* coding region was cloned downstream of the early *HOP1* promoter in a 2 μ m high-copy-number vector. As shown in Table 4, expression of *SMK1* as an early gene fully complements all of the sporulation defects of a *smk1*- Δ strain. These results strongly support the idea that the tight timing of *SMK1* transcription functions solely to ensure that sufficient levels of the gene product are present during the developmental interval when it is needed. Thus, the function of the *SMK1* pathway is

TABLE 4. Sporulation phenotypes of strains expressing *SMK1* as an early gene^a

Plasmid	Fraction of cells forming spores	Fraction of cells with resistance to:		
		Glusulase	Ether	Heat shock
2 μ m	0.00	<1.0 $\times 10^{-3}$	<1.0 $\times 10^{-3}$	<1.0 $\times 10^{-3}$
2 μ m <i>SMK1</i>	0.63	1.0	0.31	0.72
2 μ m <i>HOP1/SMK1</i>	0.53	1.2	0.85	0.45

^aThe *smk1*- Δ yeast strain MDPY10, harboring a 2 μ m control plasmid (YEp352), a 2 μ m plasmid containing the entire *SMK1* gene (PLAK40), and a 2 μ m plasmid containing the *HOP1* promoter directing the expression of *SMK1* (pJS1) were sporulated, and the fractions of cells that completed sporulation or that were resistant to the indicated treatment were assayed as described for Table 3 and in Materials and Methods. Values are averages from at least two separate experiments.

not contingent on the graded accumulation of *SMK1* mRNA during middle sporulation.

DISCUSSION

This study demonstrates that the transcriptional regulation of *SMK1* as a middle sporulation-specific gene requires two *cis*-acting DNA elements, UAS^S and MSE^S. UAS^S functions to activate expression of *SMK1*. MSE^S plays a dual role in regulating *SMK1* expression. It is required for activation of *SMK1* as a middle sporulation gene and also for repression during vegetative growth and early sporulation. Promoter mutants that express *SMK1* during vegetative growth or early in meiosis show no overt phenotypes and are still able to complete spore wall morphogenesis as long as sufficient levels of *SMK1* mRNA are present during the middle sporulation period. Mutants that reduce the level of *SMK1* expression during middle sporulation to different extents show distinct sporulation defects. The implications of these results for transcriptional control mechanisms and the regulation of development by MAP kinases are discussed below.

Abf1 and transcriptional activation by UAS^S. Our results demonstrate that UAS^S specifically interacts with the Abf1p transcription factor in vitro. It has previously been shown that an Abf1p-binding element activates expression of the early sporulation-specific *HOP1* and middle sporulation-specific *SPR3* promoters. Furthermore, many other sporulation-specific promoters contain Abf1p-binding sites (16, 27). We have shown that the Abf1p-binding sites from the early *HOP1* and middle *SMK1* promoters are functionally interchangeable. In addition, the early *HOP1* promoter can be converted to a middle gene when its URS1 is replaced with MSE^S. These results indicate that Abf1p-binding sites have a similar role in early and middle sporulation promoters and that they function to regulate the magnitude of transcription and not the timing.

The sequence requirements for Abf1p binding in vitro and transcriptional activation in vivo have been characterized in several different promoters, and in general they are closely correlated (9, 11, 16, 25). While it is clear that an Abf1p-binding element can function to activate *SMK1* expression, the sequence requirements for UAS^S function are more complex than would be predicted for Abf1p binding alone. There are two possible explanations for these results. One possibility is that there exists a transcription factor whose sequence requirements for binding are similar but not identical to those for Abf1p. The second possibility is that there may be base pairs in UAS^S which are not essential for sequence-specific recognition by Abf1p but are required for its function in vivo. For example, it has been shown that Abf1p causes a significant bend in DNA when it binds to its site and that it makes a number of phos-

phate contacts with the DNA outside the conserved recognition sequence (23, 24). DNA bending by Abf1p may be important to the mechanism by which it promotes transcription. It is possible that the mutant DNA sites that we have constructed have altered bending properties that affect the ability of Abf1p to promote transcription. Alternatively, the transcriptional activation properties of the collection of UAS^S mutants may reflect the presence of a factor that functions positively in concert with Abf1p. The suppression of the transcriptional defect of a mutation (-106A) that was outside the Abf1p-binding site consensus by substitutions within the degenerate core (-116A, -115A, and -114A) is consistent with this possibility. Abf1p is a multifunctional protein that is required for a variety of cellular processes which, in addition to transcriptional activation, include silencing of chromatin and DNA replication (11). Alterations in the properties of Abf1p-DNA complexes by accessory factors could provide additional levels of regulation for this general transcription factor. The further characterization of UAS^S may provide additional insight into the role of this ubiquitous factor.

MSE-dependent transcriptional activation and repression.

The MSE has previously been shown by others to be capable of activating the transcription of heterologous promoters during middle sporulation (7, 28). Our results show that MSE^S, in addition to activating transcription during middle sporulation, can also repress transcription in vegetative cells. Hepworth et al. previously tested whether the MSE found in the *SPS4* middle sporulation promoter could repress vegetative transcription (17). Based on reconstitution experiments using the *CYC1* promoter, it was concluded that although the *SPS4* MSE specifically binds to a factor in vegetative cells, this element is not a direct repressor of transcription. Our results show that MSE^S can repress the vegetative expression of the *CYC1* as well as the *SMK1* and *HOP1* promoters. Taken together, these data suggest that there are two different classes of MSE. The first class (the *SPS4* MSE being the founding member) activates expression during middle sporulation but does not repress vegetative expression. The second class (the *SMK1* MSE^S characterized here) activates middle sporulation-specific expression and also represses promoter activity in vegetative cells. Recently, Ndt80p has been shown to activate transcription of middle sporulation-specific genes in an MSE-dependent fashion and to specifically interact with MSE DNA in vitro (7). *NDT80* is itself expressed at high levels as a middle sporulation-specific gene. As expected based on its expression pattern, an *ndt80*- Δ mutation does not derepress *SMK1* expression in vegetative cells. These results show that MSE^S repression does not require Ndt80p and indicate that there is a distinct factor expressed in vegetative cells with an overlapping DNA-binding specificity.

MSE^S can function in a fashion strikingly similar to that of URS1 in early genes (4, 39). Both elements repress vegetative expression and lead to transient derepression as well as activation at precise intervals during sporulation. While these similarities suggest that URS1 and MSE^S could share certain components required for repression, mutations which affect URS1-dependent repression (*ume6*, *sin3*, or *rdp3*) in vegetative cells had no significant effects on MSE-dependent repression. Thus, URS1 and MSE^S appear to function through distinct repression pathways. In addition, we have shown that the Ssn6p-Tup1p corepression complex, which is required for repression of the *DIT1/DIT2* divergently transcribed mid-late gene pair (14), does not affect *SMK1* repression by MSE^S. Thus, MSE^S repression appears to function through a mechanism that is distinct from those previously described. The identification of genes required for MSE^S-dependent repression

will shed new light on the mechanisms that regulate gene expression during development.

Context-dependent URS1 effects on transcription. The presence of an early regulatory URS1 consensus site in the middle *SMK1* promoter initially presented a paradox. URS1 has been shown to only transiently derepress expression during early sporulation, and we have shown that MSE^S is required for transient derepression during middle sporulation. If both of these elements were fully functional in a single promoter, it would be inactive throughout the entire sporulation program. This paradox is resolved by the observation that pseudo-URS1^S only weakly represses the *SMK1* promoter. This element has a stronger repression activity in the context of the early *HOP1* promoter (Fig. 6), suggesting either that the *HOP1* promoter contains sequences that are required for full pseudo-URS1^S repression or that the *SMK1* promoter contains sequences that antagonize the repression activity. In this respect, we have shown that pseudo-URS1^S only weakly represses vegetative expression of a heterologous *SMK1* promoter that contains the *HOP1* Abf1p-binding site (data not shown). In contrast, the MSE is fully able to repress this promoter. Thus, the inability of pseudo-URS1^S to function efficiently as a vegetative repressor of *SMK1* is not dependent on the Abf1p element.

What is the function of pseudo-URS1^S in the *SMK1* promoter? The fact that *SMK1* expressed from the early *HOP1* promoter can complement a *smk1*- Δ mutant is consistent with the possibility that during its evolutionary history *SMK1* was once regulated as an early sporulation gene. Thus, the pseudo-URS1^S in *SMK1* may be vestigial. However, analysis of *NDT80* suggests a function for a consensus URS1 in middle promoters. While *NDT80* is expressed predominantly as a middle gene, it also has a function early in sporulation, where it has been proposed to respond to meiotic recombination checkpoint controls (7, 17a, 43). *NDT80* expression is dependent on *IME1* (which positively regulates early transcription through the URS1 element). In the case of the *NDT80* promoter, it is possible that a low level of early URS1-dependent *NDT80* expression is required to initiate a positive MSE-dependent autoregulatory loop that leads to full expression of middle genes. In an *ndt80* strain, *SMK1* transcription is activated early in sporulation (7). This observation is consistent with the early peak of *SMK1* expression seen in the *mse*^S promoter mutant and suggests that pseudo-urs1^S can function when Ndt80 activity is low.

Functional significance of *SMK1* transcriptional regulation. Our previous results demonstrate that *SMK1* is required for coordination of spore morphogenesis and that regulated increases in *SMK1* activity during spore development play a role in sequentially activating distinct steps required for spore morphogenesis (20, 41, 42). The tightly regulated increase in *SMK1* transcription that occurs during spore morphogenesis raised the question of whether *SMK1* transcriptional regulation is required to coordinate the morphogenetic program. Our results show that the constitutive expression of *SMK1* does not affect vegetative growth, nor does it interfere with meiotic development or spore morphogenesis. In addition, promoter mutations which advance the timing of *SMK1* expression to early sporulation make spores which are indistinguishable from wild-type spores. Thus, the developmental function of *SMK1* is not contingent on its expression as a sporulation-specific middle gene. These results imply that mechanisms in addition to transcription regulate *SMK1* activity during sporulation.

Phenotypic analysis of the *smk1* promoter mutants show that different quantitative defects can lead to different qualitative end stage phenotypes. The UAS^S point mutants show that it is

possible to reduce expression of *SMK1* by 90% with no detectable sporulation phenotype. Thus, under optimal sporulation conditions, the level of *SMK1* activity exceeds the requirements. However, when the level of *SMK1* expression is reduced below a critical threshold (roughly 10% of the level expressed by wild-type cells), multiple distinct sporulation phenotypes that correlate with the degree to which *SMK1* is expressed are observed. These results show that the *SMK1* MAP kinase is required for the execution of multiple events during spore development and that the execution of distinct steps can require different levels of *SMK1* activity.

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