MOLECULAR AND CELLULAR BIOLOGY, Sept. 2002, p. 6417–6429 Vol. 22, No. 18
0270-7306/02/$04.00+0 DOI: 10.1128/MCB.22.18.6417–6429.2002

Regulation of the Premiddle and Middle Phases of Expression of the *NDT80* Gene during Sporulation of *Saccharomyces cerevisiae*

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Received 5 April 2002/Returned for modification 7 June 2002/Accepted 21 June 2002

The *NDT80* gene of *Saccharomyces cerevisiae*, which encodes a global activator of transcription of middle sporulation-specific genes, is first expressed after the activation of early meiotic genes but prior to activation of middle sporulation-specific genes. Both upstream repression sequence 1 (URS1) and mid-sporulation element (MSE) sites are present in the promoter region of the *NDT80* gene; these elements have been shown previously to contribute to the regulation of expression of early and middle sporulation-specific genes, respectively, by mediating repression in growing cells and activation at specific times during sporulation. In this study, we have shown that the overlapping windows of URS1- and MSE-mediated repression and activation are responsible for the distinctive premiddle expression pattern of the *NDT80* gene. Our data suggest that a Sum1-associated repression complex bound at the *NDT80* MSE sites prevents Ime1 tethered at the *NDT80* URS1 sites from activating transcription of the *NDT80* gene at the time that Ime1-dependent activation of early URS1-regulated meiotic genes is occurring. We propose that a decrease in the efficiency of Sum1-mediated repression as cells progress through the early events of the sporulation program allows the previously inactive Ime1 tethered at the URS1^{NDT80} sites to promote a low level of expression of the *NDT80* gene. This initial phase of URS1-dependent *NDT80* expression is followed by Ndt80-dependent upregulation of its own expression, which requires the MSE^{NDT80} sites and occurs concomitantly with Ndt80-dependent activation of a set of middle MSE-regulated sporulation-specific genes. Mutation of *IME2* prevents expression of *NDT80* in sporulating cells. We show in this study that *NDT80* is expressed and that middle genes are activated in cells of an Δ*ime2*/Δ*ime2* Δ*sum1*/Δ*sum1* strain in sporulation medium. This suggests that Ime2 activates expression of *NDT80* by eliminating Sum1-mediated repression.

The sporulation program of the yeast *Saccharomyces cerevisiae* provides a simple model system to study the temporal control of gene expression during development. On entry into the sporulation program, which is triggered by starvation, a diploid **a**/α cell completes one round of premeiotic DNA replication and then progresses through a lengthy prophase during which a high level of recombination occurs and homologous chromosomes pair. Cells then undergo the reductional and equational meiotic divisions, which result in a single four-lobed nucleus that contains the four haploid complements of chromosomes. The nuclear lobes are engulfed and ultimately pinched off by the prospore membranes that extend from the spindle pole bodies. The deposition of spore wall material within the prospore membrane completes the sporulation process, generating four mature spores arranged tetrahedrally within the ascus.

Studies aimed at identifying genes that are differentially expressed as cells progress through sporulation or that serve sporulation-specific roles have defined four temporally distinct classes of sporulation-specific genes: early, middle, mid-late, and late (reviewed in references 25 and 34). An additional three temporal classes have been identified by analysis of expression profiles obtained by a DNA microarray approach (6, 38). Progression through sporulation depends, at least in part, on the sequential completion of key genetic and morphological events that serve to regulate this transcriptional cascade. Various studies have identified transcriptional regulators that coordinate the expression of subsets of sporulation-specific genes (reviewed in reference 49) and have implicated other gene products as regulators of the transition from expression of one class to expression of the subsequent class (e.g., see references 10 and 46).

An understanding of some of the mechanisms that control the coordinate expression of early and middle sporulation-specific genes has been achieved through the characterization of promoter elements and identification of transcriptional regulatory factors (reviewed in reference 49). Expression of the early set of meiotic genes requires Ume6 and Ime1. The latter protein serves as a global regulator of entry into the sporulation program (43) and its expression is regulated both transcriptionally and posttranscriptionally by nutritional and mating-type signals (reviewed in references 25, 34, and 49). Ume6, which binds to the upstream repression sequence 1 (URS1) site that is present in the regulatory region of most early meiotic genes, prevents mitotic expression of these genes by recruitment of the Sin3-Rpd3 histone deacetylase complex (20, 21) and the Isw2 chromatin remodeling complex (13). Mutation of *UME6* leads to an intermediate level of expression of early meiotic genes in cells during vegetative growth. At the onset of the sporulation program, the transcriptional activator Ime1 accumulates, interacts with URS1-bound Ume6 in a Rim11-, Mck1-dependent manner, and activates expression of early meiotic genes (2, 33, 40, 44, 50). Other general regula-

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TABLE 1. *S. cerevisiae* strains used in this study

Strain	Genotype	Source
SK1 derivatives		
DKB98	*MAT***a**/*MAT*α *lys2/lys2 ho::LYS2/ho::LYS2 ura3/ura3 leu2::hisG/leu2::hisG his4X::LEU2/his4B::LEU2 arg4-Nsp*I*/arg4-Bgl*II	D. Bishop (32)
DKB407	*MAT*α *ho::LYS2 lys2 ura3 leu2 his4X-ADE2-his4B ade2*	D. Bishop (32)
DKB408	*MAT***a** *ho::LYS2 lys2 ura3 leu2 his4X-ADE2-his4B ade2*	D. Bishop (32)
NKY2296	*MAT***a**/*MAT*α *ho::LYS2/ho::LYS2 lys2/lys2 ura3/ura3 leu2/leu2* Δ*ndt80::LEU2/*Δ*ndt80::LEU2*	N. Kleckner (52)
JPY141	*MAT*α *ho::LYS2 lys2 ura3 leu2 his4X-ADE2-his4B ade2* Δ*sum1::kanMX6*	This study
JPY187	*MAT***a** *ho::LYS2 lys2 ura3 leu2 his4X-ADE2-his4B ade2* Δ*sum1::kanMX6*	This study
JPY214	*MAT***a**/*MAT*α *ho::LYS2/ho::LYS2 lys2/lys2 ura3/ura3 leu2/leu2 his4X-ADE2-his4B/his4X-ADE2-his4B ade2/ade2* Δ*sum1::kanMX6/*Δ*sum1::kanMX6*	This study
JPY215	*MAT***a**/*MAT*α *ho::LYS2/ho::LYS2 lys2/lys2 ura3/ura3 leu2/leu2 HIS4/*[a] *ADE2/*[b] Δ*ndt80::LEU2/*Δ*ndt80::LEU2* Δ*sum1::kanMX6/*Δ*sum1::kanMX6*	This study
L213	*MAT***a**/*MAT*α *ho::LYS2/ho::LYS2 lys2/lys2 ura3/ura3 leu2/leu2 trp1/trp1 ime2::2::LEU2/ime2::2::LEU2*	B. Byers (8)
JPY211	*MAT***a**/*MAT*α *ho::LYS2/ho::LYS2 lys2/lys2 ura3/ura3 leu2/leu2 TRP1/*[c] *HIS4/*[a] *ADE2/*[b] *ime2::2::LEU2/ime2::2::LEU2* Δ*sum1::kanMX6/*Δ*sum1::kanMX6*	This study
W303 derivatives		
LNY315	*MAT***a** *ade2-1 trp1-1 his3-11,15 can1-100 ura3-1 leu2-3,112*	E. Winter (51)
JXY34	*MAT*α *ade2-1 trp1-1 his3-11,15 can1-100 ura3-1 leu2-3,112* Δ*ume6::HIS3*	E. Winter (51)
JXY3	*MAT***a** *ade2-1 trp1-1 his3-11,15 can1-100 ura3-1 leu2-3,112* Δ*sum1::kanMX4*	E. Winter (51)
JXY15	*MAT***a** *ade2 ade6 trp1-1 his3-11,15 can1-100 ura3-1 leu2-3,112* Δ*ume6::HIS3* Δ*sum1::kanMX4*	E. Winter (51)

[a] This strain may carry *his4X-ADE2-his4B.*
[b] This strain may carry *ade2.*
[c] This strain may carry *trp1.*

tors, such as the RSC chromatin remodeling complex (54) and Abf1 (11), whose DNA-binding site is present in the promoter regions of several early meiotic genes, contribute to maximal expression. *IME2*, which encodes a protein kinase and is itself an early meiotic gene, is required for the normal kinetics and full expression of early meiotic genes and is essential for expression of middle sporulation-specific genes (reviewed in reference 34).

Ndt80 is a global activator of middle sporulation-specific gene expression that also upregulates its own expression (5, 6, 17). This activator binds to a short regulatory element (5), the mid-sporulation element (MSE) site (16, 36), which is found in the promoter region of 70% of the 158 genes that belong to the middle class of sporulation-specific genes as well as in the promoter region of the premiddle genes, *SMK1* and *NDT80* (5, 6, 37). *NDT80*, which was initially characterized as a gene required for exit from the pachytene stage of meiotic prophase (52), is regulated by the meiotic recombination checkpoint. Activation of this checkpoint by defects in recombination or synaptonemal complex formation reduces transcription of the *NDT80* gene and leads to inactivation of Ndt80 as an activator of MSE-dependent gene expression (5, 17, 47). Thus, there is a low level of checkpoint-insensitive expression of *NDT80* in checkpoint-arrested cells, but there is no Ndt80-dependent auto-upregulation of *NDT80* expression or activation of middle sporulation-specific gene expression (5, 17).

A subset of MSEs confers repression during vegetative growth (16, 37, 51). *SUM1* and *HST1* were identified recently in a screen for genes that are required to maintain mitotic repression of a reporter gene containing the MSE site from the promoter of the *SMK1* gene (51). *SUM1* was also identified (J. Pak, unpublished data) in further analysis of mutant strains that had been isolated on the basis of allowing mitotic expression of a normally repressed reporter gene containing four copies of the MSE from the *SPS4* gene (17). Originally identified as a dominant allele, *SUM1-1*, which bypasses the requirement for the *SIR* genes in silencing the *HM* mating-type loci (4, 22, 26, 28, 30), *SUM1* has been shown more recently to encode an MSE-binding protein (51). *HST1* is one of four genes that encode Sir2-related proteins (3, 7). Recently, Hst1 has been shown to have NAD^+-dependent histone deacetylase activity and to interact with Sum1 (41, 45). Thus, Sum1 may compete with Ndt80 for binding to MSE sites (51) and, when bound, may lead to Hst1-mediated deacetylation of adjacent histones (41, 45), generating an inaccessible chromatin structure and effectively preventing transcription.

In the present study we have examined the regulation of expression of the *NDT80* gene, the founding member of the premiddle class of sporulation-specific genes. Expression of the *NDT80* gene begins after transcripts of the early meiotic genes are first detected but before transcripts of middle genes begin to accumulate. Our data suggest that early meiotic expression of *NDT80* is prevented by Sum1 bound at the MSE sites that are present in the promoter region of the *NDT80* gene. Ime2, the product of an early meiotic gene, however, leads to inactivation of Sum1, allowing a checkpoint-insensitive phase of *NDT80* expression to proceed. This expression is promoted by the URS1 sites that are also present in the promoter region of the gene. Once this URS1-dependent phase of *NDT80* expression has been initiated, a checkpoint-sensitive phase of expression occurs in which Ndt80 upregulates its own expression in an MSE-dependent manner.

MATERIALS AND METHODS

Yeast strains and genetic procedures. Table 1 lists the *S. cerevisiae* strains used in this study. W303-derived strains were used for the experiment shown below in Fig. 3. SK1-derived strains were used for all other experiments. Strain JPY141 was derived from DKB407 by integrative transformation with a PCR product

that replaced *SUM1* with the kanMX6 cassette (31). Strain JPY187 (*MAT***a** Δ*sum1::kanMX6*) was derived as a haploid spore segregant of the diploid strain obtained by mating JPY141 and DKB408. JPY215 was obtained by first mating a haploid *MAT***a** version of NKY2296 with JPY141. The resultant diploid strain was sporulated, and haploid *MAT***a** Δ*sum1::kanMX6* Δ*ndt80::LEU2* and *MAT*α Δ*sum1::kanMX6* Δ*ndt80::LEU2* segregants were identified and mated to give the diploid JPY215. JPY211 was derived in a similar manner; a haploid *MAT***a** segregant of L213 was mated with JPY141 and the resultant diploid strain was sporulated giving rise to haploid *MAT***a** Δ*sum1::kanMX6 ime2::LEU2* and *MAT*α Δ*sum1::kanMX6 ime2::LEU2* segregants, which were mated to generate the diploid strain JPY211.

Yeast transformations were performed by the lithium acetate method (12). Standard genetic methods were used for mating, sporulation, and random spore disruption.

Media and growth conditions. SD medium is a minimal medium (2% glucose, 0.7% yeast nitrogen base without amino acids) supplemented with 40 μg of adenine sulfate per ml, 20 μg of L-arginine per ml, 20 μg of L-histidine per ml, 60 μg of L-leucine per ml, 30 μg of L-lysine (mono-HCl) per ml, 20 μg of L-methionine per ml, 50 μg of L-phenylalanine per ml, 200 μg of L-threonine per ml, 40 μg of L-tryptophan per ml, 30 μg of L-tyrosine per ml, and 20 μg of uracil per ml. SD-X medium refers to SD medium that lacks supplement X. Rich medium (yeast-extract-peptone-dextrose [YEPD]), presporulation medium (yeast extract-peptone-acetate [YEPA]), and sporulation medium (SPO) were as described previously for SK1-derived strains (17). All yeast cultures were grown at 30°C.

Sporulation of SK1-derived strains was performed as follows. Cells were taken from a YEPA plate and were grown to late log phase in SD-uracil, and this culture was used to inoculate YEPA medium (1:100 dilution). When the YEPA culture reached a density of 1.0×10^7 to 2.0×10^7 cells per ml, the cells were harvested by centrifugation, washed once in 1% potassium acetate, and resuspended in SPO medium at a density of 2.0×10^7 cells per ml. The time of transfer of cells to sporulation medium is referred to as 0 h. The efficiency of ascus formation was assessed by examination of 200 or more cells by light microscopy after 24 h or more in SPO medium.

Plasmids. A low-copy-number plasmid containing a truncated version of the *NDT80* gene was constructed as follows. First, cSC131-1 was constructed by cloning the *Not*I-*Cla*I fragment spanning the *GAL1-NDT80* fusion gene of cSC131 (5) into pRS316. Then, a PCR-amplified fragment that contained 505 bp of the sequence upstream of the initiator ATG of the *NDT80* gene was generated with primers N80-505T (5′GGGGCGGCCGCCCATCAAGCGCTCCAAGC3′) and N80-1B (5′GGGGCATATGTTTAAGCGCTTTTTATAATATTGT3′) and pNKY1212 (52) as template. This PCR product was cloned into the *Sma*I site of pRS425 and then recovered from this plasmid as a *Nde*I-*Not*I fragment. This *Nde*I-*Not*I fragment was then used to replace the *Nde*I-*Not*I fragment of cSC131-1, which contains the *GAL1* promoter region. The resultant plasmid, p1-1, contained 505 bp of sequence upstream of the *NDT80* initiator ATG codon and the entire *NDT80* coding region and downstream sequence. The initiator ATG codon of the *NDT80* gene was within an engineered *Nde*I site. This plasmid was digested with *Eco*RI, and the resultant 304-bp and 6,262-bp fragments were recovered and religated to generate a plasmid, pRS316(−505)ndt80, that contained a truncated *ndt80* gene which lacked the last 1,040 bp of the open reading frame (ORF).

Versions of pRS316(−505)ndt80 in which URS1 or MSE sites in the upstream region of the *ndt80* gene were deleted were constructed as follows. The *Nde*I-*Not*I fragment containing the promoter region of the *NDT80* gene was replaced with equivalent fragments, with the exception that a URS1 element or an MSE-1 element was deleted. These fragments were generated by the PCR-based overlap extension method (18). The outside primers were N80-505T and N80-1B (see above). The internal primers for deletion of 9 bp from the URS1-1 element to generate the $(-505^{\Delta U1})ndt80$ minigene were N80-311T (5′CTTTACATTGTTACTATTTGACG3′) and N80-280B (5′CGTCAAATAGTAACAATGTAAAG3′); the internal primers for deletion of the 9 bp from the MSE-1 element to generate the $(-505^{\Delta M1})ndt80$ minigene were N80-230T (5′AGGCCGTATGAGTAGAAAAC3′) and N80-202B (5′GTTTTCTACTCATACGGCCT3′); the internal primers for deletion of 8 bp from the URS1-2 element to generate the $(-505^{\Delta U2})ndt80$ minigene were N80-176T (5′TCCTCTATACAGCTCTCTGA3′) and N80-149B (5′TCAGAGAGCTGTATAGAGGA3′); and the internal primers for deletion of 9 bp from the MSE-2 element to generate the $(-505^{\Delta M2})ndt80$ minigene were N80-94T (5′GCCCTCCAACCTATTTAAGC3′) and N80-66B (5′GCTTAAATAGGTTGGAGGGC3′). See Fig. 1 and the text for a description of these elements and minigenes.

The $(-505^{\Delta U1\Delta U2})ndt80$ minigene that lacked both the URS1-1 and URS1-2 elements was constructed by ligating a 638-bp *Nae*I-*Bgl*II fragment from the plasmid containing the $(-505^{\Delta U1})ndt80$ minigene with a 5,910-bp *Nae*I-*Bgl*II fragment from the plasmid containing the $(-505^{\Delta U2})ndt80$ minigene. A plasmid containing the $(-505^{\Delta M1\Delta M2})ndt80$ minigene was constructed in the same manner from the plasmids containing the $(-505^{\Delta M1})ndt80$ minigene and the $(-505^{\Delta M2})ndt80$ minigene. The plasmids containing the $(-505^{\Delta U1\Delta M1\Delta M2})ndt80$ minigene and the $(-505^{\Delta M1\Delta U2\Delta M2})ndt80$ minigene were constructed by using the overlap extension method and the same primers that were used to delete the URS1-1 element and the URS1-2 element (see above), respectively, and the plasmid containing the $(-505^{\Delta M1\Delta M2})ndt80$ minigene as template. The resultant *Nde*I-*Not*I fragments were used to replace the equivalent fragment in pRS316(−505)ndt80.

The multicopy plasmid pLGΔ312(Bgl) containing a *CYC1-lacZ* reporter gene has been described previously (16). pCYC1-(URS1-1-MSE-1)^{NDT80}-lacZ contains a 91-bp fragment extending from just upstream of the URS1-1^{NDT80} site to just downstream of the MSE-1^{NDT80} site (Fig. 1) inserted into the *Bgl*II site of pLGΔ312(Bgl). The insert fragment was obtained by PCR amplification of the *NDT80* sequence with the primers N80-298T-*Bgl*II (5′GGGAGATCTCTTCCGCGGCTATTTGACG3′) and N80-207B-*Bgl*II (5′GGGAGATCTCTACTCTTTTGTGTCATACGG3′), which had additional sequence at their 5′ ends introducing *Bgl*II sites in order to facilitate cloning.

pP_{HOP1}-*NDT80* contains the *HOP1* promoter fused to the *NDT80* ORF and was constructed as follows. pNH59-2 (19) was cut with *Eco*RI and the ends were filled in with Klenow. This linearized DNA was then cut with *Nde*I, releasing a fragment containing 990 bp of sequence upstream of the *HOP1* ORF. This fragment was ligated to 1,688-bp and 4,764-bp fragments generated by cutting cSC131-1 with *Not*I, filling in with Klenow, and cutting with *Nde*I.

The integrity of all PCR-generated DNAs was confirmed by sequencing.

Assay for β-galactosidase activity. β-Galactosidase expression from *lacZ* reporter genes was monitored by use of a colony overlay assay (1). Ten milliliters of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)-containing agar (0.5% agar, 0.5 M potassium phosphate [pH 7.0], 6% dimethyl formamide, 0.1% sodium dodecyl sulfate, 0.2 mg of X-Gal per ml) was poured over colonies on plates, and the plates were incubated at 30°C until blue color development was evident.

RNA isolation and Northern analysis. RNA preparation from yeast and Northern blot analysis were performed as described previously (17). Gene-specific probes were prepared with the following templates: *NDT80*, a 1.2-kb *Eco*47III-*Bam*HI fragment from pNKY1212 (52); *CLB1*, a 500-bp *Eco*RV-*Eco*RV fragment from pMT417 (provided by M. Tyers); *SMK1*, an 800-bp *Sty*I-*Sty*I fragment from pLAKK40 (Krisak et al. [24]); *SPS1*, a 550-bp *Cla*I-*Eco*RV fragment from pSPS1-URA3 (10); *SPS4*, a 521-bp *Mlu*I-*Cla*I fragment from

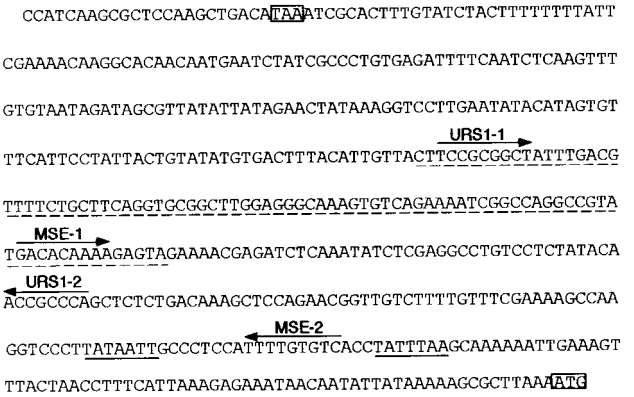

FIG. 1. Sequence of the 5′-flanking region of the *NDT80* gene. The sequence upstream of the initiator ATG codon of the *NDT80* gene is given. All numbering in the text is with respect to the A of this ATG codon being nucleotide +1. This ATG codon and the stop codon of the upstream gene, *EPT1*, are boxed. Putative TATA boxes of the *NDT80* gene are underlined. Arrows are over sequences that correspond to the consensus for a URS1 or an MSE and indicate the orientation of the element. See the text for the coding of the sites as URS1-1, URS1-2, MSE-1, and MSE-2. The *NDT80* sequence inserted into pLGΔ312(Bgl) to create the *CYC1-(URS1-1-MSE-1)*^{NDT80}*-lacZ* reporter gene (see Fig. 3) is underlined with a dashed line.

p4LE159 (16); and pC4, which contains an uncharacterized gene whose expression is similar in growing cells and through sporulation (27).

RESULTS

A model for the temporal regulation of expression of the *NDT80* gene. Expression of *NDT80*, a premiddle sporulation-specific gene, is tightly regulated and requires its own gene product for upregulation of its expression (5, 17). Transcripts are first detected after the activation of early meiotic genes, such as *HOP1* and *IME2*, but prior to the activation of middle sporulation-specific genes, such as *SPS1* and *SPS4* (5, 17). Transcript accumulation is maximal at 6 h after transfer of cells to sporulation medium and then declines such that transcripts are barely detectable at 15 h of sporulation. In setting out to study the temporal regulation of expression of the *NDT80* gene, we focused on four candidate regulatory sites present in the ≈500-bp interval between the initiator methionine codon of the *NDT80* gene and the stop codon of the upstream ORF. Sequence inspection revealed two URS1 sites, which we refer to as URS1-1^{NDT80} and URS1-2^{NDT80}, present at nucleotide (nt) −295 and nt −165, respectively, and two MSE sites, which we refer to as MSE-1^{NDT80} and MSE-2^{NDT80}, present at nt −221 and nt −86, respectively (Fig. 1) (5). The latter MSE is located between two potential TATA box sequences.

Based on the presence of both URS1 and MSEs in the upstream region of the *NDT80* gene and the known roles of these elements in regulating early and middle sporulation-specific gene expression (see above), we put forth the following model for the temporal regulation of expression of this gene (Fig. 2). We suggest that in vegetatively growing cells, both Ume6 bound to URS1^{NDT80} and Sum1 bound to MSE^{NDT80} assemble repression complexes that prevent expression of the *NDT80* gene (13, 20, 21, 51) (Fig. 2A). We infer that early during sporulation, at the time that Ime1 is activating the expression of early meiotic genes, including *IME2*, Ime1 is also recruited to the putative Ume6-URS1^{NDT80} complex. We propose that activation of the *NDT80* gene by the Ime1-Ume6-URS1^{NDT80} complex is initially prevented, however, by the continued presence of Sum1 at the MSE^{NDT80} sites (Fig. 2B). Data presented in this study suggest that Ime2 promotes the first phase of *NDT80* expression by inhibiting the repression activity of Sum1 and thereby allowing Ime1 to direct a low level of expression of the *NDT80* gene (Fig. 2C). This is the premiddle phase of *NDT80* expression. The newly synthesized Ndt80 would then compete with Sum1 for binding to the MSE^{NDT80} sites and upregulate its own expression, leading to the middle phase of *NDT80* expression (Fig. 2D). This would result in the peak of *NDT80* transcript accumulation, coinciding with that of transcripts from MSE-regulated middle sporulation-specific genes. The transient decrease in the level of Sum1 that occurs during sporulation (29) may contribute to the regulated expression of the *NDT80* gene. Finally, as the ability of Ime1 and Ndt80 to promote transcription is sequentially downregulated in a manner that is dependent on progression of the cells through the sporulation program (17, 35, 42), expression of *NDT80* is shut off. As described below, we tested this model by assessing the contributions of the URS1^{NDT80} sites and of the MSE^{NDT80} sites to the expression of the *NDT80* gene.

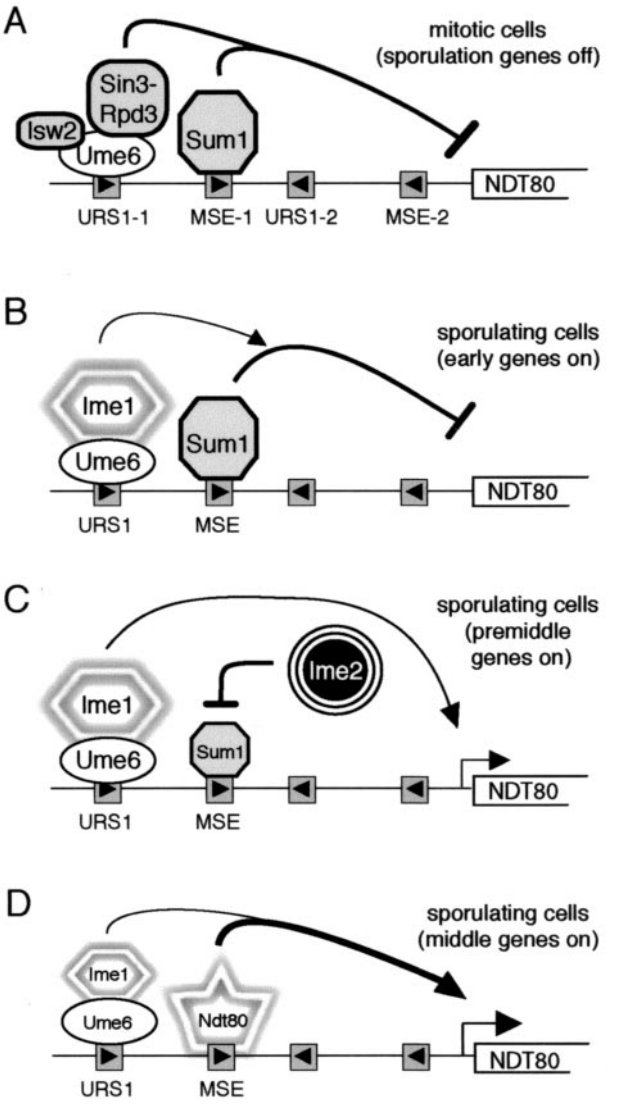

FIG. 2. Model for the temporal regulation of expression of the *NDT80* gene. See text for details. The horizontal line represents the *NDT80* promoter, with the URS1 and MSE sites depicted as shaded boxes with arrowheads (see Fig. 1). For simplicity, regulatory molecules are shown only at the URS1-1 and MSE-1 sites. Curved lines ending in an arrowhead indicate activation of transcription; curved lines ending with a bar indicate repression of transcription. URS1- or MSE-bound proteins, or complexes, that mediate repression are represented in gray; proteins, or complexes, that mediate activation are represented by shapes outlined with shadows. The reductions in size of the Sum1 symbol (C) and the Ime1-Ume6 symbols (D) denote a reduction in activity. (A) The *NDT80* promoter in mitotic cells. (B to D) The *NDT80* promoter in sporulating cells at the time that early meiotic genes, including *IME2*, are being expressed (B), after early genes, but before middle sporulation-specific genes have been activated (C), or at the time of middle sporulation-specific gene expression (D).

Role of Ume6 and Sum1 in mitotic repression of *NDT80*. The observation that the chromosomal *NDT80* gene is derepressed in vegetatively growing *sum1 ume6* cells (51) implicates the URS1 sites and the MSE sites in the promoter region of the *NDT80* gene as operator sites. We therefore tested the

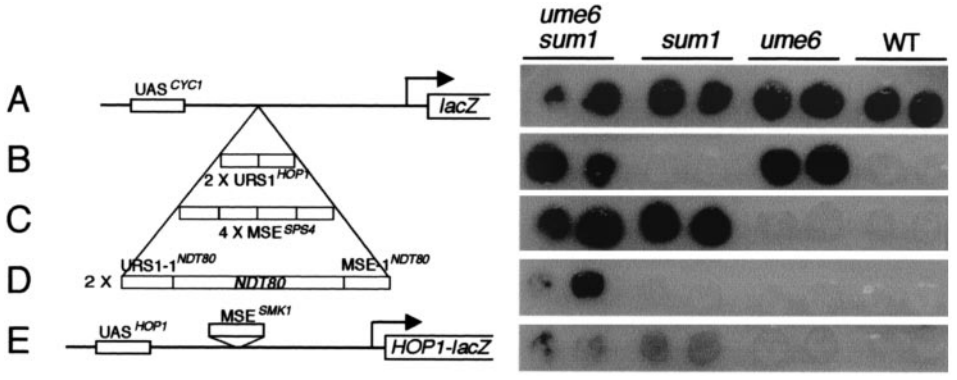

FIG. 3. Derepression of a *CYC1-(URS1-1-MSE-1)^{NDT80}-lacZ* reporter gene in mitotic cells requires mutation of both *UME6* and *SUM1*. Duplicate patches of cells of a *ume6 sum1* strain (first column), a *sum1* strain (second column), a *ume6* strain (third column), and a wild-type strain (fourth column) contained the following plasmids: (A) pLGΔ312(Bgl), which contains the *CYC1-lacZ* reporter gene described by Hepworth et al. (16); (B) pAV138-2, which contains two tandem copies of the URS1 site of the *HOP1* gene inserted between the *CYC1* UAS sites and TATA box in pLGΔ312(Bgl) (48); (C) pCYC1-SPS4-lacZ, which contains four tandem copies of a 29-bp fragment that spans the MSE site of the *SPS4* gene inserted between the *CYC1* UAS sites and TATA box in pLGΔ312(Bgl) (16, 17); (D) pCYC1-(URS1-1-MSE-1)^{NDT80}-lacZ, which contains a 91-bp fragment that extends from the URS1-1 site to the MSE-1 site in the promoter region of the *NDT80* gene (see Fig. 1), inserted between the *CYC1* UAS sites and TATA box in pLGΔ312(Bgl) (see Materials and Methods); (E) pJX43, which contains a fragment spanning the MSE of the *SMK1* promoter inserted into the URS1 site of a *HOP1-lacZ* reporter gene (37). The patches of cells were overlaid with X-Gal-containing agar (see Materials and Methods).

NDT80 sequence extending from nt −298, just upstream of the URS1-1^{NDT80} site, to nt −207, just downstream of the MSE-1^{NDT80} site (Fig. 1), for its ability to prevent expression of a heterologous reporter gene in vegetative cells. We cloned two copies of this fragment between the upstream activation sequence (UAS) and TATA box of a plasmid-borne *CYC1-lacZ* reporter gene and then monitored expression of the resultant *CYC1-(URS1-1-MSE-1)^{NDT80}-lacZ* gene in wild-type, *ume6*, *sum1*, and *ume6 sum1* cells (Fig. 3D). As controls, we also examined expression of a *HOP1-lacZ* reporter gene in which the URS1^{HOP1} site had been replaced with the MSE of the *SMK1* gene (Fig. 3E) (37), a *CYC1-lacZ* reporter gene containing four copies of the MSE from the *SPS4* gene (Fig. 3C) (16), and a *CYC1-lacZ* reporter gene containing two copies of the URS1 element from the *HOP1* gene (Fig. 3B) (48). Each insert prevented expression of the plasmid-borne reporter gene in wild-type cells growing vegetatively as tested in a colony overlay assay for β-galactosidase expression (Fig. 3, WT column). As expected, mutation of *UME6*, but not mutation of *SUM1*, allowed expression of the *CYC1-URS1^{HOP1}-lacZ* reporter gene (Fig. 3B) (48, 51); similarly, mutation of *SUM1*, but not mutation of *UME6*, allowed expression of the *CYC1-4xMSE^{SPS4}-lacZ* reporter gene (Fig. 3C) (17; J. Pak, unpublished observation) and the *HOP1-MSE^{SMK1}-lacZ* reporter gene (Fig. 3E) (51). However, mutation of both *UME6* and *SUM1* was required for expression of the *CYC1-(URS1-1-MSE-1)^{NDT80}-lacZ* reporter gene in mitotic cells (Fig. 3D). This experiment showed that Ume6 and Sum1 serve redundant functions, presumably through URS1-1 and MSE-1, respectively, in supporting the ability of the *NDT80*-derived region extending from −298 to −207 to repress UAS^{CYC1}-driven expression in mitotic cells.

Expression of a plasmid-borne version of *NDT80* parallels expression of the chromosomal *NDT80* gene. We used a truncated *ndt80* gene present on a low-copy plasmid to investigate the contributions that the URS1^{NDT80} and MSE^{NDT80} sites make to the expression of the *NDT80* gene during sporulation. This reporter minigene, termed *(−505^{WT})ndt80*, which contained the *NDT80* sequence from nt −505 to nt +880, with +1 being the start of the ORF, did not complement the sporulation deficiency of Δ*ndt80*/Δ*ndt80* cells (data not shown). However, the presence of a full-length, plasmid-borne *NDT80* gene with 505 bp of upstream region allowed Δ*ndt80*/Δ*ndt80* cells to form spores (data not shown), indicating that 505 bp of the upstream region was sufficient to activate expression of the gene. We next monitored expression of the plasmid-borne *(−505^{WT})ndt80* minigene by Northern blot analysis of RNA from diploid cells during vegetative growth and at various times after transfer to sporulation medium. Stable, minigene-encoded transcripts accumulated during sporulation, and these transcripts could be readily distinguished on the basis of size from transcripts of the wild-type *NDT80* gene (Fig. 4A). This allowed us to compare expression of the plasmid-borne *(−505^{WT})ndt80* minigene with expression of the chromosomal *NDT80* gene in the same cells.

We found that the temporal pattern of expression of the plasmid-borne *(−505^{WT})ndt80* minigene paralleled that of the chromosomal *NDT80* gene (Fig. 4A, lanes 1 to 8). Transcripts were not detected in vegetative cells (Fig. 4A, lane 1). A low level of transcripts could first be detected between 3 and 4 h of sporulation (Fig. 4A, lanes 3 and 4), with maximal transcript accumulation occurring at 6 h (Fig. 4A, lane 5). This is the time at which Ndt80 is activating expression of middle sporulation-specific genes (6, 17). By 10 h of sporulation, transcript levels had declined to low levels (Fig. 4A, lane 7). At all times the amount of plasmid-borne minigene-derived transcripts that was present in the cells was similar to the amount of chromosome-derived *NDT80* transcripts. Quantitation of the transcript levels at 6 h of sporulation indicated that transcript accumulation from the plasmid-borne minigene was 91% that of chromosome-derived *NDT80* transcripts. The close correlation that we observed between the temporal patterns of ex-

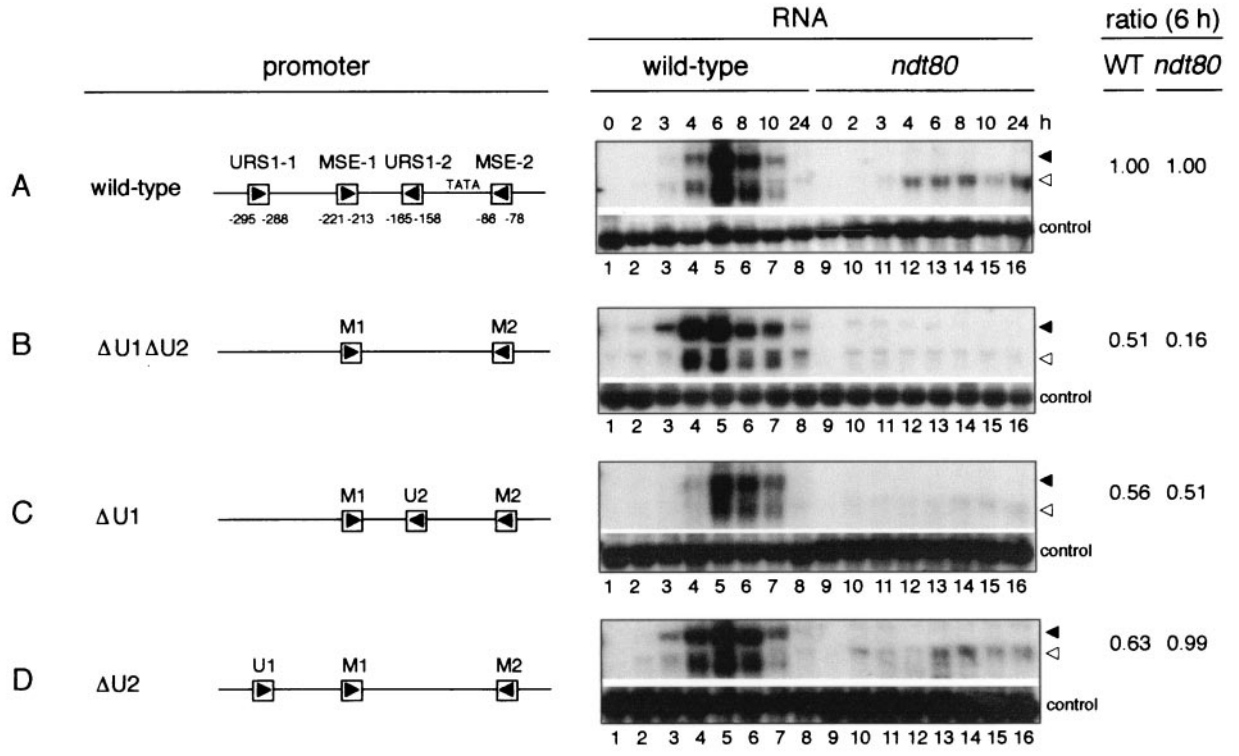

FIG. 4. The URS1 sites are responsible for initial expression of the *NDT80* gene. The Northern filters represented under the RNA heading contained RNA extracted from wild-type cells (lanes 1 to 8) and Δ*ndt80*/Δ*ndt80* cells (lanes 9 to 16) harvested during vegetative growth (0 h) or at the indicated times, as noted above the top panel (in hours), after transfer of cells to sporulation medium. Cells used for the experiment of each panel harbored the following plasmid-borne *ndt80* minigenes: (A) (-505^{WT})*ndt80*; (B) $(-505^{\Delta U1\Delta U2})$*ndt80*; (C) $(-505^{\Delta U1})$*ndt80*; and (D) $(-505^{\Delta U2})$*ndt80*. A schematic diagram of the promoter region of each of these minigenes is given under the promoter heading. Abbreviations: U1, URS1-1; M1, MSE-1; U2, URS1-2; M2, MSE-2. Δ denotes that the specified element has been deleted. The Northern filters were hybridized with a radioactively labeled *NDT80*-specific probe (top portion of each panel) and a control probe (bottom portion of each panel) prepared with pC4 (see Materials and Methods). The closed and open arrowheads denote the full-length chromosome-derived *NDT80* transcripts and the truncated *ndt80* minigene-derived transcripts, respectively. The ratio column presents a normalized ratio for the expression of each plasmid-borne (-505^{mutant})*ndt80* minigene relative to the chromosomal *NDT80* gene in the same cells, determined as follows. The WT column gives the intensity of the hybridization signal for transcripts derived from each plasmid-borne (-505^{mutant})*ndt80* minigene at 6 h of sporulation (lane 5) relative to the intensity of the hybridization signal for transcripts derived from the chromosomal *NDT80* gene in the same cells at 6 h of sporulation, normalized to the same ratio obtained for transcripts derived from cells containing the (-505^{WT})*ndt80* plasmid-borne minigene (in panel A). The *ndt80* ratio column gives the intensity of the hybridization signal for transcripts derived from each (-505^{mutant})*ndt80* minigene in Δ*ndt80*/Δ*ndt80* cells at 6 h after transfer to sporulation medium (lane 13), normalized to the loading control, relative to the amount of chromosome-derived *NDT80* transcripts in wild-type cells at 6 h of sporulation (lane 5), normalized to the control probe, and then normalized to the same ratio obtained for transcripts derived from the (-505^{WT})*ndt80* minigene (in panel A). Relative intensities of hybridization signals were determined by quantitation of phosphorimages obtained with a Molecular Dynamics STORM 860 PhosphorImager. The images were analyzed with ImageQuant (Molecular Dynamics, Inc.) and IPLab Gel (Signal Analytics Corporation) software. The images of autoradiograms presented in this figure are scans obtained with Adobe Photoshop 5.0 LE software and assembled with the use of Adobe Illustrator 10 and PowerPoint 98.

pression of the plasmid-borne (-505^{WT})*ndt80* minigene and the chromosomal *NDT80* gene allowed us to use expression of the chromosomal gene as an internal control in monitoring the timing of expression of *ndt80* minigenes containing promoter mutations. This internal comparison allowed us to ignore any small temporal differences in the overall pattern of gene expression that occurred between experiments. Such differences could result from variations in the timing of key regulatory events or in the synchrony of cells as the sporulation program proceeded.

We next monitored expression of the (-505^{WT})*ndt80* minigene in Δ*ndt80*/Δ*ndt80* cells transferred to sporulation medium (Fig. 4A, lanes 9 to 16). These cells, which lack functional Ndt80, arrest at pachytene (52) and do not express middle sporulation-specific genes (5, 6, 17). The onset of minigene expression in Δ*ndt80*/Δ*ndt80* cells was the same as in wild-type cells, between 3 and 4 h after transfer of cells to sporulation medium, but there was no subsequent upregulation of expression (Fig. 4A, lanes 9 to 16). This pattern of expression was consistent with the notion that the initial phase of *NDT80* expression is Ndt80 independent and that the subsequent upregulation of expression is Ndt80 dependent (5, 17). The continued presence of a low level of minigene transcripts in Δ*ndt80*/Δ*ndt80* cells, even after 24 h in sporulation medium, was consistent with previous observations that early and premiddle genes continue to be expressed in cells that are unable to activate middle genes (e.g., references 17, 35, and 42).

The URS1 sites are required for the early phase of *NDT80* gene expression. Our model for the regulation of expression of the *NDT80* gene proposed that the URS1 sites were responsible for the initial expression of the gene on transfer of cells to

sporulation medium (Fig. 2C). Consistent with this model, expression of the plasmid-borne *(−505$^{\Delta U1\Delta U2}$)ndt80* minigene, which lacked both the URS1-1^{NDT80} and URS1-2^{NDT80} elements, appeared to be delayed relative to expression of the chromosomal wild-type gene (Fig. 4B, lanes 3 and 4). Transcripts from the wild-type chromosomal gene could be detected at 3 h after transfer of cells to sporulation medium, whereas minigene-derived transcripts were not readily detected in these cells until 4 h of sporulation (Fig. 4B, lane 4). We inferred that in the absence of URS1-driven expression, activation of the *(−505$^{\Delta U1\Delta U2}$)ndt80* minigene was delayed until Ndt80, expressed from the chromosomal *NDT80* gene, promoted its expression. Consistent with this notion, expression of the plasmid-borne *(−505$^{\Delta U1\Delta U2}$)ndt80* minigene was barely detectable in Δ*ndt80*/Δ*ndt80* cells (Fig. 4B, lanes 9 to 16), whereas the *(−505^{WT})ndt80* minigene was expressed at a low level in these cells (Fig. 4A, lanes 9 to 16). In addition to regulating the time of onset of expression of the *ndt80* minigene, the URS1^{NDT80} elements also contributed to its maximal expression. Transcript accumulation from the *(−505$^{\Delta U1\Delta U2}$)ndt80* minigene was 50% of that from the *(−505^{WT})ndt80* minigene at 6 h of sporulation (Fig. 4A and B, lane 5). Both URS1-1^{NDT80} and URS1-2^{NDT80} contributed to maximal transcript accumulation in wild-type cells (Fig. 4C and D, lanes 1 to 8).

Sum1 prevents premature expression of the *NDT80* gene. As discussed above, we inferred that in vegetatively growing cells URS1^{NDT80}-bound Ume6 and MSE^{NDT80}-bound Sum1 assembled repression complexes that prevented expression of the *NDT80* gene (Fig. 2A). Early during sporulation, Ime1 would presumably be recruited to the Ume6-URS1^{NDT80} complexes in the promoter region of the *NDT80* gene at the same time that it was recruited to similar complexes in the promoter region of early meiotic genes. Whereas early meiotic genes would be expressed immediately, we proposed that activation of the *NDT80* gene by the Ime1-Ume6-URS1^{NDT80} complex was initially prevented by the continued presence of the Sum1 repression complexes at the MSE^{NDT80} sites (Fig. 2B). We tested this idea by assessing the effect of the deletion of *SUM1* on the expression profile of *NDT80*. We note that *SUM1* has been shown previously to have no essential role in spore formation (29).

Examination of the temporal profile of gene expression during sporulation of a Δ*sum1*/Δ*sum1* strain showed that both the chromosomal *NDT80* gene and the *(−505^{WT})ndt80* minigene were expressed prematurely in the absence of Sum1 (Fig. 5A, lanes 17 to 24). Whereas *NDT80* transcripts were generally first detected at 3 to 4 h in wild-type cells (Fig. 4, lanes 3 to 6; chromosomal transcript), transcripts from the chromosomal *NDT80* gene and the *(−505^{WT})ndt80* minigene could be readily detected at 2 h after transfer of Δ*sum1*/Δ*sum1* cells to sporulation medium (Fig. 5A, lane 18). Similarly, transcripts of the *(−505^{WT})ndt80* minigene accumulated earlier in cells of the Δ*ndt80*/Δ*ndt80* Δ*sum1*/Δ*sum1* strain than in cells of the Δ*ndt80*/Δ*ndt80* strain on transfer to sporulation medium (Fig. 5A, compare lanes 9 to 12 with lanes 1 to 4). We also monitored expression of the premiddle gene, *SMK1*, and the middle sporulation-specific genes, *CLB1* and *SPS4*, whose expression is maximal around 6 to 8 h of sporulation in wild-type cells (5, 17). As reported previously, we found that *SMK1* was expressed in mitotic Δ*sum1*/Δ*sum1* cells (Fig. 5B, lanes 9 and 17) (29, 51) and in Δ*ndt80*/Δ*ndt80* cells in sporulation medium, albeit at a reduced level (Fig. 5B, lanes 1 to 8) (5, 17). The middle sporulation-specific genes, *CLB1* and *SPS4*, were expressed in Δ*sum1*/Δ*sum1* cells but not in Δ*ndt80*/Δ*ndt80* cells (Fig. 5C and D, lanes 1 to 8 and 17 to 24) (5, 17, 51) or in Δ*ndt80*/Δ*ndt80* Δ*sum1*/Δ*sum1* cells in sporulation medium (Fig. 5C and D, lanes 9 to 16). This analysis supported our suggestion that Sum1 prevents premature expression of the *NDT80* gene in sporulating cells.

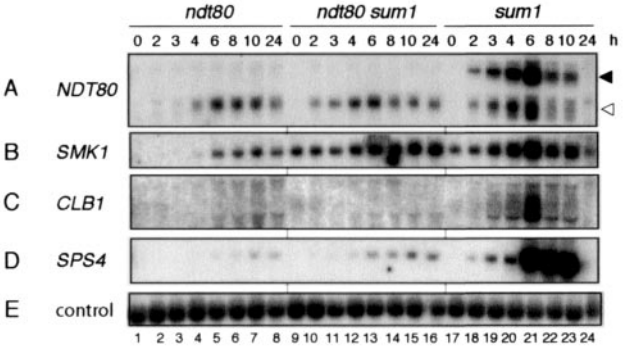

FIG. 5. *SUM1* prevents premature expression of the *NDT80* gene. Northern filters were prepared with RNA from Δ*ndt80*/Δ*ndt80* cells (lanes 1 to 8), Δ*ndt80*/Δ*ndt80* Δ*sum1*/Δ*sum1* cells (lanes 9 to 16), and Δ*sum1*/Δ*sum1* cells (lanes 17 to 24) that contained the plasmid-borne *(−505^{WT})ndt80* minigene. Cells were harvested during vegetative growth (0 h) or at the indicated time (in hours), as noted above panel A, after transfer of cells to sporulation medium. The filter was hybridized sequentially with the gene-specific probes denoted on the left. The closed and open arrowheads on the right of panel A denote the full-length chromosome-derived *NDT80* transcripts and the truncated *ndt80* minigene-derived transcripts, respectively.

The MSE sites prevent premature expression of the *NDT80* gene. To test the notion that Sum1 prevents early expression of the *(−505^{WT})ndt80* minigene by maintaining repression through the MSE^{NDT80} sites, we monitored expression of a minigene in which both MSEs had been deleted. As noted above, expression of the plasmid-borne *(−505^{WT})ndt80* minigene was coincident with expression of the chromosomal *NDT80* gene (Fig. 6A). Deletion of both MSEs advanced transcript accumulation to a modest extent; transcript accumulation from the *(−505$^{\Delta M1\Delta M2}$)ndt80* minigene was slightly higher than that from the chromosomal *NDT80* gene at 3 h of sporulation (Fig. 6B, lane 3, and data not shown). A longer autoradiographic exposure indicated that this was also the case at 2 h of sporulation (data not shown). The earlier onset of expression of the *(−505$^{\Delta M1\Delta M2}$)ndt80* minigene relative to the *(−505^{WT})ndt80* minigene was particularly evident in Δ*ndt80*/Δ*ndt80* cells (Fig. 6, compare panels A and B, lanes 9 to 16). We note that the temporal expression profiles of the MSE-less *(−505$^{\Delta M1\Delta M2}$)ndt80* minigene in wild-type cells (Fig. 6B, lanes 1 to 8) and in Δ*ndt80*/Δ*ndt80* cells (Fig. 6B, lanes 9 to 16) were similar to those of the *(−505^{WT})ndt80* minigene in Δ*sum1*/Δ*sum1* cells (Fig. 5A, lanes 17 to 24) and in Δ*ndt80*/Δ*ndt80* Δ*sum1*/Δ*sum1* cells (Fig. 5A, lanes 9 to 16), respectively. These observations support our suggestion (Fig. 2B) that Sum1 acts through the MSEs to prevent premature expression of the *NDT80* gene in sporulating cells. In addition, the MSE sites are

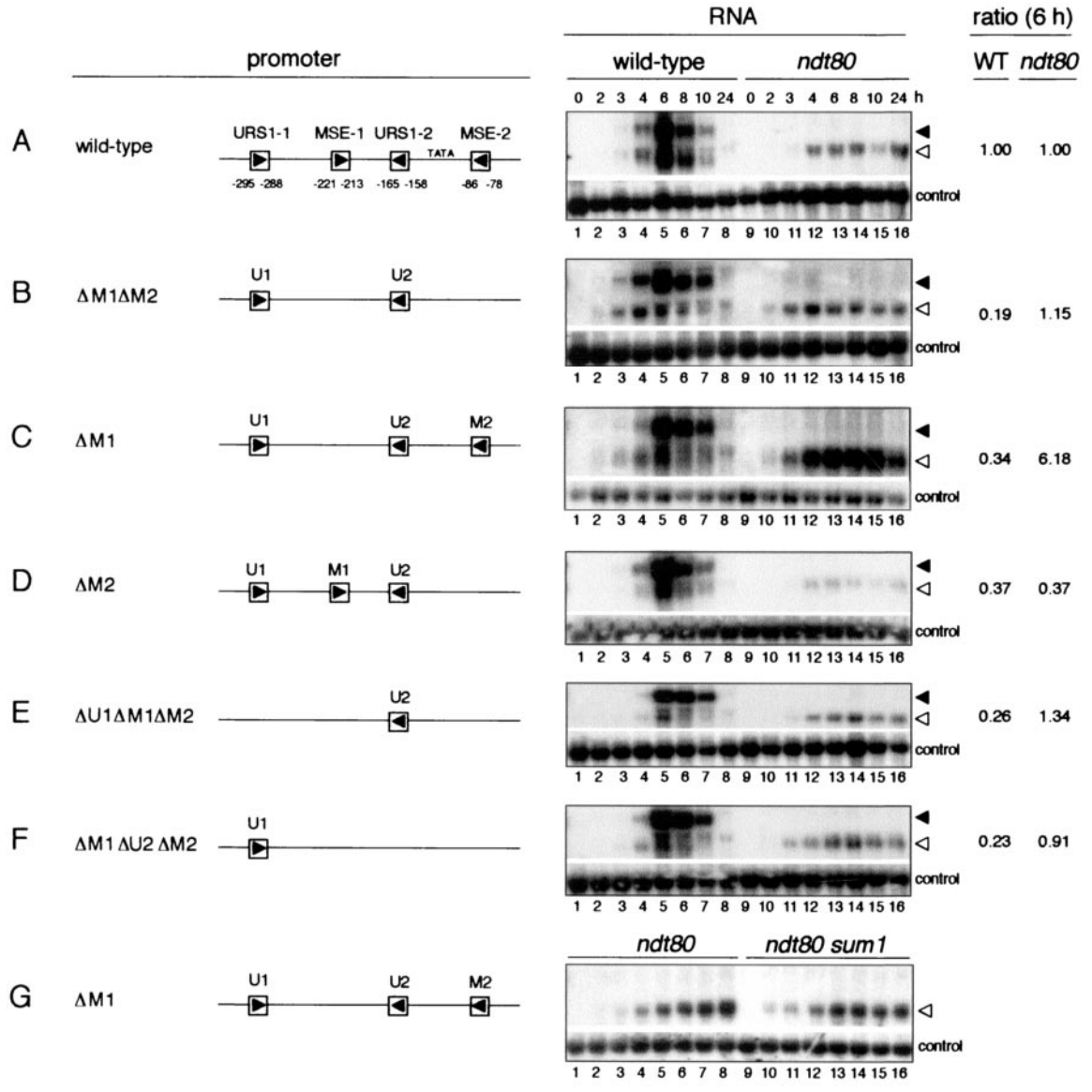

FIG. 6. The MSE sites prevent premature expression of the *NDT80* gene and mediate upregulation of its expression midway through sporulation. (A to F) The Northern filters represented under the RNA column heading contained RNA extracted from wild-type cells (lanes 1 to 8) and Δ*ndt80*/Δ*ndt80* cells (lanes 9 to 16) harvested during vegetative growth (0 h) or at the indicated times, as noted above the top panel (in hours), after transfer of cells to sporulation medium. (G) The Northern filter contained RNA extracted from Δ*ndt80*/Δ*ndt80* cells (lanes 1 to 8) and Δ*ndt80*/Δ*ndt80* Δ*sum1*/Δ*sum1* cells (lanes 9 to 16). Cells used for the experiment of each panel harbored the following plasmid-borne *ndt80* minigenes: (A) $(-505^{WT})ndt80$; (B) $(-505^{\Delta M1\Delta M2})ndt80$; (C and G) $(-505^{\Delta M1})ndt80$; (D) $(-505^{\Delta M2})ndt80$; (E) $(-505^{\Delta U1\Delta M1\Delta M2})ndt80$; (F) $(-505^{\Delta M1\Delta U2\Delta M2})ndt80$. The closed and open arrowheads on the right of panel A denote the full-length chromosome-derived *NDT80* transcripts and the truncated *ndt80* minigene-derived transcripts, respectively. For experimental details and explanations of nomenclature, see the legend to Fig. 4. The filter represented in panel A is the same as that shown in Fig. 4A.

required for Ndt80 to upregulate expression of the *NDT80* gene. The maximal level of expression of the plasmid-borne $(-505^{\Delta M1\Delta M2})ndt80$ minigene was only one-fifth that of the $(-505^{WT})ndt80$ minigene (Fig. 6A and B, lane 5).

Comparison of the expression patterns of *ndt80* minigenes lacking either MSE-1 or MSE-2 suggested that only MSE-1^{NDT80} had a role in preventing premature expression of the *NDT80* gene in wild-type cells, whereas both MSEs contributed to Ndt80-dependent upregulation of minigene expression (Fig. 6, compare panels A to D, lanes 1 to 8). Transcript accumulation from the $(-505^{\Delta M1})ndt80$ minigene (Fig. 6C, lane 5) and from the $(-505^{\Delta M2})ndt80$ minigene (Fig. 6D, lane 5) was approximately one-third that from the $(-505^{WT})ndt80$ minigene in wild-type cells at 6 h of sporulation (Fig. 6A, lane 5). We also monitored the effect of deletion of each URS1 element on expression of the $(-505^{\Delta M1\Delta M2})ndt80$ minigene. The levels of expression of the $(-505^{\Delta U1\Delta M1\Delta M2})ndt80$ minigene (Fig. 6E, lanes 1 to 8) and the $(-505^{\Delta M1\Delta U2\Delta M2})ndt80$ minigene (Fig. 6F, lanes 1 to 8) in wild-type cells were similar to each other and to that of the $(-505^{\Delta M1\Delta M2})ndt80$ minigene

(Fig. 6B, lanes 1 to 8). This was also the case for expression in Δ*ndt80*/Δ*ndt80* cells (Fig. 6B, E, and F, lanes 9 to 16). This suggested, as noted above, that both URS1^{NDT80} sites were able to promote expression of the *NDT80* gene; we could not, however, distinguish the individual contributions made by each URS1^{NDT80} site. It is possible that we could not reliably quantify the low levels of expression observed in Δ*ndt80*/Δ*ndt80* cells.

Unexpectedly, we found that not only was the *(−505$^{\Delta M1}$)ndt80* minigene expressed prematurely in Δ*ndt80*/Δ*ndt80* cells but also that it was expressed at a sixfold-higher level than the *(−505^{WT})ndt80* minigene in Δ*ndt80*/Δ*ndt80* cells (Fig. 6, compare panels A and C, lanes 9 to 16). This high level of expression of the *(−505$^{\Delta M1}$)ndt80* minigene required that MSE-2^{NDT80} be present (Fig. 6B and C, lanes 9 to 16). Thus, MSE-1^{NDT80} appeared to act as an operator that prevented Ndt80-independent activation from MSE-2^{NDT80}. To test the possibility that Sum1 might form a novel activation complex at MSE-2^{NDT80} in the absence of Ndt80, we compared expression of the *(−505$^{\Delta M1}$)ndt80* minigene in Δ*ndt80*/Δ*ndt80* cells and in Δ*ndt80*/Δ*ndt80* Δ*sum1*/Δ*sum1* cells in sporulation medium. We found that the minigene was expressed at similar levels in both Δ*ndt80*/Δ*ndt80* cells and in Δ*ndt80*/Δ*ndt80* Δ*sum1*/Δ*sum1* cells (Fig. 6G). Thus, Sum1 was not required for the Ndt80-independent, MSE-2^{NDT80}-mediated activation that was revealed on removal of the MSE-1^{NDT80} site. We have not explored this unusual expression pattern further.

The requirement for *IME2* for expression of the *NDT80* gene can be bypassed by mutation of *SUM1*. *IME2* is an early sporulation-specific gene that encodes a kinase (23, 53) that regulates several meiotic events, including the correct timing of premeiotic DNA synthesis (8, 9, 14), maximal expression of early meiotic genes (35, 42), and expression of middle sporulation-specific genes (35, 42) and of *NDT80* (17). We have investigated further the role of *IME2* in controlling the expression of *NDT80*.

Because it has been suggested that Ime2 promotes premeiotic DNA synthesis in early sporulating cells by causing the destruction of Sic1, a Cdk inhibitor, we first tested whether the absence of Sic1 might bypass the need for *IME2* not only for DNA synthesis (8) but also for activation of expression of *NDT80*. Northern blot analysis showed that *NDT80* is not expressed in Δ*ime2*/Δ*ime2* Δ*sic1*/Δ*sic1* cells after transfer to sporulation medium (data not shown). Thus, mutation of *SIC1* did not allow Ime2-independent expression of *NDT80.* Control experiments showed, as expected, that *NDT80* was expressed in Δ*sic1*/Δ*sic1* cells and was not expressed in Δ*ime2*/Δ*ime2* cells on transfer to sporulation medium (data not shown).

Taking into consideration our suggestion that the first phase of *NDT80* expression depended on a reduction in Sum1-mediated repression, we next tested whether the requirement for *IME2* in *NDT80* expression might reflect a role for Ime2 as a regulator of the activity of Sum1. We therefore monitored gene expression in cells of an Δ*ime2*/Δ*ime2* Δ*sum1*/Δ*sum1* strain. Whereas *NDT80* was expressed at a low level only after extended incubation of Δ*ime2*/Δ*ime2* cells in sporulation medium (Fig. 7A, lanes 1 to 8), *NDT80* transcripts could be detected at 2 h in Δ*ime2*/Δ*ime2* Δ*sum1*/Δ*sum1* cells (Fig. 7A, lanes 9 to 16), as was also the case in Δ*sum1*/Δ*sum1* cells (Fig. 7A, lanes 17 to 24; Fig. 5A, lanes 17 to 24), and by 8 h

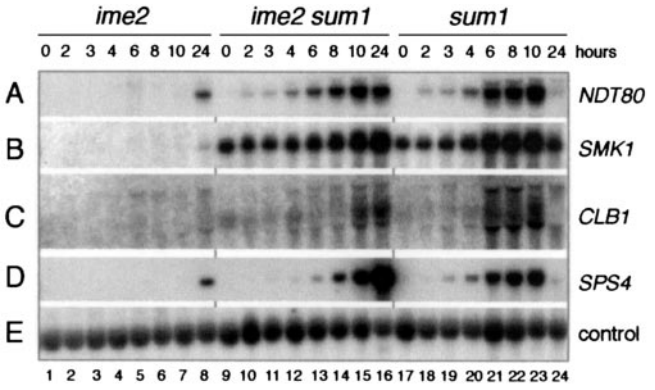

FIG. 7. *IME2* activates middle sporulation-specific gene expression by alleviating Sum1-mediated repression of the *NDT80* gene. A Northern filter was prepared with RNA from *ime2*/*ime2* cells (lanes 1 to 8), *ime2*/*ime2* Δ*sum1*/Δ*sum1* cells (lanes 9 to 16), and Δ*sum1*/Δ*sum1* cells (lanes 17 to 24) harvested during vegetative growth (lanes 1, 9, and 17) or at the indicated time after transfer of cells to sporulation medium, as noted above panel A. The filter was hybridized sequentially with the gene-specific probes denoted on the right.

significant transcript accumulation had occurred (Fig. 7A, lane 14). This suggested that Ime2 promoted the early phase of *NDT80* expression by inhibiting Sum1-dependent repression. Additionally, the Ndt80 that was expressed in the Δ*ime2*/Δ*ime2* Δ*sum1*/Δ*sum1* cells was active, as both *CLB1* and *SPS4* were also expressed in these cells but not in Δ*ime2*/Δ*ime2* cells in sporulation medium (Fig. 7C and D). Consistent with the observation that Ime2 plays multiple roles during sporulation (reviewed in reference 25), Δ*ime2*/Δ*ime2* Δ*sum1*/Δ*sum1* cells did not form spores. We concluded that *IME2* activates middle gene expression, at least in part, by relieving Sum1-mediated repression of *NDT80*, as noted in Fig. 2C.

The activity of Ndt80 is posttranslationally inhibited on activation of the meiotic recombination checkpoint (5, 17, 47). Although Ime2 is a candidate kinase for posttranslational activation of Ndt80, the experiment described above (Fig. 7) suggested that regulation of *NDT80* by *IME2* occurs primarily at the transcriptional level. Moreover, ectopically produced Ndt80 is capable of activating MSE-dependent gene expression in vegetatively growing cells, which do not express *IME2* (5). To test further the suggestion that Ime2 has no essential role in posttranscriptional regulation of Ndt80 in sporulating cells, we constructed a *HOP1-NDT80* fusion gene in which the ORF of the *NDT80* gene was under the control of the promoter of the early meiotic gene, *HOP1* (see Materials and Methods). Neither Ime2 nor Ndt80 are required for expression of *HOP1*, although Ime2 is involved in upregulation of its expression (reviewed in reference 49). We found that expression of *NDT80* in an Ime2-independent manner from the *HOP1-NDT80* fusion gene (Fig. 8A, lanes 10 to 18) was sufficient to activate expression of the middle sporulation-specific gene, *SPS4*, in cells of a Δ*ime2*/Δ*ime2* strain in sporulation medium (Fig. 8B, lanes 10 to 18). We note that although *NDT80* transcripts derived from the *HOP1-NDT80* gene could be detected at 2 to 3 h in the Δ*ime2*/Δ*ime2* strain, maximal accumulation of these transcripts occurred only at 10 h. This is consistent with the known requirement for *IME2* for the normal kinetics and

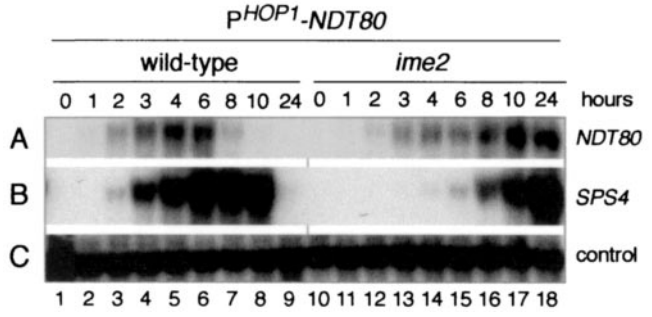

FIG. 8. Ndt80 that is ectopically expressed in *ime2/ime2* cells in sporulation medium is active. A Northern filter was prepared with RNA extracted from wild-type cells (lanes 1 to 9) and *ime2/ime2* cells (lanes 10 to 18) during vegetative growth (lanes 1 and 9) or at the indicated time after transfer of cells to sporulation medium, as noted above panel A. Both the wild-type cells and the *ime2/ime2* cells harbored a plasmid that contained a *HOP1-NDT80* fusion gene, in which the *NDT80* coding region was fused to the promoter region (see Materials and Methods). The filter was hybridized sequentially with the gene-specific probes denoted on the right.

full expression of early meiotic genes (35, 42). We infer that the delayed expression of Ndt80 accounted, at least in part, for the delayed expression of the Ndt80-dependent middle sporulation-specific gene *SPS4* in this experiment. Although it is possible that Ime2-dependent phosphorylation of Ndt80 contributes to its full activity, our results indicated that Ime2 kinase is not essential for the transcription factor activity of Ndt80.

DISCUSSION

Roles of the URS1^{NDT80} and MSE^{NDT80} sites in generating the premiddle expression profile of the *NDT80* gene. In this study we have examined the role of the URS1 and MSE sites that are present in the promoter region of the *NDT80* gene in regulation of its expression. We postulated that the overlapping windows of URS1- and MSE-mediated repression and activation were responsible for the distinctive premiddle expression pattern of the *NDT80* gene (Fig. 2). Our experiments showed that a plasmid-borne *ndt80* minigene lacking the URS1 elements was expressed in *NDT80/NDT80* cells with the same kinetics as a middle gene; the premiddle phase of expression did not occur and expression of the URS1-less minigene was entirely Ndt80 dependent. In contrast, a plasmid-borne *ndt80* minigene lacking the MSEs was expressed prematurely and was not significantly upregulated. Indeed, the levels of expression of the MSE-less minigene in wild-type cells and of the *(−505^{WT})ndt80* minigene in Δ*ndt80*/Δ*ndt80* cells were similar. We also found that the *(−505^{WT})ndt80* minigene was expressed prematurely on mutation of *SUM1* and that this early expression was independent of *NDT80*.

These observations support the idea that a Sum1-associated repression complex bound at MSE^{NDT80} prevents URS1^{NDT80}-tethered Ime1 from activating transcription of the *NDT80* gene at the time that Ime1 is activating the expression of early meiotic genes. Our data are consistent with the idea that a decrease in the efficiency of Sum1-mediated repression as cells progress through the early events of the sporulation program allows the previously inactive Ime1 tethered at the URS1^{NDT80} sites to promote a low level of expression of the *NDT80* gene. This initial expression would account for the Ime1 dependence of *NDT80* expression (5). This proposal is also supported by the observations that a transient reduction in the level of Sum1 protein occurs from 6 to 8 h of sporulation (29) and that mutation of *SUM1* leads to premature expression of *NDT80* (this study). This initial phase of URS1-dependent *NDT80* expression is followed by Ndt80-dependent upregulation of its own expression, which requires the MSE^{NDT80} sites and occurs concomitantly with Ndt80-dependent activation of a set of middle MSE-regulated sporulation-specific genes. The presumed replacement of Sum1 by Ndt80, particularly at the MSE-1^{NDT80} site, as cells progress into the middle phase of sporulation could be a reflection of a higher affinity of MSE-1^{NDT80} for Ndt80 than for Sum1 as well as a reduction in the level of Sum1 (29, 51).

We note that although Xie et al. (51) reported that MSE-2^{NDT80} acts as a more efficient Sum1-dependent operator than does MSE-1^{NDT80}, we found that MSE-1^{NDT80}, but not MSE-2^{NDT80}, was responsible for preventing expression of the *(−505^{WT})ndt80* minigene at early times of sporulation. It is possible that differing experimental approaches account for these differing observations. Whereas Xie et al. (51) monitored repression by testing the ability of MSE elements to substitute for the URS1^{HOP1} operator element in preventing expression of a *HOP1-lacZ* reporter gene in mitotic cells, we monitored the effect of deletion of the MSE on the temporal expression pattern of the *(−505)ndt80* minigene during sporulation. We note that examination of the role of MSE-2^{NDT80} in its natural context is complicated by its close juxtaposition to the putative TATA box of the *NDT80* gene. We cannot exclude the possibility that deletion of MSE-2^{NDT80} may have indirectly affected the function of the promoter (see below). However, we were also unable to detect operator activity for MSE-2^{NDT80} in the context of a *CYC1-lacZ* reporter gene in mitotic cells (data not shown).

As cells progress beyond the middle portion of the sporulation program, *NDT80* expression is downregulated in an *NDT80*-dependent manner. This downregulation does not occur in cells that arrest at pachytene (17). The turn-down of *NDT80* expression could occur in several ways. For example, it is possible that the transcriptional activation function or DNA-binding function of Ndt80 is inactivated by the product of a middle sporulation-specific gene. Alternatively, MSE-bound Ndt80 could once again be replaced by Sum1 or by a yet-to-be identified MSE-binding or MSE-tethered repressor that is encoded by a middle gene.

Additional regulators of expression of the *NDT80* gene? Although the key aspects of our model are supported by our data, some aspects of our data remain unexplained. For example, the observation that the *(−505$^{\Delta M1}$)ndt80* minigene was expressed to a relatively high level in Δ*ndt80*/Δ*ndt80* cells in sporulation medium, a situation which has little biological relevance, was unexpected. This unusual expression pattern, which was observed only with this minigene, depended on the MSE-2 site and did not occur in *NDT80/NDT80* cells. Because Δ*ndt80*/Δ*ndt80* cells, which arrest at pachytene, fail to turn down Ime1-mediated expression of early meiotic genes, it is possible that continued URS1-mediated expression of this

(−505$^{\Delta M1}$)ndt80 minigene allowed continued transcript accumulation. The dependence on MSE-2 might be a fortuitous reflection of the close juxtaposition of this site to the putative TATA element of the *NDT80* gene. As mentioned above, it is possible that deletion of MSE-2 might nonspecifically reduce the strength of the promoter. Although none of our experiments directly addressed this possibility, we note that several versions of the *ndt80* minigenes that lacked the MSE-2 site were expressed and therefore contained a functional promoter, but none was expressed at a high level. It is also possible that MSE-2 functions as an Ndt80-independent UAS. We have ruled out the possibility that Sum1 served a novel role as an activator when bound at MSE-2; expression of the (*−505$^{\Delta M1}$*)*ndt80* minigene was high in both Δ*ndt80*/Δ*ndt80* cells and Δ*ndt80*/Δ*ndt80* Δ*sum1*/Δ*sum1* cells.

In the course of this study we were surprised to find that multiple copies of a 21-bp fragment spanning URS1-1^{NDT80} or URS1-2^{NDT80} or a 22-bp fragment spanning MSE-1^{NDT80} or MSE-2^{NDT80} failed to act as operator elements when inserted into the *CYC1-lacZ* reporter gene (data not shown). The observation that mutation of both *UME6* and *SUM1* was required to allow expression of the *CYC1-(URS1-1-MSE-1)^{NDT80}-lacZ* reporter gene in mitotic cells (Fig. 3) suggested that both URS1-1^{NDT80} and MSE-1^{NDT80} promoted the assembly of a repression complex. It is possible that repression complexes were efficiently assembled at these sites only in the context of the *NDT80*-derived sequence present in the 91-bp fragment. For example, it is possible that an expanded site provided increased affinity for binding of the protein or an accessory factor relative to the 21-bp or 22-bp sites. It is also possible that an additional site within the 91-bp *NDT80*-derived sequence acted in conjunction with either the URS1-1^{NDT80} site or the MSE-1^{NDT80} site to direct efficient repression.

It is clear that there are additional complexities in the *NDT80* promoter that remain to be elucidated. As suggested by Xie et al. (51), the relative affinities of Ndt80 and Sum1 for MSE sites could be sequence and context dependent. The DNA-binding and regulatory activities of Ndt80 and Sum1 could be affected by posttranslational modifications as well as by cofactors. Other DNA elements, including cryptic UAS sites in the vector sequence and sequences within the first 880 bp of the coding region of *NDT80*, could influence expression of the minigene. Also, unidentified regulators may exist that bind to the URS1, MSE, or other elements in the promoter region of the *NDT80* gene and contribute to its regulated expression.

Coordinate regulation of expression of the *NDT80* and *SMK1* genes. The sporulation-specific gene *SMK1*, which encodes a mitogen-activated protein kinase that is required for spore wall development, was initially considered to be expressed as a middle gene (24, 37). Other studies, however, indicate that *SMK1* is a member of the premiddle class of sporulation genes. *SMK1* transcripts are first detected after the onset of expression of early meiotic genes and prior to activation of middle sporulation-specific genes and the expression of *SMK1* is subsequently upregulated in an Ndt80-dependent manner (5, 17). Consistent with their pattern of coregulation, both *NDT80* and *SMK1*, but not middle sporulation-specific genes, are expressed at a low level in cells that arrest at pachytene in response to defects in meiotic recombination (5, 17, 29).

Three regulatory elements have been identified in the promoter region of the *SMK1* gene: an Abf1-binding site, a URS1 element, and an MSE (37). The Abf1-binding site acts nonspecifically to upregulate expression of the *SMK1* gene (37). The MSE^{SMK1} site prevents *SMK1* expression in mitotic and early meiotic cells and upregulates its expression midway into the sporulation program (37). On mutation of the MSE^{SMK1} site, the URS1^{SMK1} site acts early during sporulation to direct a low level of expression of the *SMK1* gene (37). Thus, for both the *NDT80* gene and the *SMK1* gene, a combination of URS1 and MSE sites appears to be responsible for setting the premiddle pattern of gene expression. It will be interesting to analyze in greater detail the expression pattern of other sporulation-specific genes that contain both URS1 and MSE sites in their promoter region (6, 38)

Roles of *IME2* and *SUM1* in *NDT80* expression. *IME2* encodes a protein kinase that is required for multiple processes during sporulation, including full expression and subsequent downregulation of expression of early meiotic genes (35), the degradation of Sic1 (8), the correct timing of premeiotic DNA replication (9, 14), expression of *NDT80* (17), and activation of middle sporulation-specific gene expression (35, 42). We have shown that the requirement for Ime2 in the activation of middle sporulation-specific genes is a consequence of its key role in expression of *NDT80*. The middle sporulation-specific gene *SPS4* was expressed in Δ*ime2*/Δ*ime2* cells that ectopically expressed *NDT80* from the *HOP1* promoter. Based on our observation that *NDT80* is expressed in Δ*ime2*/Δ*ime2* Δ*sum1*/Δ*sum1* cells, we have speculated that Ime2 inactivates Sum1. Moreover, our observation that mutation of *SUM1* bypasses the requirement for Ime2 for both the expression and transcriptional activity of *NDT80* indicates that Ime2 has no essential role as a direct activator of Ndt80. It remains possible, however, that Ime2-dependent phosphorylation of Ndt80 contributes to its full activity.

How does Ime2 inactivate Sum1? Lindgren et al. (29) demonstrated that although the level of *SUM1* mRNA remains constant through sporulation, the level of Sum1 protein fluctuates, reaching its lowest level around the time that middle sporulation-specific genes are induced. This suggests that Ime2 might be a regulator of degradation of Sum1, with Sum1 being stabilized in Δ*ime2*/Δ*ime2* cells. It is tantalizing to suggest that Ime2 might serve this function directly by phosphorylating Sum1 in a manner that targets it for degradation. This is analogous to the recent observation that the availability of Ime1 is regulated by Ime2-dependent phosphorylation targeting it for degradation (15).

Regulation of *NDT80* expression and activity by the meiotic recombination checkpoint. Defects in certain aspects of meiotic recombination and chromosome synapsis during sporulation generate a checkpoint signal that is transmitted to downstream targets and leads to arrest at pachytene, prior to entry into the meiotic divisions (reviewed in reference 39). *NDT80* is one of the targets of this checkpoint. The expression of *NDT80* is reduced and the ability of Ndt80 to activate expression of middle sporulation-specific genes is inhibited when the meiotic recombination checkpoint is triggered (5, 17, 52). On the basis of the present study, we suggest that the low level of *NDT80* expression that is observed in checkpoint-arrested cells reflects URS1^{NDT80}-dependent expression.

Our data suggest that Sum1 is regulated by Ime2 (see above). Interestingly, Sum1 is also a target of the meiotic recombination checkpoint; on activation of this checkpoint, Sum1 is stabilized (29). An intriguing possibility is that Ime2 transmits the checkpoint signal to Sum1; in this case, the checkpoint would prevent Ime2 from acting to destabilize Sum1. If this were the case, then putative checkpoint-mediated inactivation of Ime2 would occur only after Ime2 had served its role in promoting the initial phase of *NDT80* expression. Subsequent checkpoint-mediated stabilization of Sum1 would allow Sum1-dependent repression of *NDT80* gene expression to be reestablished. Additionally, any Ndt80 that had been synthesized would have its transcription factor activity inhibited. Further study will lead to a more complete understanding of the regulatory events that coordinate the sporulation-specific transcriptional cascade with progression through the sporulation program.

ACKNOWLEDGMENTS

We thank Ed Winter and his coworkers for communicating results prior to publication and for helpful discussions. We thank Helena Friesen and Ghadeer Shubassi for their helpful comments on the manuscript.

This work was supported by a Canadian Institute of Health Research grant (MOP-6826) to J.S. J.P. was supported in part by an Ontario Government Scholarship for Science and Technology and by a University of Toronto Open Fellowship.

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