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NDT80 and the Meiotic Recombination Checkpoint Regulate Expression of Middle Sporulation-Specific Genes in *Saccharomyces cerevisiae*

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Distinct classes of sporulation-specific genes are sequentially expressed during the process of spore formation in *Saccharomyces cerevisiae*. The transition from expression of early meiotic genes to expression of middle sporulation-specific genes occurs at about the time that cells exit from pachytene and form the meiosis I spindle. To identify genes encoding potential regulators of middle sporulation-specific gene expression, we screened for mutants that expressed early meiotic genes but failed to express middle sporulation-specific genes. We identified mutant alleles of *RPD3*, *SIN3*, and *NDT80* in this screen. Rpd3p, a histone deacetylase, and Sin3p are global modulators of gene expression. Ndt80p promotes entry into the meiotic divisions. We found that entry into the meiotic divisions was not required for activation of middle sporulation genes; these genes were efficiently expressed in a *clb1 clb3 clb4* strain, which fails to enter the meiotic divisions due to reduced Clb-dependent activation of Cdc28p kinase. In contrast, middle sporulation genes were not expressed in a *dmc1* strain, which fails to enter the meiotic divisions because a defect in meiotic recombination leads to a *RAD17*-dependent checkpoint arrest. Expression of middle sporulation genes, as well as entry into the meiotic divisions, was restored to a *dmc1* strain by mutation of *RAD17*. Our studies also revealed that *NDT80* was a temporally distinct, pre-middle sporulation gene and that its expression was reduced, but not abolished, on mutation of *DMC1*, *RPD3*, *SIN3*, or *NDT80* itself. In summary, our data indicate that Ndt80p is required for expression of middle sporulation genes and that the activity of Ndt80p is controlled by the meiotic recombination checkpoint. Thus, middle genes are expressed only on completion of meiotic recombination and subsequent generation of an active form of Ndt80p.

Sporulation is a process of cellular differentiation that is initiated in diploid **a**/α cells of the yeast *Saccharomyces cerevisiae* in response to nitrogen starvation in the presence of a nonfermentable carbon source. As a cell progresses through the events of meiosis and spore wall formation, a coordinated series of genetic and morphological events generates a tetrad of haploid spores within an ascus (reviewed in references 9 and 30). The sporulation program begins when starved cells exit the mitotic cell cycle and undergo premeiotic DNA replication. This is followed by a lengthy prophase in which homologous chromosomes pair, the synaptonemal complex (SC) is elaborated, and a high level of genetic recombination occurs. At pachytene, the penultimate stage of prophase and the last stage at which cells can return to mitotic growth, the chromosomes are fully synapsed, and the spindle pole bodies lie side-by-side in the nuclear envelope. As cells exit from pachytene, the SC disassembles, chiasmata appear, and the spindle pole bodies separate to form the meiosis I spindle. The two meiotic divisions occur in rapid succession, leading first to segregation of homologous chromosomes and then to segregation of sister chromatids, giving rise to a four-lobed nucleus. The prospore walls, which initiate as a membranous outgrowth from a modified outer plaque of the spindle pole bodies, expand around each nuclear lobe. Ultimately, a haploid nucleus and a portion of maternal cytoplasmic material is engulfed within each prospore. Maturation of the spore wall into a multilayered structure completes the process of spore formation. The successive events of meiosis and spore formation are dependent on the ordered expression of at least four classes of genes, referred to as early, middle, mid-late, and late based on their time of expression during sporulation (reviewed in references 30 and 39). This temporal pattern of gene expression during sporulation in *S. cerevisiae* provides a simple model for studying the mechanisms by which morphological and genetic changes might control gene expression during development.

Expression of early meiotic genes, such as *HOP1* and *DMC1*, which are involved in pairing and synapsis of homologous chromosomes and meiotic recombination, is regulated by the transcription factors Ime1p and Ume6p (reviewed in reference 30). Repression of early meiotic genes in vegetative cells is mediated by Ume6p, which binds to an URS1 promoter element and recruits a Sin3p-Rpd3p-containing repression complex to the DNA (23). *IME1*, a key inducer of meiosis, is rapidly upregulated when diploid cells are transferred to sporulation medium. An Ime1p-Rim11p activation complex, which replaces the Sin3p-Rpd3p repression complex (7, 36, 52, 63), then acts in conjunction with other upstream activation sequence (UAS)-bound proteins, such as Abf1p (15), to promote expression of early meiotic genes. Ime2p kinase, an early meiotic gene product, acts in a second pathway to bring about full activation of early meiotic genes (reviewed in reference 39).

As cells enter the meiotic divisions, expression of early meiotic genes ceases and expression of middle sporulation genes begins. Activation of middle sporulation genes, such as *SPS1* and *SMK1*, which contribute to spore wall formation (14, 29),

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

Strain	Genotype	Source
SK1 derivatives		
DKB414	*MAT***a** *ho::LYS2 lys2 ura3 leu2 his4X-ADE2-his4B ade2 spo13::hisG*	D. Bishop (4)
DKB414-1	DKB414 + pRS315-MATα	This study
DKB414B	*MAT*α derivative of DKB414	This study
DKB414C	DKB414 × DKB414B	This study
SRH415B	Δ*rpd3::URA3* derivative of DKB414B	This study
SRH416B	Δ*sin3::URA3* derivative of DKB414B	This study
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 (35)
DKB608	*MAT***a**/*MAT*α *lys2*/*lys2 ho::LYS2*/*ho::LYS2 ura3*/*ura3 leu2::hisG*/*leu2::hisG arg4-Nsp*I/*arg4-Nsp*I Δ*dmc1::ARG4*/Δ*dmc1::ARG4*	D. Bishop (35)
DKB1170	*MAT***a**/*MAT*α *lys2*/*lys2 ho::LYS2*/*ho::LYS2 ura3*/*ura3 leu2::hisG*/*leu2::hisG arg4-Bgl*II/*arg4-Nsp*I *his4B::LEU2*/*his4X::LEU2-URA3* Δ*rad17::hisG*/Δ*rad17::hisG* Δ*dmc1::ARG4*/Δ*dmc1::ARG4*	D. Bishop (35)
NKY2296	*MAT***a**/*MAT*α *ho::LYS2*/*ho::LYS2 lys2*/*lys2 ura3*/*ura3 leu2*/*leu2* Δ*ndt80::LEU2*/Δ*ndt80::LEU2*	N. Kleckner (79)
NKY2296B	Haploid *MAT*α derivative of NKY2296	This study
W303 derivatives		
W3031A	*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*	S. Lindquist (47)
W3031B	*MAT*α *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*	S. Lindquist (47)
LP112	W3031A × W3031B	S. Lindquist (47)
YSH2	*ume6::LEU2*/*ume6::LEU2* derivative of LP112	This laboratory (19)
YKE2	*ime2::LEU2*/*ime2::LEU2* derivative of LP112	This laboratory (19)
YKE2α	*ime2::LEU2* derivative of W3031B	This laboratory (19)
CD140	*clb1::URA3*/*clb1::URA3 clb3::TRP1*/*clb3::TRP1 clb4::HIS3*/*clb4::HIS3* derivative of LP112	B. Futcher (13)

is dependent on Ime2p kinase activity (40, 62). A sporulation-specific activation element, referred to as the middle sporulation element (MSE), has been defined in the promoters of the *SPS4* and *SPR3* genes, and sequence inspection reveals that similar sites are present in the 5′-flanking sequence of other middle sporulation genes (19, 43).

The transition from expression of early meiotic genes to expression of middle sporulation genes occurs at about the time that cells exit from prophase and form the meiosis I spindle. Commencement of the meiotic divisions, which is dependent on activation of maturation-promoting factor (MPF), is an important regulatory point in the meiotic cell cycle of many organisms (reviewed in reference 44). MPF refers to the complex of cyclin-dependent kinase with its regulatory partner, a B-type cyclin. Several yeast mutants that are defective at intermediate stages of recombination arrest in pachytene, at the end of prophase (2, 4, 37, 38, 48, 68, 70). During meiotic recombination, recombination complexes provide a signal that activates the meiotic recombination checkpoint, preventing exit from pachytene. This pachytene arrest requires the mitotic DNA damage checkpoint genes *RAD17*, *RAD24*, and *MEC1* (35) and the meiosis-specific recombination genes *RED1* and *MEK1* (78). Completion of recombination events removes this signal, allowing entry into meiosis I (4, 35, 78, 79). Exit from pachytene also appears to be regulated by other factors (34, 49, 71); for example, mutation of *NDT80* leads to pachytene arrest in the absence of any significant defects in meiotic recombination (79).

In this study, we identified *SIN3*, *RPD3*, and *NDT80* in a genetic screen for regulators of middle sporulation gene expression. The proposal by Xu and colleagues (79) that Ndt80p might control entry into meiosis by modulating the activity of MPF prompted us to test whether MPF activity itself was required for activation of middle sporulation genes. We found that middle sporulation genes were efficiently expressed in cells of a *clb1 clb3 clb4* strain, which are deficient in MPF activity and are unable to enter meiosis (13, 18). Thus, entry into meiosis is not a prerequisite for activation of middle sporulation genes. In contrast, we found that mutation of *DMC1*, which leads to a defect in the strand exchange step of meiotic recombination and triggers pachytene arrest (4, 78), blocked activation of middle sporulation genes. Preventing pachytene arrest by mutation of the meiotic recombination checkpoint gene *RAD17* restored middle sporulation gene expression to *dmc1* cells. These results suggest that the meiotic recombination checkpoint regulates both entry into meiosis and activation of middle sporulation gene expression. The recent demonstration that Ndt80p is a transcriptional activator that binds to the MSE element (10) accounts for our isolation of *NDT80* in a screen for regulators of middle gene expression.

MATERIALS AND METHODS

Yeast strains and genetic procedures. Table 1 lists the *S. cerevisiae* strains used in this study. DKB414-1 is a derivative of the *MAT***a** *spo13* strain DKB414 (provided by D. Bishop) that contained the plasmid pRS315-MATα and was used to screen for mutants. We found that an SK1-derived strain was more proficient than our standard W303-derived laboratory strain at carrying out haploid meiosis and at promoting a high level of expression of a UAS^{SPS4}-*lacZ* reporter gene during sporulation. However, cells from an SK1 strain background lost the ability to grow on acetate more frequently than did cells from a W303 strain background; since growth on nonfermentable carbon is a prerequisite for sporulation, we routinely checked putative mutants for their ability to grow on acetate-containing medium. NKY2296 (79) (provided by N. Kleckner) was transformed with pNKY1212, a plasmid that contains *NDT80* (79), and sporulated to obtain the haploid *MAT*α Δ*ndt80* strain NKY2296B. The wild-type diploid strain DKB98 and its isogenic *dmc1*/*dmc1* and *dmc1*/*dmc1 rad17*/*rad17* derivatives, DKB608 and DKB1170, respectively, were provided by D. Bishop (35). Construction of *ime2*/*ime2* (YKE2) (19) and *clb1*/*clb1 clb3*/*clb3 clb4*/*clb4* (CD140; provided by B. Futcher) (13) derivatives of W303 have been described previously.

Isogenic Δ*rpd3::URA3* and Δ*sin3::URA3* derivatives of DKB414B were obtained by integrative transformation (51) with *Xba*I-linearized pMV130 and *Sma*I-linearized pMV117, respectively (provided by R. Gaber) (73, 74). The Δ*spo11::hisG* allele was introduced into various strains by integrative transformation with *Xba*I- and *Bgl*II-digested pGB518 (provided by C. Giroux), which contains a Δ*spo11::hisG-URA3-hisG* allele. Appropriate integrative transformation was confirmed by Southern blot analysis of genomic DNA from Ura^+ transformants. Strains with the Δ*spo11::hisG-URA3-hisG* allele were grown on SD medium (see below) containing 5-fluoro-orotic acid (5-FOA) to select for cells that had lost the *URA3* gene (1). The Δ*rad17::hisG* allele was introduced in the same manner by using *Bam*HI-digested pWL7, which contains the Δ*rad17::hisG-URA3-hisG* allele (provided by T. Weinert).

Yeast transformations were performed by the lithium acetate method (17). Standard genetic methods were used for mating, sporulation, and tetrad analysis (57, 58). Isogenic strains of the opposite mating type were obtained by using

pGAL-HO (22) to induce mating-type switching (12). Diploids were obtained by prototrophic selection, taking advantage of either chromosomal markers or markers introduced on plasmids for this purpose.

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 (YEPD), presporulation medium (YEPA), and sporulation medium (SPO) were as described previously (19) with the exception that for SK1-derived strains YEPA contained 2% potassium acetate, 2% Bacto Peptone, and 1% yeast extract and SPO medium contained 2% potassium acetate supplemented with adenine sulfate, histidine, leucine, lysine, tryptophan, and uracil at the same concentrations as those in SD medium. All yeast cultures were grown at 30°C.

Sporulation of cells of W303-derived strains in liquid culture was described previously (19). For sporulation of SK1-derived strains, cells taken from a YEPA plate were grown to late log phase in YEPD and this culture was used to inoculate YEPA medium (1:100 dilution). When the YEPA culture reached a density of 1.5×10^7 to 2.0×10^7 cells per ml, the cells were harvested by centrifugation, washed once in water, and resuspended in SPO medium at a density of 1.5×10^7 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 300 or more cells by light microscopy.

Plasmids. The multicopy *CYC1-lacZ*-containing plasmids pLGΔ312(Bgl), pLGΔ312SΔSS(Bgl), and pCYC1-SPS4-lacZ [previously referred to as 29×4/pLGΔ312(Bgl)], have been described previously (19). pSPS4-lacZ, which contains four copies of fragment 29 (nucleotides −145 to −116 of the *SPS4* gene) adjacent to a *CYC1-lacZ* gene that lacks a UAS, was constructed as follows. A *Sal*I-*Sal*I fragment recovered from pCYC1-SPS4-lacZ was cloned into the *Bgl*II site of pLGΔ312SΔSS(Bgl) after the restriction endonuclease-generated ends of the vector and the insert had been filled in with the Klenow form of DNA polymerase. Forward orientation of the insert was confirmed by dideoxy sequence analysis. pRS315-MATα was constructed by cloning a 4.2-kb *MAT*α-containing *Hin*dIII fragment from pJM9 (provided by A. Mitchell) into the *Hin*dIII site of pRS315 (61). pAV79B contains a *HOP1-lacZ* translational fusion gene (provided by A. Vershon) (72). The YCp50-based plasmids pMV1 and pMV34 contain *RPD1* and *RPD3*, respectively (provided by R. Gaber) (73, 74). p(SPO13)8 is a YCp50-based plasmid which contains the *SPO13* gene (provided by C. Giroux) (8). All plasmids were amplified in *Escherichia coli* DH5α.

Assay for β-galactosidase activity. β-Galactosidase expression from *lacZ* reporter genes was monitored by use of a colony overlay assay (3). Ten milliliters of X-Gal-containing agar (0.5% agar, 0.5 M potassium phosphate [pH 7.0], 6% dimethyl formamide, 0.1% sodium dodecyl sulfate, 0.1 to 0.4 mg of 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside [X-Gal] per ml) was poured over colonies on plates, and the plates were incubated at 30°C until blue color development was evident.

Screens for mutants defective in expression of *SPS4*. One screen, aimed at the identification of mutants that failed to activate a *UAS^{SPS4}-lacZ* reporter gene during sporulation, was carried out with strain DKB414-1 containing pSPS4-lacZ. Cells that had been mutagenized to ~50% survival by exposure to ethyl methanesulfonate (EMS) in accordance with the procedure of Lawrence (33) were plated on SD−Leu−Ura medium at 200 to 500 cells per plate and incubated for 4 to 5 days. About 32,000 colonies were patched onto SD−Leu−Ura medium, incubated for 1 to 2 days, and then replica plated to SPO medium. The SPO plates were incubated for 14 to 18 h, and the patches were then overlaid with X-Gal-containing agar (see above). We recovered 258 mutants that failed to form blue patches in this assay and that retained the ability to grow on acetate-containing YEPA medium. To perform secondary tests, these mutant strains were grown on SD medium containing 5-FOA to select for cells that had lost pSPS4-lacZ (5). Each mutant was then cotransformed in duplicate with pRS315-MATα and either pSPS4-lacZ or pAV79B, which contains *HOP1-lacZ*. The transformants that grew readily on YEPA medium were identified, patched onto SD−Leu−Ura medium, grown for 1 to 2 days, and then patched in duplicate onto SPO medium to retest for expression of *UAS^{SPS4}-lacZ* and to test for expression of *HOP1-lacZ*. Most mutants were discarded because they expressed *UAS^{SPS4}-lacZ* or because they showed a reduced or undetectable level of *HOP1-lacZ* expression on transfer to sporulation medium. Three mutant strains, m51, m312, and m320, that had the desired phenotype were identified; they expressed *HOP1-lacZ* at a high level and *UAS^{SPS4}-lacZ* at a reduced or undetectable level. These strains were found subsequently to contain the mutant alleles *das1-1*, *das3-1*, and *das1-2*, respectively. The gene designation refers to defective in activation of *SPS4*. We found that *DAS1* was identical to *NDT80* and that *DAS3* was identical to *SIN3*.

Another screen was aimed at the identification of mutants that expressed a *CYC1-UAS^{SPS4}-lacZ* reporter gene in vegetative cells. Mutagenized cells of strain DKB414-1 that contained pCYC1-SPS4-lacZ were plated onto SD−Leu−Ura medium and incubated for 3 to 4 days to give about 97,000 colonies that were then overlaid with X-Gal-containing agar. We recovered cells from 115 colonies that expressed a high level of β-galactosidase. These strains were grown on SD−Leu−Ura medium, retested for expression of the *CYC1-UAS^{SPS4}-lacZ* reporter gene, and then grown on SD medium containing 5-FOA to select for cells that had lost pCYC1-SPS4-lacZ (5). In secondary tests, 113 of the resulting strains were cotransformed in triplicate with pRS315-MATα and one of the following plasmids: pCYC1-SPS4-lacZ, to retest for expression of the *CYC1-UAS^{SPS4}-lacZ* gene in vegetative cells; pLGΔ312SΔSS(Bgl), to test for expression of a *CYC1-lacZ* gene that lacks a UAS in vegetative cells; or pSPS4-lacZ, to test for expression of a *UAS^{SPS4}-lacZ* gene in cells transferred to sporulation medium. These tests eliminated 97 strains: 30 were false positives, 2 expressed an elevated level of the *CYC1-lacZ* gene that lacked a UAS, and 65 expressed a high level of *UAS^{SPS4}-lacZ* and formed spores on transfer to sporulation medium. Of the remaining 16 strains which were defective in expression of *UAS^{SPS4}-lacZ*, 2 (m83 and m96) were transformed with pAV79B and were found to express the *HOP1-lacZ* reporter gene on transfer to sporulation medium. These strains were found subsequently to contain the mutant alleles *das4-1* and *das4-2*, respectively, with *DAS4* being identical to *RPD3*.

Genetic analysis. Mutants were placed into complementation groups by using standard techniques (57). Allelism with *IME2*, *SIN3*, *RPD3*, and *NDT80* was determined by mating *das* mutants with YKE2α, SRH416B, SRH415B, and NKY2296B, respectively, and testing the resultant diploids for spore formation.

Cloning *DAS4* by complementation. An **a**/α *das4-1*/*das4-1* strain that contained pSPS4-lacZ was transformed with a pSB32-based (*CEN ARS LEU2*) library that consisted of a partial *Sau*3A digest of genomic DNA (constructed by P. Hieter and provided by B. Andrews) (50). Transformants were selected on SD−Leu−Ura medium and then replica plated to SPO medium. After incubation for 14 to 16 h, colonies that expressed a high level of β-galactosidase were identified by an overlay assay. These strains were retested for expression of *UAS^{SPS4}-lacZ* and examined for spore formation. After complemented strains had been grown on medium containing 5-FOA to select for loss of pSPS4-lacZ, library plasmids were recovered by introducing total yeast genomic DNA into *E. coli*. Purified library plasmids were retested for their ability to complement the Spo$^-$ phenotype of the *das4-1*/*das4-1* strain. Complementing plasmids were subjected to partial dideoxy sequence analysis with primers that flanked the site of insertion of genomic yeast DNA into pSB32. The resultant sequence was used in a BLAST search of the Saccharomyces Genome Database (http://genome-www.stanford.edu/Saccharomyces) to identify the chromosomal region that was present in the complementing plasmid.

Transposon disruption mutagenesis. pDAS4-32, which contained yeast genomic DNA that complemented the sporulation defect of an **a**/α *das4-1*/*das4-1* strain, was mutagenized by transposon-mediated disruption with a *HIS3*-tagged version of Tn*1000* (γδ) as described previously (41, 56). Donor and recipient *E. coli* strains were provided by M. Donoviel and B. Andrews. The site of transposon insertion in a plasmid that was unable to complement the **a**/α *das4-1*/*das4-1* mutant was determined by dideoxy sequence analysis with primers that annealed to the ends of the transposon (41, 56). The resultant sequence was used in a BLAST search of the Saccharomyces Genome Database (see above).

FACS analysis. The relative DNA content of propidium iodide-stained cells was determined by fluorescence-activated cell sorter (FACS) analysis as previously described (14). For each experiment, 10,000 gated events were analyzed with LYSYS II software.

DAPI staining and microscopy. Cells to be stained with 4′,6-diamidino-2-phenylindole (DAPI) were harvested from about 1 ml of culture, fixed by resuspension in 70% ethanol, and stored at 4°C. Just prior to use, cells were pelleted by centrifugation and resuspended in 50 μl of water. Three microliters of this cell suspension was placed on a glass slide and mixed with 3 μl of a solution that contained 0.6 mg of DAPI per ml of mounting medium (90% glycerol and 10% antiquenching solution [10 mg of *p*-phenylenediamine per ml of phosphate-buffered saline, pH 9.0]). To monitor the meiotic divisions, the cells were then examined with a Nikon Microphot FXA microscope with Nomarski and fluorescence optics.

RNA isolation and Northern analysis. RNA was prepared from yeast as described previously (46), except for the experiment shown in Fig. 6. In that case, cells harvested from 50-ml cultures were disrupted by a freeze-thaw procedure (55) and RNA was purified as described previously (55) with the addition of a lithium chloride and an ethanol precipitation of RNA. Northern blot analysis was performed as previously described (19). Gene-specific probes were prepared with the following templates: *SMK1*, an 800-bp *Sty*I-*Sty*I fragment from pLAKK40 (29); *NDT80*, a 1.2-kb *Eco*47III-*Bam*HI fragment from pNKY1212 (79); *SPS100*, a 750-bp *Bam*HI-*Nco*I fragment from pE18-B8a (32); and pC4, an uncharacterized control gene (32). Other templates were as described previously (19).

RESULTS

Isolation of mutants that are defective in expression of the *SPS4* gene. We used the haploid meiosis strain DKB414-1, which is a *spo13* derivative of SK1, to screen for mutations in potential regulators of expression of *SPS4*, a middle sporulation gene. We previously defined a 15-bp MSE, which we refer to as UAS^{SPS4}, in the promoter region of this gene that is sufficient to direct sporulation-specific gene expression (19). The presence of a *MAT*α-containing plasmid in the *MAT***a**

strain DKB414-1 enables cells to initiate meiosis in response to nutrient starvation. The *spo13* mutation causes the cells to bypass what would be a lethal meiosis I division and to enter directly into meiosis II, a mitosis-like division in which sister chromatids are segregated (reviewed in reference 30). Subsequent formation of spore walls generates a dyad of viable haploid spores within each ascus. Strain DKB414-1, therefore, allows identification of recessive mutations affecting sporulation-specific events.

We carried out two screens aimed at the identification of mutants that fail to activate middle sporulation genes. In the first screen, we monitored the expression of the plasmid-borne *UAS^{SPS4}-lacZ* reporter gene, which has four copies of a UAS^{SPS4}-containing fragment upstream of a *CYC1-lacZ* gene that lacks a UAS (see Materials and Methods). This gene was activated midway through sporulation in wild-type cells (data not shown) (see reference 19). Colonies derived from 32,000 survivors of EMS mutagenesis were transferred to sporulation medium and tested for expression of the reporter gene by using an X-Gal overlay assay to monitor β-galactosidase activity (see Materials and Methods). Three mutants, m51, m312, and m320, were chosen for further study because their phenotype was as expected for mutants that entered the sporulation program but could not activate expression of middle sporulation genes: the strains responded appropriately to nutrient starvation and activated early meiotic genes as assessed by expression of the *IME1*-dependent reporter gene, *HOP1-lacZ*; they reproducibly failed to activate expression of the *UAS^{SPS4}-lacZ* reporter gene; and they were defective in spore formation (see Materials and Methods) (Fig. 1 and data not shown). Because *SPS4* is not required for spore formation (16), we hypothesized that a general defect in expression of middle sporulation genes caused the asporogenous phenotype of the mutant strains.

The second screen took advantage of our previous observation that the *CYC1-UAS^{SPS4}-lacZ* reporter gene, which has four copies of a UAS^{SPS4}-containing fragment inserted between the UAS^{CYC1} and the TATA^{CYC1}, is not expressed in vegetatively growing cells (19). Because the ability of UAS^{SPS4} to inhibit expression of the reporter gene is position dependent, we hypothesized that a protein that is present in vegetative cells binds to UAS^{SPS4} and prevents UAS^{CYC1}-bound activators from functioning via an indirect mechanism such as steric hindrance (19). We therefore screened EMS-mutagenized cells for mutants that allowed expression of the *CYC1-UAS^{SPS4}-lacZ* reporter gene in vegetative cells; we anticipated that such mutants might also be defective in activation of the *UAS^{SPS4}-lacZ* reporter gene during sporulation. Indeed, in a screen of 97,000 colonies, we identified two mutants, m83 and m96, that expressed the *CYC1-UAS^{SPS4}-lacZ* reporter gene in vegetative cells and also fulfilled our criteria for potential regulatory mutants: the two mutants expressed *HOP1-lacZ* on transfer to sporulation medium but failed to activate the *UAS^{SPS4}-lacZ* reporter gene and were defective in spore formation (see Materials and Methods) (Fig. 1 and data not shown).

Three complementation groups of *DAS* genes. The five mutants selected for further study fell into three complementation groups, which we designated *DAS1*, *DAS3*, and *DAS4* to indicate that the mutants were defective in activation of *SPS4* (see Materials and Methods). Two strains, m51 and m320, contained mutant alleles of *DAS1*, referred to as *das1-1* and *das1-2*, respectively; m312 contained a mutant allele of *DAS3* referred to as *das3-1*; and m83 and m96 contained mutant alleles of *DAS4*, referred to as *das4-1* and *das4-2*, respectively. We next constructed and sporulated **a**/α *spo13*/*SPO13 das*/*DAS* diploid strains. The spore progeny from each of 10 tetrads analyzed for each mutant gave 2:2 segregation for a Spo$^+$:Spo$^-$ phenotype on mating with the original *das* strain or its *MAT***a** derivative (see Materials and Methods). This suggests that the phenotype of each *das* strain was caused by a single mutation.

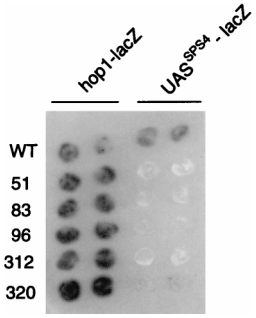

FIG. 1. Mutant strains express a *HOP1-lacZ* reporter gene but not an *SPS4-lacZ* reporter gene. Duplicate patches of cells from the wild-type strain (DKB414-1) and from mutant m51, m83, m96, m312, and m320 strains were incubated for 18 h on sporulation medium and then overlaid with X-Gal-containing agar to monitor β-galactosidase expression (see Materials and Methods). As indicated at the top of the figure, one set of cells harbored pAV79B, which contains a *HOP1-lacZ* reporter gene, and another set harbored pSPS4-lacZ, which contains a *UAS^{SPS4}-lacZ* reporter gene.

***das* mutants complete premeiotic DNA synthesis but remain mononucleate.** We next monitored the ability of the *das* mutants to undergo premeiotic DNA synthesis and to complete meiosis. Prior studies have shown that premeiotic DNA synthesis is required for expression of meiotic genes (25, 31). DNA synthesis was assessed by flow cytometric determination of the relative DNA content of cells that had been stained with propidium iodide. For this analysis, we compared the wild-type diploid strain (**a**/α *spo13*/*spo13 DAS*/*DAS*) with homozygous mutant diploid strains (**a**/α *spo13*/*spo13 das*/*das*). The FACS scans of cells of the wild-type and mutant strains during vegetative growth gave the expected distribution of cells in the G_1 (2N) and G_2 (4N) stages of the cell cycle (Fig. 2A, panels VEG). The FACS scans of cells that had been incubated in sporulation medium for 8 h revealed that most cells of the wild-type strain and of the mutant *das1-1*/*das1-1*, *das3-1*/*das3-1*, and *das4-1*/*das4-1* strains had a 4N DNA content, indicating that they had completed premeiotic DNA synthesis (Fig. 2A, panels SPO).

We next monitored the ability of the wild-type and mutant strains to complete meiosis by using the fluorescent DNA-specific stain DAPI to visualize the segregation of chromosomes. We examined the microscopic appearance of wild-type (**a**/α *spo13*/*spo13 DAS*/*DAS*) cells and mutant *das*/*das* cells at 24 h after transfer to liquid sporulation medium. In the absence of a functional copy of the *SPO13* gene, ~90% of wild-type cells became binucleate and formed a dyad of spores (Fig. 2B and data not shown). No spores were detected in any of the *das* mutants, and only 2.6, 4.8, and 12% of *das1-1*/*das1-1*, *das3-1*/*das3-1*, and *das4-1*/*das4-1* cells, respectively, appeared binucleate (Fig. 2B and data not shown). To ascertain that the failure of the mutant strains to complete meiosis did not depend on mutation of *SPO13*, we introduced a plasmid-borne version of *SPO13* into the mutant strains. Whereas cells of a *spo13*/*spo13 DAS*/*DAS* strain containing this plasmid became tetranucleate on transfer to sporulation medium, albeit not as efficiently as cells of a *SPO13*/*SPO13* strain, most cells of the *spo13*/*spo13 das1-1*/*das1-1*, *spo13*/*spo13 das3-1*/*das3-1*, and *spo13*/*spo13 das4-1*/*das4-1* strains containing a plasmid-borne version of *SPO13* remained mononucleate on sporulation medium (data not shown).

In summary, this analysis indicated that all three mutant

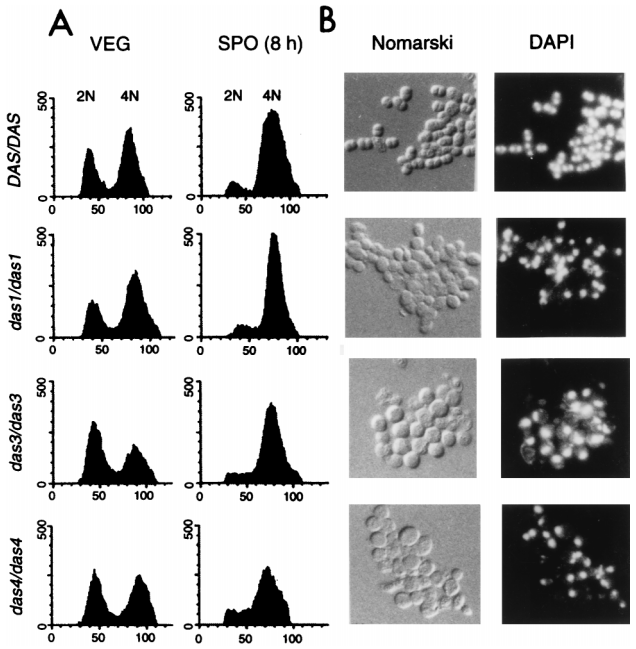

FIG. 2. Mutant strains complete premeiotic DNA synthesis but do not segregate their chromosomes. (A) FACS analysis of wild-type and mutant cells during vegetative growth and after transfer to sporulation medium. Aliquots of wild-type *DAS/DAS spo13/spo13* cells (DKB414C) and mutant *das1-1/das1-1 spo13/spo13*, *das3-1/das3-1 spo13/spo13*, and *das4-1/das4-1 spo13/spo13* cells were harvested during logarithmic growth in YEPA medium (VEG) and after incubation for 8 h in sporulation medium (SPO). Cells were fixed and stained with propidium iodide prior to analysis in a Becton-Dickinson FACScan. The peaks marked 2N and 4N represent cells in G_1 and G_2 portions of the cell cycle, respectively. Relative DNA content (*x* axis) is plotted versus cell number (*y* axis). (B) Microscopic appearance of wild-type and mutant cells on transfer to sporulation medium. Photographs are shown of wild-type *DAS/DAS spo13/spo13* cells (DKB414C) and mutant *das1-1/das1-1 spo13/spo13*, *das3-1/das3-1 spo13/spo13*, and *das4-1/das4-1 spo13/spo13* cells that had been incubated in sporulation medium for 24 h, fixed, and then stained with DAPI (see Materials and Methods). Cells were viewed with Nomarski (Nomarski) or fluorescence (DAPI) optics.

strains chosen for study because they were defective in activation of the UAS^{SPS4}-*lacZ* reporter gene completed premeiotic DNA replication but did not complete meiosis.

***das* mutants are defective in activation of middle sporulation genes.** We next tested the hypothesis that the failure of the mutant strains to express the UAS^{SPS4}-*lacZ* reporter gene and to form spores reflected a general defect in middle sporulation gene expression. We monitored transcript accumulation by Northern blot analysis of RNA from wild-type diploid cells (**a**/α *DAS/DAS spo13/spo13*) and from mutant *das1-1/das1-1*, *das3-1/das3-1*, and *das4-1/das4-1* cells during vegetative growth and at various times after transfer to sporulation medium. Blots were probed for the presence of transcripts from the early meiotic genes *IME1*, *IME2*, and *HOP1*, the middle sporulation genes *SPS1*, *SPS4*, and *SMK1*, and the late sporulation gene *SPS100*.

The early meiotic genes *IME1*, *IME2*, and *HOP1* were activated in the wild-type and mutant strains shortly after transfer to sporulation medium (Fig. 3). In the wild-type strain, maximal transcript accumulation occurred at about 4 h, and transcript levels then declined from 6 to 11 h of sporulation (Fig. 3). In the *das3-1/das3-1* and *das4-1/das4-1* strains, the levels of *IME1*, *IME2*, and *HOP1* transcripts remained relatively constant from 2 to 24 h of sporulation (Fig. 3). In the *das1-1/das1-1* strain, *IME2* transcripts accumulated to a higher level than in the wild-type strain and maximal accumulation of *HOP1* and *IME2* transcripts was delayed until about 6 to 8 h of sporulation (Fig. 3). We note that a very low level of *IME1* and *IME2* transcripts could be detected in most strains during vegetative growth.

In the wild-type strain, the middle sporulation genes *SPS1*, *SPS4*, and *SMK1* were activated at about 4 to 6 h with maximal transcript accumulation occurring at about 8 h of sporulation. The late sporulation gene *SPS100* was activated at about 8 h in the wild-type strain with maximal transcript accumulation occurring at about 15 h after transfer of cells to sporulation medium (Fig. 3). Transcripts from these middle and late genes did not accumulate in the *das1-1/das1-1*, *das3-1/das3-1*, or *das4-1/das4-1* strains, with the exception that a very low level of *SMK1*-derived transcripts could be detected in RNA from cells of the *das1-1/das1-1* and *das4-1/das4-1* strains at 8 to 11 h of sporulation (Fig. 3).

In summary, this analysis of transcript accumulation revealed that the *das1-1/das1-1*, *das3-1/das3-1*, and *das4-1/das4-1* strains had similar transcriptional defects during sporulation.

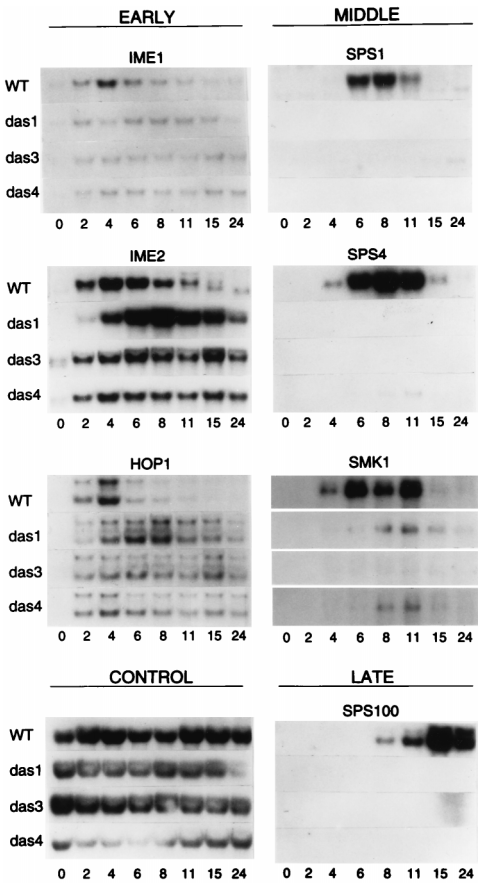

FIG. 3. Expression of sporulation-specific genes in wild-type and *das* strains. RNA was purified from cells of wild-type strain DKB414C (WT) and mutant *das1-1/das1-1* (das1), *das3-1/das3-1* (das3), and *das4-1/das4-1* (das4) strains, as indicated to the left of the panels. Cells were harvested during logarithmic growth in YEPA medium (0 h) and at various times after transfer to sporulation medium, as indicated (in hours) below the panels. Northern blots of these RNAs were sequentially hybridized with radioactively labeled gene-specific probes (see Materials and Methods), as indicated at the top of the panels. One set of filters was hybridized with probes for transcripts from *SPS4*, *IME2*, *HOP1*, *SPS1*, *IME1*, and the control gene. The control probe was prepared with pC4. Two *HOP1*-hybridizing transcripts have been described previously (20). A duplicate set of filters was used for hybridization with probes for transcripts from *SMK1* and *SPS100*. Only the portions of the autoradiograms that revealed hybridization with the probes are shown.

The most striking defect was the failure to activate expression of the middle sporulation genes *SPS1*, *SPS4*, and *SMK1* and the late sporulation gene *SPS100*. A minor defect was the continued expression of the early meiotic genes *IME1*, *IME2*, and *HOP1* at a time at which their expression was turned down in wild-type cells. Although it has been suggested previously that downregulation of *IME1* might be a prerequisite for activation of middle sporulation gene expression (40), a recent study indicates that continued expression of *IME1* through sporulation does not impede spore formation (34).

***DAS1* is *NDT80*, *DAS3* is *SIN3*, and *DAS4* is *RPD3*.** Having confirmed that the *das1-1*, *das3-1*, and *das4-1* mutant strains were defective in activation of middle sporulation genes, we proceeded to identify the wild-type *DAS* genes. We first tested two candidate genes, *IME2* and *NDT80*, for allelism with the *das* genes. We tested *IME2* because it is known to be essential for middle sporulation gene expression (40, 62), including expression of *SPS4* (19). We tested *NDT80*, a key regulatory gene required for the transition from pachytene into the meiosis I division (79), because we had found that the transcriptional defect of the *das* mutants correlated with a failure to complete meiosis.

We found that *das1-1*/Δ*ime2*, *das3-1*/Δ*ime2*, and *das4-1*/Δ*ime2* cells that had been transferred to sporulation medium expressed the UAS^{SPS4}-*lacZ* reporter gene and formed spores efficiently, indicating that the *das* mutations were in genes other than *IME2* (data not shown). To test for allelism with *NDT80*, we introduced a plasmid-borne version of this gene into the *das* strains. The plasmid-borne *NDT80* gene restored both UAS^{SPS4}-*lacZ* expression and spore formation to *das1-1*/*das1-1* cells transferred to sporulation medium, suggesting that *das1-1* was an allele of *NDT80* (data not shown; see below).

We next isolated complementing plasmids for *das4* by screening *das4-1*/*das4-1* cells that had been transformed with a yeast genomic library for restoration of expression of the UAS^{SPS4}-*lacZ* reporter gene during sporulation. We identified two plasmids with overlapping inserts that promoted expression of the UAS^{SPS4}-*lacZ* reporter gene in *das4-1*/*das4-1* and *das4-2*/*das4-2* cells on transfer to sporulation medium and that inhibited expression of the *CYC1*-UAS^{SPS4}-*lacZ* reporter gene in vegetative cells (data not shown). Transposon mutagenesis of one of the plasmids indicated that mutation of *RPD3*, one of three genes present in the genomic DNA insert, led to a loss in the ability of the plasmid to complement the *das4* mutation (data not shown; see Materials and Methods). As expected, we found that a plasmid-borne version of *RPD3* complemented the *das4-1* mutation (data not shown).

Mutations in *RPD3*, which encodes a histone deacetylase (53, 69; reviewed in reference 77), have been shown previously to cause elevated expression of *IME2* in vegetative cells and to lead to an asporogenous phenotype (6, 74). Because *SIN3*/*UME4*/*RPD1* is in the same genetic pathway as *RPD3* (64, 73), with *sin3* and *rpd3* strains having similar phenotypes (73, 74), we tested *SIN3* for the ability to complement a mutant allele of *DAS3*. We found that introduction of a plasmid-borne version of *SIN3* into a *das3-1*/*das3-1* strain restored both UAS^{SPS4}-*lacZ* expression and spore formation in cells that had been transferred to sporulation medium (data not shown).

To confirm that *NDT80*, *SIN3*, and *RPD3* were identical to *DAS1*, *DAS3*, and *DAS4*, respectively, and not low-copy suppressors of the *das* alleles, we constructed *das1-1*/Δ*ndt80::LEU2*, *das3-1*/Δ*sin3::URA3*, and *das4-1*/Δ*rpd3::URA3* strains (see Materials and Methods). We found that cells of all three diploid strains were unable to form spores on transfer to sporulation medium (data not shown). This complementation analysis supported the assignments of *das1-1* as a mutant allele of *NDT80*, *das3-1* as a mutant allele of *SIN3*/*UME4*/*RPD1*, and *das4-1* as a mutant allele of *RPD3*.

In summary, the isolation of mutant alleles of *DAS3* and *DAS4* in this screen, and their assignment as *SIN3* and *RPD3*, respectively, confirmed previous observations that mutation of these genes leads to misregulation of expression of early meiotic genes and prevents completion of meiosis (6, 65, 73, 74). In addition, the identification of *DAS1* as *NDT80*, a gene that is required for the transition from pachytene into the meiotic divisions, suggests that entry into meiosis and activation of middle sporulation genes might be coregulated.

The B-type cyclins Clb1p, Clb3p, and Clb4p are not required for activation of middle sporulation gene expression. The middle sporulation genes *SPS1* and *SMK1* have been characterized in detail and have been shown to play an essential role in spore wall morphogenesis (14, 29). Because spore wall maturation is a postmeiotic event, with the initial prospore membranes emanating from the spindle pole bodies and growing around the four lobes of the postmeiotic nucleus (42; reviewed in reference 30), we considered that the sequence of events during sporulation could be as follows: DNA replication → meiotic recombination → meiotic divisions → middle sporulation genes → spore wall formation. In this cascade, entry into meiosis I would provide a regulatory signal for activation of middle sporulation genes. However, the fact that the mutants that we isolated as defective in expression of middle sporulation genes were also defective in the meiotic divisions suggested that a common regulatory event might promote both entry into meiosis I and the activation of middle genes. To distinguish between these possibilities, we tested whether middle genes were expressed in a strain that arrests at pachytene and does not enter meiosis I due to a reduction in the activity of MPF.

Previous studies have shown that Clb1p, and to a lesser extent Clb3p and Clb4p, is the major meiotic activator of Cdc28p, the kinase component of yeast MPF (13, 18). We therefore monitored gene expression in cells of a *clb1*/*clb1 clb3*/*clb3 clb4*/*clb4* strain after transfer to sporulation medium. Because Clb2p is sufficient to regulate the G_2/M transition during the mitotic cell cycle, these cells are viable (13). Consistent with previous studies (13, 18), we found that at 36 h of sporulation, a time at which 69% of wild-type cells had formed mature asci, only 2.5% of *clb1*/*clb1 clb3*/*clb3 clb4*/*clb4* cells had formed asci (data not shown). As assessed by DAPI staining, 57% of wild-type cells were bi- or tetranucleate at 24 h after transfer to sporulation medium; only 11% of *clb1*/*clb1 clb3*/*clb3 clb4*/*clb4* cells were binucleate, and none were tetranucleate at this same time. Despite this defect in meiosis, the expression patterns of *IME1*, *IME2*, *NDT80*, and *SPS4* in the *clb1*/*clb1 clb3*/*clb3 clb4*/*clb4* strain were indistinguishable from their expression patterns in the wild-type strain (Fig. 4). We therefore concluded that *CLB1*-, *CLB3*-, or *CLB4*-dependent MPF activity and subsequent entry into the meiotic divisions were not required for expression of middle sporulation genes. This suggested that an event occurring prior to entry into meiosis I provided a signal for activation of middle sporulation gene expression.

Mutation of *DMC1* prevents middle sporulation gene expression. Our experiments indicated that middle sporulation genes were not expressed in cells that arrested in pachytene and failed to enter the meiotic divisions due to mutation of *NDT80*, a putative regulator of MPF activity (79). In contrast, we found that middle sporulation genes were expressed in *clb1 clb3 clb4* cells that failed to enter the meiotic divisions due to reduced MPF activity. Thus, MPF activity and entry into the meiotic divisions are not required for activation of middle sporulation genes. This suggested that pachytene arrest itself might pre-

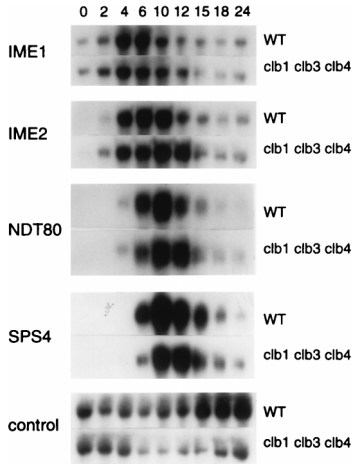

FIG. 4. Middle sporulation genes are expressed in a *clb1 clb3 clb4* strain. RNA was purified from cells of the wild-type strain (LP112) (WT) and from its isogenic *clb1*/*clb1 clb3*/*clb3 clb4*/*clb4* derivative (CD140), as indicated to the right of the figure. Northern blot analysis was performed as described in the legend to Fig. 3. The times at which cells were harvested are indicated (in hours) at the top of the figure; the hybridization probes are indicated to the left of the figure; the control transcript was from the *PYK1* gene. Only the portions of the autoradiograms that revealed hybridization with the probes are shown.

vent activation of middle sporulation genes. To test this idea, we examined gene expression in a *dmc1* strain, which also arrests in pachytene. Whereas pachytene arrest occurs in the *das1-1* (*ndt80*) strain in the absence of any significant defect in meiotic recombination (79), in a *dmc1* strain a defect in meiotic recombination results in accumulation of DNA double-strand breaks that triggers the meiotic recombination checkpoint and subsequent pachytene arrest (4).

Transcripts from the early genes *IME1*, *IME2*, and *HOP1* began to accumulate in both the wild-type strain and in the *dmc1*/*dmc1* strain shortly after transfer of cells to sporulation medium (Fig. 5). Transcript accumulation occurred over a longer time period in the *dmc1* mutant strain, and peak levels, which occurred at a later time than they did in wild-type cells, were higher. In contrast, accumulation of transcripts from *NDT80* and *SMK1* was markedly reduced in the *dmc1*/*dmc1* strain, and transcripts from *SPS1* and *SPS4* were not detected (Fig. 5). We next monitored gene expression in a *dmc1*/*dmc1 rad17*/*rad17* strain; mutation of the checkpoint gene *RAD17* prevents activation of the meiotic recombination checkpoint, thereby allowing *dmc1* cells to proceed through the two meiotic divisions with near-normal kinetics (35). In the *dmc1 rad17* mutant, expression of all genes tested was similar to that in wild-type cells except that the time period of transcript accumulation was extended and the time of maximal transcript accumulation of middle sporulation genes was delayed by 2 to 3 h (Fig. 5).

In summary, we found that activation of the *RAD17*-dependent meiotic recombination checkpoint by mutation of *dmc1* prevented both entry into meiosis I and activation of middle sporulation genes. We also found that expression of *NDT80*, which we showed was essential for expression of middle sporulation genes (Fig. 3), was reduced, but nonetheless readily detectable, in a *dmc1* strain. This suggested that the Ndt80p produced in the *dmc1* strain was unable to activate expression of middle sporulation genes. It is possible that the level of Ndt80p was below a critical threshold in the *dmc1*-arrested strain or that activation of the meiotic recombination checkpoint in this strain prevented Ndt80p from serving its role in expression of middle sporulation genes (see Discussion and reference 10).

Meiotic arrest of *sin3* and *rpd3* strains is not suppressed by mutation of *SPO11* or *RAD17*. We tested whether mutation of *SPO11* would overcome the apparent arrest that was observed in *das3* (*sin3*) and *das4* (*rpd3*) strains. Mutation of *SPO11* prevents initiation of recombination, thus bypassing defects at intermediate stages of recombination that normally lead to pachytene arrest (2, 4, 27, 37, 38, 48, 68, 70); for example, a *spo11*/*spo11 dmc1*/*dmc1* strain is able to form spores, albeit inviable ones (4). Whereas cells of a *DAS*/*DAS spo11*/*spo11 spo13*/*spo13* strain formed ~90% dyads on transfer to sporulation medium, cells of isogenic *das3-1*/*das3-1 spo11*/*spo11 spo13*/*spo13* and *das4-1*/*das4-1 spo11*/*spo11 spo13*/*spo13* strains were unable to form spores (data not shown). This suggests that mutation of *SIN3* and mutation of *RPD3* prevent the meiotic divisions by a defect other than, or in addition to, a defect in meiotic recombination. Consistent with this, abrogation of the meiotic recombination checkpoint by mutation of the checkpoint gene *RAD17* did not allow efficient meiosis to occur in either the *das* mutants or a Δ*ndt80* strain (data not shown).

Unique regulation of expression of *NDT80* and *SMK1*. Close examination of the gene expression patterns that we obtained during the course of this study suggested that *NDT80* and *SMK1* belong to a temporally distinct class of sporulation genes. Activation of these genes occurs after the early meiotic genes are turned on and before middle sporulation genes are first expressed. Additionally, *NDT80* and *SMK1* are regulated in a unique manner.

Comparison of transcript accumulation from *IME2* and *NDT80* by Northern blot analysis (Fig. 6A) showed that activation of *NDT80* occurred after activation of early meiotic genes, as has been noted previously (79). At 4 h of sporulation, transcripts from the middle sporulation gene *SPS1* could not be detected and transcripts from *SPS4* were just beginning to appear. At this time, transcript accumulation from *NDT80*, however, was already close to the maximal level reached at 6 to 11 h of sporulation (Fig. 6A).

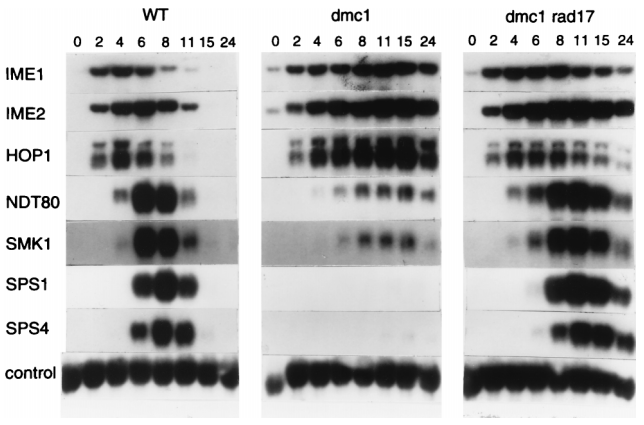

FIG. 5. Sporulation-specific gene expression in wild-type (WT), *dmc1*/*dmc1* (dmc1), and *dmc1*/*dmc1 rad17*/*rad17* (dmc1 rad17) strains. RNA was purified from cells of the wild-type strain (DKB98) and its isogenic *dmc1*/*dmc1* (DKB608) and *dmc1*/*dmc1 rad17*/*rad17* (DKB1170) derivatives. Northern blot analysis was performed as described in the legend to Fig. 3. The times at which cells were harvested are indicated (in hours) above the panels. One filter was sequentially hybridized with radioactivity labeled probes for *IME1* and *IME2* transcripts, and a duplicate filter was sequentially hybridized with probes for *SPS1*, *SPS4*, *HOP1*, *NDT80*, *SMK1*, and a control transcript. The control probe was prepared with pC4. Only the portions of the autoradiograms that revealed hybridization with the probes are shown.

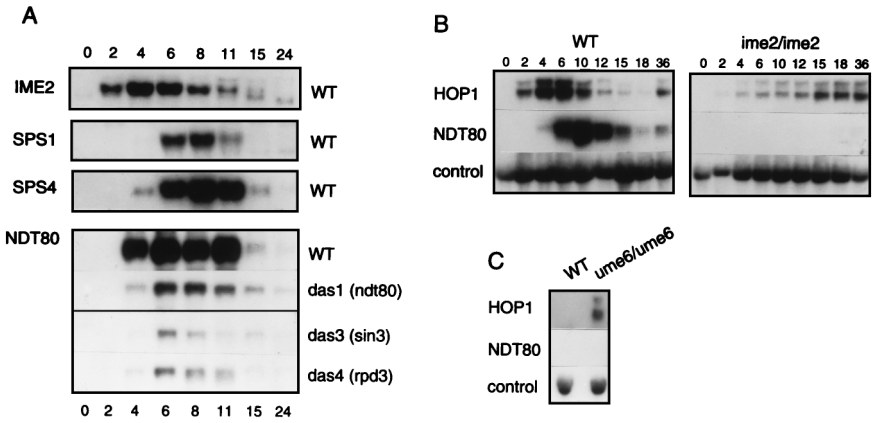

FIG. 6. Expression of *NDT80*, a pre-middle sporulation-specific gene, depends on *IME2*. (A) *NDT80* is expressed prior to middle sporulation-specific genes. A set of filters from the experiment shown in Fig. 3, containing RNAs purified from cells of the wild-type strain (DKB414C) (WT) and from mutant *das1-1*/*das1-1* [das1 (ndt80)], *das3-1*/*das3-1* [das3 (sin3)], and *das4-1*/*das4-1* [das4 (rpd3)] strains, was stripped after hybridization with the probe for *SMK1*-derived transcripts and hybridized with a probe for *NDT80*-derived transcripts. Northern blot analysis was performed as described in the legend to Fig. 3. The autoradiograms depicting accumulation of *IME2*, *SPS2*, and *SPS4* transcripts in the wild-type strain are from Fig. 3. The times at which cells were harvested are indicated (in hours) at the top and bottom of the figure. (B) *IME2* is required for expression of *NDT80* in sporulating cells. RNAs that had been purified previously (19) from cells of the wild-type strain (LP112) (WT) and its isogenic *ime2*/*ime2* derivative (YKE2) (ime2/ime2), as indicated above the panels, were used to prepare filters for Northern blot analysis. The times at which cells were harvested are indicated (in hours) above the panels. The filters were sequentially hybridized with probes for detection of *HOP1*-, *NDT80*-, and control *PYK1*-derived transcripts. Only the portions of the autoradiograms that revealed hybridization with the probes are shown. (C) *UME6* is not required for repression of *NDT80* in vegetative cells. RNAs that had been purified from cells of the wild-type strain (LP112) (WT) and its isogenic *ume6*/*ume6* derivative (YSH2) (ume6/ume6) growing in YEPD medium, as indicated above the panel, were subjected to Northern blot analysis (19). The autoradiogram of the filter that was hybridized with a probe for *HOP1*-derived transcripts is from the work of Hepworth et al. (19). A duplicate filter was sequentially hybridized with probes for *NDT80*- and control *PYK1*-derived transcripts. Only the portions of the autoradiograms that revealed hybridization with the probes are shown.

We also examined expression of *NDT80* in the *das1-1*/*das1-1* (*ndt80*), *das3-1*/*das3-1* (*sin3*), and *das4-1*/*das4-1* (*rpd3*) strains. *NDT80* was first expressed in the three mutant strains at about the same time that it was in the wild-type strain. Strikingly, however, *NDT80*-derived transcripts did not accumulate to any significant level in the three mutant strains (Fig. 6A). The absence of high-level expression of *ndt80* in the *das1-1*/*das1-1* (*ndt80*) strain can be explained by the recent observation that Ndt80p upregulates its own expression (10); we cannot exclude the possibility, however, that the *das1-1* allele of *NDT80* contained a mutation in its promoter or a mutation that destabilizes its mRNA.

The expression patterns of *NDT80* and *SMK1* could also be distinguished from those of middle sporulation genes on examination of transcript accumulation in the *dmc1*/*dmc1* and *dmc1*/*dmc1 rad17*/*rad17* strains (Fig. 5). Whereas *NDT80* and *SMK1* were expressed at a low level in the *dmc1*/*dmc1* strain, no transcripts could be detected from *SPS1* or from *SPS4*. In the *dmc1*/*dmc1 rad17*/*rad17* strain, *NDT80* and *SMK1* transcripts could be detected at 4 h and had accumulated to a significant level by 6 h, a time at which *SPS1* and *SPS4* transcripts could barely be detected (Fig. 5).

Taking these observations into consideration, we designate *NDT80* and *SMK1* as pre-middle sporulation genes. Although expression of *SMK1* prior to expression of middle genes was not obvious in all of our experiments (e.g., that shown in Fig. 3), a distinction in the time of expression, albeit minor, could be noted in several experiments (Fig. 5) (see reference 75). Because regulation of this temporally distinct class of genes has not been studied, we tested the roles of *IME2*, a key regulator of middle sporulation gene expression (40), and of *UME6*, a key regulator of early meiotic gene expression (66), in expression of *NDT80*. The early meiotic gene *HOP1* was expressed in an *ime2*/*ime2* strain after transfer to sporulation medium, although transcript accumulation was delayed and reduced compared to that seen in wild-type cells (Fig. 6B) (19). *NDT80* transcripts, however, were not detected in the *ime2*/*ime2* strain (Fig. 6B). Similarly, transcripts from *HOP1* were readily detected in RNA from a *ume6*/*ume6* strain growing vegetatively (19), whereas transcripts of *NDT80* were not present (Fig. 6C). The findings that Ume6p (Fig. 6C), and Rpd3 and Sin3p (Fig. 6A), were not needed to prevent expression of *NDT80* in vegetative cells and that Ime2p was needed for its activation during sporulation suggest that regulation of *NDT80* is distinct from the regulation of early meiotic genes.

In summary, we suggest that *NDT80* and *SMK1* define a class of pre-middle sporulation genes whose transcription is regulated by two mechanisms. An initial low level of *IME2*-dependent transcription occurs after the activation of the early meiotic genes. Once cells have proceeded through the *RAD17*-dependent checkpoint, expression of these genes is upregulated.

DISCUSSION

The *SPS4* gene of *S. cerevisiae* belongs to a group of middle sporulation-specific genes that are expressed during the meiotic divisions. A 15-bp regulatory element from the promoter region of *SPS4*, referred to as UAS^{SPS4}, suffices for activation of a heterologous gene midway through sporulation (19). In this study, we identified mutants that had defects in regulation through UAS^{SPS4}. The five mutants that we studied in detail defined three complementation groups, referred to as *DAS1*, *DAS3*, and *DAS4*, with similar mutant phenotypes. Cells of the mutant strains completed premeiotic DNA synthesis and expressed early meiotic genes on transfer to sporulation medium but did not complete the meiotic divisions or activate expression of middle sporulation genes. We found that *DAS1*, *DAS3*, and *DAS4* were identical to the previously characterized genes *NDT80*, *SIN3*, and *RPD3*, respectively.

The roles of these three genes have been well characterized. Mutation of *NDT80* has been shown to cause cells to arrest in pachytene in the absence of any defects in meiotic recombination (79). Because the arrest phenotype of an *ndt80* mutant is

similar to the meiotic phenotype of a *cdc28*(Ts) mutant (59), Xu and colleagues (79) proposed that *NDT80* might encode a regulator of MPF activity. MPF, which consists of Cdc28p kinase and a B-type cyclin as its regulatory partner, controls entry into meiosis I and meiosis II. Indeed, Chu and Herskowitz (10) have recently shown that Ndt80p is a transcriptional activator that induces expression of *CLB1*, *CLB3*, and *CLB4* during sporulation in addition to activating middle sporulation gene expression.

SIN3 and *RPD3* have been identified repeatedly in mutational analyses as modifiers of gene transcription [e.g., as *RPD1* (*SIN3*) and *RPD3* (73, 74); as *SIN3* (76); as *UME4* (*SIN3*) (65; reviewed in reference 77)]. Recently, it has been shown that Rpd3p, a histone deacetylase, is part of a large multiprotein complex that includes Sin3p (26; reviewed in references 45 and 67). In a mechanism that is conserved among eukaryotes, this complex is recruited to promoters by sequence-specific DNA-binding factors (23). Subsequent histone deacetylation is thought to generate a chromatin structure that represses transcription (24, 54).

Clearly, our screen for regulators of middle sporulation gene expression was not saturating; some genes that might have been identified, such as *IME2* and *DMC1*, were not found. Because expression of the *HOP1-lacZ* reporter gene is reduced in an *ime2* strain (data not shown; see also Fig. 6B), it is possible that any *ime2* strain that might have been recovered as a potential *das* mutant was discarded on the basis of reduced expression of this early meiotic gene. Two of the three genes that we identified, however, were isolated twice. We attribute this bias to the sensitivity of our UAS^{SPS4}-*lacZ* reporter gene. It is likely that strains with mutations that gave a leaky phenotype would have escaped detection. Similarly, mutations in genes that contribute to, but are not essential for, activation of *SPS4* would not have been detected.

Pachytene arrest blocks activation of middle sporulation genes. The observations presented in this study have allowed us to distinguish between two models that we had considered for coordination of the meiotic divisions and middle sporulation gene expression. The first model postulated that entry into the meiotic divisions was required for activation of middle sporulation genes; in this case, expression of middle sporulation genes would depend on activation of MPF. We tested this model by monitoring gene expression in a *clb1 clb3 clb4* strain. This strain, which has almost no MPF activity and fails to complete meiosis (13, 18), was proficient in expression of middle sporulation genes (Fig. 4). We therefore conclude that Clb1p-, Clb3p-, and Clb4p-dependent MPF activity and entry into the meiotic divisions is dispensable for activation of middle sporulation genes. Moreover, the observation that *CLB1*, which encodes the primary meiotic B-type cyclin (13, 18), is expressed as a middle sporulation gene (10) makes it unlikely that MPF would itself serve in activation of middle genes.

The second model postulates that a common signal generated prior to entry into meiosis regulates both commencement of the meiotic divisions and activation of middle sporulation genes. The recovery of *NDT80*, which is required for the transition from pachytene into meiosis I (79), as a *DAS* gene was consistent with the notion that entry into meiosis and expression of middle sporulation genes were coordinately regulated. We found that a *dmc1* strain, which arrests at pachytene because an accumulation of recombination intermediates activates the meiotic recombination checkpoint (4, 78), failed to express middle sporulation genes. In contrast, a *dmc1 rad17* strain, which progresses through the meiotic divisions because of mutation of the checkpoint gene *RAD17* (35), did express middle sporulation genes (Fig. 5). For unknown reasons, mature spores do not form in the *dmc1 rad17* strain (35; also this study). We propose that the pachytene arrest signal generated by the accumulation of recombination complexes, which prevents entry into the meiotic divisions, also prevents activation of middle sporulation genes.

We note that Clancy and coworkers (11) previously reported that a sporulation-specific glucoamylase activity (SAG) that is encoded by the sporulation gene *SGA1* (28, 80) is not expressed in cells that arrest at pachytene. These investigators found that SAG activity was present in a mutant that initiates meiosis I but is blocked in spindle development [*spo1*(Ts)] and in a mutant that is defective in nuclear migration after meiosis II (*spo3*(Ts)]. However, SAG activity was not detected in a mutant that arrests at pachytene [*pac1*(Ts)] nor in a mutant that is defective in premeiotic DNA synthesis [*cdc4*(Ts)] (11).

Why does mutation of *SIN3* or *RPD3* confer a meiosis I block? As mentioned above, *SIN3* and *RPD3* have been identified repeatedly in mutational analyses as modifiers of gene expression (reviewed in reference 77). Rpd3p, which has histone deacetylase activity, and Sin3p are components of a multiprotein regulatory complex that is recruited to promoters by sequence-specific DNA-binding proteins (26; reviewed in references 45 and 67). Mutation of *SIN3* or *RPD3* and the resultant histone hyperacetylation could have effects on expression of sporulation genes, on recombination, and on the structure of chromatin and chromosomes. We suggest that a combination of such defects leads to pachytene arrest and that this arrest blocks middle sporulation gene expression. Because neither mutation of *SPO11* nor mutation of *RAD17* bypassed the arrest caused by mutation of *SIN3* or *RPD3*, we suggest that this arrest is mediated by a mechanism that differs, at least in some aspect, from the meiotic recombination checkpoint (for examples, see references 49 and 71). We note that we found that mutation of *SIN3* or *RPD3* in some SK1-derived backgrounds led to a delay in meiosis and a reduced efficiency of spore formation rather than a complete block (unpublished observations).

Why does mutation of *RPD3* (*DAS4*) lead to expression of the *CYC1-*UAS^{SPS4}*-lacZ* reporter gene in vegetative cells? We identified *RPD3* (*DAS4*) in a screen that took advantage of our previous observation that the *CYC1-*UAS^{SPS4}*-lacZ* reporter gene, which has four copies of a 29-bp, UAS^{SPS4}-containing fragment inserted between the UAS^{CYC1} and the $TATA^{CYC1}$, is not expressed in vegetatively growing cells (19). This 29-bp fragment, which contains UAS^{SPS4} and 14 bp of upstream sequence, promotes 10-fold-higher expression during sporulation than does a 15-bp UAS^{SPS4}-containing fragment (19). We hypothesized that a protein that is present in vegetative cells binds to the 29-bp fragment and prevents UAS^{CYC1}-bound activators from functioning via an indirect mechanism such as steric hindrance (19). Although the *CYC1-*UAS^{SPS4}*-lacZ* reporter gene is expressed in *rpd3* cells, as well as in *sin3* (*das3*) cells (data not shown), that are growing vegetatively, the *SPS4* gene is not expressed in these cells (Fig. 3, *SPS4* panel, 0 time point). We speculate, therefore, that the 29-bp fragment contains a sequence that allows Sin3p-Rpd3p-dependent repression to be established in vegetative cells and that this repression, although effective on a heterologous UAS in vegetative cells, is not required for repression of *SPS4* itself. Indeed, we have no evidence that the *SPS4* gene is subject to negative control in vegetative cells (19). It is possible, however, that the putative DNA-binding protein that contributes to Sin3p-Rpd3p-dependent repression in vegetative cells is also responsible for enhancing Ndt80p-dependent activation of *SPS4* during sporulation. We note that this putative DNA-binding protein is not Ume6p, since vegetative repression of the *CYC1-*UAS^{SPS4}*-lacZ*

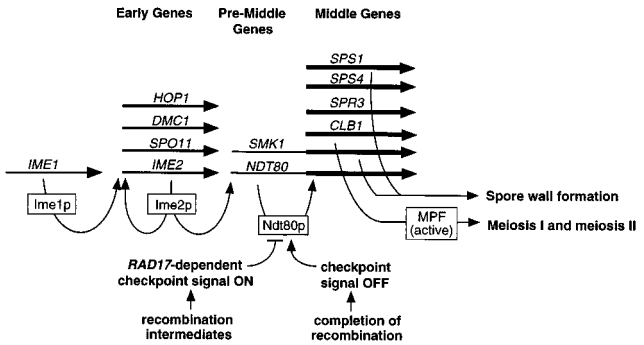

FIG. 7. Model for the role of Ndt80p in regulation of expression of middle sporulation-specific genes. The long horizontal arrows indicate the relative order of expression of various sporulation-specific genes; curved arrows refer to known or putative regulatory events; boxes indicate gene products. Upon transfer of diploid cells to sporulation medium, Ime1p rapidly activates expression of *IME2* and other early meiotic genes. Ime2p has an early role in *IME1*-independent activation of early meiotic genes and a later role in activation of middle sporulation genes. We propose that the major role of the later function of Ime2p is to promote a low level of expression of pre-middle sporulation genes, such as *NDT80*. Our results suggest that activation of the *RAD17*-dependent meiotic recombination checkpoint inhibits the activity of Ndt80p and that an active form of Ndt80p is generated only after cells have completed most prophase events. In particular, meiotic recombination must be completed and any defects in meiotic chromosome metabolism that generate a pachytene arrest signal must be resolved before active Ndt80p is produced. We propose that the active form of Ndt80p is then responsible for upregulation of expression of pre-middle sporulation genes, denoted by a thickening of the horizontal arrows, and for activation of middle sporulation genes, such as *SPS1* and *SPS4*.

reporter gene is maintained in a *ume6* strain (data not shown). Similarly, as would be expected, vegetative repression of the *CYC1-UAS*^{SPS4}*-lacZ* reporter gene is maintained in an *ndt80* strain (data not shown).

***NDT80*, a pre-middle sporulation gene.** In the course of our studies, we observed that regulation of expression of *NDT80* and *SMK1* was distinct from that of middle sporulation genes. In some of our time course experiments, we could readily detect transcripts from *NDT80* prior to the appearance of transcripts from *SPS4* (for an example, see Fig. 6A). A temporal and regulatory distinction was also apparent on analysis of transcript accumulation in *dmc1* and *dmc1 rad17* strains (Fig. 5). In the pachytene-arrested *dmc1* strain, *SPS1* and *SPS4* were not expressed but a low level of transcripts could be detected from *NDT80* and *SMK1* (Fig. 5). In the *dmc1 rad17* strain, transcripts from *NDT80* and *SMK1* could be detected prior to the appearance of transcripts from *SPS1* and *SPS4* (Fig. 5).

Regulation of *NDT80* is clearly distinct from that of early meiotic genes. Whereas repression of early meiotic genes in vegetative cells depends on recruitment of a Sin3p-Rpd3p-containing complex to URS1-bound Ume6p (23), we found that mutation of *UME6*, *SIN3* (*DAS3*), or *RPD3* (*DAS4*) did not lead to expression of *NDT80* in vegetatively growing cells (Fig. 6C and A, NDT80 panel, 0 h time point).

We concluded that *NDT80* and *SMK1* are members of a temporally distinct class of sporulation-specific genes that is activated after the early meiotic genes but prior to the middle sporulation genes. In this case, the requirement for *IME2*, an early meiotic gene, for expression of middle sporulation genes could be indirect, reflecting a role of *IME2* in expression of *NDT80*.

Model for coordination of middle sporulation gene expression and the meiotic divisions. We present the following model for the sequence of events that leads to expression of middle sporulation genes (Fig. 7). We propose that Ime1p-dependent activation of early meiotic genes is followed by Ime2p-dependent activation of pre-middle sporulation genes, such as *NDT80* and *SMK1*. Ime2p, a serine-threonine protein kinase, has been shown to serve initially in *IME1*-independent activation of early meiotic genes and subsequently in activation of middle sporulation genes (reviewed in reference 39). The manner in which Ime2p carries out these temporally distinct functions is not clear; however, the two roles can be separated genetically (60). We suggest that the primary role of the late function of Ime2p is initial activation of pre-middle sporulation genes, such as *NDT80*. Because a modest level of *NDT80* transcripts accumulated in a *dmc1* strain, but middle sporulation genes were not expressed in this strain, we hypothesize that the early *NDT80* transcripts are either not translated or, if they are translated, that active Ndt80p is not generated until cells have completed those aspects of meiotic recombination (78) that are monitored by the *RAD17*-dependent meiotic checkpoint (35). The active form of Ndt80p that we speculate is generated once the cells have progressed through the meiotic recombination checkpoint could result from translation of previously nontranslated transcripts, a posttranslational modification of Ndt80p, interaction with a cofactor, or some other sporulation-specific event. Finally, active Ndt80p would lead to upregulation of expression of pre-middle sporulation genes, including *NDT80* itself, and activation of expression of middle sporulation genes, including *CLB1* (10). Clb1p would then contribute to activation of MPF and subsequent entry into meiosis I. Hence, the meiotic divisions would be dependent on prior activation of middle sporulation genes, and activation of middle sporulation genes would be dependent on the absence of an arrest signal generated by intermediates of meiotic recombination. In this way, Ndt80p would coordinate entry into meiosis with spore wall formation. This model is consistent with the recent demonstration by Chu and Herskowitz (10) that Ndt80p is an MSE-binding transcriptional activator that activates its own expression. These investigators also noted that the activity of Ndt80p might be regulated by the meiotic recombination checkpoint machinery (10). The proposed autoregulatory loop for *NDT80* expression (10) is also consistent with the low level of expression of *ndt80* in our *das1-1* (*ndt80*) strain (Fig. 6A). Interestingly, the *mei4*$^{+}$ gene of *Schizosaccharomyces pombe*, which encodes a meiosis-specific transcription factor that is required for meiosis and sporulation-specific gene expression, also appears to be autoregulated (21).

Because the signal for pachytene arrest in *S. cerevisiae* appears to be maintained as long as recombination complexes persist (35, 78), Xu et al. (78, 79) have suggested that the meiotic recombination complex emits an inhibitory signal that prevents entry into meiosis until the recombination process is complete or near completion; it may be that this putative inhibitory signal prevents generation of the proposed active form of Ndt80p (10, 78, 79; also this study). It is interesting to note that meiotic recombination per se is not required to generate an active form of Ndt80p; for example, middle sporulation genes must be expressed in *spo11* cells, which enter meiosis I in the absence of recombination.

It will be interesting to investigate whether the meiotic recombination machinery and its checkpoint activities do indeed control the activity of Ndt80p. Future experiments will also determine whether distinct *NDT80* promoter elements are required for its initial Ime2p-dependent expression and for its subsequent Ndt80p-dependent upregulation. Finally, investigation of the role of *IME2* in the first phase of *NDT80* expression may provide insights into how Ime2p serves its key role in regulating spore formation.

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