

The Mating-Type Proteins of Fission Yeast Induce Meiosis by Directly Activating *mei3* Transcription

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Cell type control of meiotic gene regulation in the budding yeast *Saccharomyces cerevisiae* is mediated by a cascade of transcriptional repressors, $a1-\alpha2$ and Rme1. Here, we investigate the analogous regulatory pathway in the fission yeast *Schizosaccharomyces pombe* by analyzing the promoter of *mei3*, the single gene whose expression is sufficient to trigger meiosis. The *mei3* promoter does not appear to contain a negative regulatory element that represses transcription in haploid cells. Instead, correct regulation of *mei3* transcription depends on a complex promoter that contains at least five positive elements upstream of the TATA sequence. These elements synergistically activate *mei3* transcription, thereby constituting an on-off switch for the meiosis pathway. Element C is a large region containing multiple sequences that resemble binding sites for M_c , an HMG domain protein encoded by the mating-type locus. The function of element C is extremely sensitive to spacing changes but not to linker-scanning mutations, suggesting the possibility that M_c functions as an architectural transcription factor. Altered-specificity experiments indicate that element D interacts with P_m , a homeodomain protein encoded by the mating-type locus. This indicates that P_m functions as a direct activator of the meiosis pathway, whereas the homologous mating-type protein in *S. cerevisiae* ($\alpha2$) functions as a repressor. Thus, despite the strong similarities between the mating-type loci of *S. cerevisiae* and *S. pombe*, the regulatory logic that governs the tight control of the key meiosis-inducing genes in these organisms is completely different.

The differentiation of a diploid somatic cell into haploid sex cells via a meiotic cell cycle is a hallmark of eukaryotic organisms. Gene regulation in haploid and diploid cells is tightly regulated, although in distinct manners. Haploids need tight control of the intracellular signals and genes that trigger a meiotic cell cycle, because meiosis is a lethal event. In contrast, diploids induce a meiotic cell cycle only upon an appropriate environmental signal(s) or a developmental program.

The meiotic regulatory cascade has been well studied in the budding and fission yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. In both organisms, there is a complex pathway that starts from the mating-type loci and involves meiosis-specific genes and other genes that have multiple cellular roles (20, 29). Both organisms contain three copies of mating-type cassettes; one of these is active and two are silent. There are two mating-type alleles (a and α for *S. cerevisiae* and P and M for *S. pombe*), and each cassette expresses two distinct proteins. Finally, two of the mating-type proteins, *S. cerevisiae* $\alpha2$ and *S. pombe* P_m , contain homeodomains that are 41% identical and are likely to recognize similar DNA sequences (15, 28).

In *S. cerevisiae*, cell type control of meiotic gene regulation is mediated by transcriptional repression (20). Diploid a/α cells specifically express the $a1-\alpha2$ heterodimer that directly represses transcription of *RME1* by binding to its promoter (5). Rme1 directly represses transcription of *IME1*, the key gene whose product directly activates a set of genes that are required for meiosis (6). Although *IME1* transcription is necessary for meiosis, it is not sufficient to trigger a meiotic cell cycle without other cellular signals (13, 23). In starved haploid cells,

IME1 transcription is toxic but not lethal (21). Thus, the *S. cerevisiae* mating-type loci activate *IME1* transcription and the meiotic pathway by an indirect mechanism, i.e., by repressing a repressor.

Meiotic regulation in *S. pombe* can be reduced to the transcriptional status of just one gene, *mei3* (28). Transcription of *mei3* is required for meiosis and is sufficient to trigger meiosis independently of ploidy and nutritional stress (19). In wild-type cells, *mei3* is transcribed only transiently in nitrogen-starved diploids (19). Mei3 is not homologous to Ime1 (23), and it mediates its effect on meiosis by a different mechanism. Mei3 binds to the Pat1 (Ran1) kinase and blocks its autophosphorylation and the phosphorylation of Ste11 and Mei2, the physiological substrates (17, 25). Pat1 kinase activity is required for a mitotic cell cycle; the lack of Pat1 kinase activity leads to a meiotic cell cycle independently of ploidy and nutritional stress. Because Mei3 is necessary and sufficient to induce meiosis, it is critical that *mei3* transcription is tightly controlled in haploid cells.

Genetic experiments indicate that *mei3* is regulated by the coexpression of the four mating-type proteins (29). Two of the mating-type proteins, M_c and P_c , are expressed in vegetatively growing cells, whereas the other two proteins, M_m and P_m , are induced specifically by nitrogen starvation (15). Thus, in wild-type haploids, *mei3* is not expressed, due to the lack of either M_m or P_m . Nitrogen-starved diploids in which the pheromone receptor is bound by pheromone are the only cells in which both M_m and P_m are expressed (2). In principle, the mating-type proteins could bind directly to the *mei3* promoter and activate transcription. Alternatively, these proteins could indirectly activate *mei3* transcription via another, as of yet unidentified, gene(s). By analogy with meiotic regulation in *S. cerevisiae*, one possibility is that the mating-type proteins directly repress transcription of a currently unidentified repressor which directly represses *mei3* transcription.

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TABLE 1. *S. pombe* strains

Strain	Genotype	Source
WP3 (SP67)	<i>h⁹⁰ ade6-210 leu1-32</i>	<i>S. pombe</i> strain bank
WP16	<i>h⁹⁰ ade6-210 ura4-595 leu1-32 mei3::LEU2⁺</i>	This work
WP17	<i>h⁺ ade6-D1 ura4-595 leu1-32</i>	This work
WP20	<i>h⁹⁰ ade6-210 leu1-32 mei3::LEU2⁺ ura4-595::mei3⁺::ura4⁺</i>	This work
WP45	<i>h⁻ ade6-D1 ura4-595 pro2-1</i>	This work
WP54	<i>h⁺/h⁻ ade6-210/ade6-216 leu1⁺/leu1-32</i> (diploid)	This work
WP62	<i>h⁻ ade6-D1 ura4-595::mei3::ade6::ura4⁺ pro2-1</i>	This work
WP64	<i>h⁺ ade6-D1 ura4-595::mei3::ade6::ura4⁺ leu1-32</i>	This work
WP66	<i>h⁻ ade6-D1 ura4-595::mei3::ade6::ura4⁺ leu1-32 mei3::LEU2⁺</i>	This work
WP151	<i>h⁺ pro2-1 ade6-D1 leu1-32 mei3::LEU2⁺ ura4-595</i>	This work
WP162	<i>h⁻ ade6-D1 leu1-32 mei3::LEU2⁺ ura4-(595 or 294) lys1-131</i>	This work
WP356	<i>h⁻ ade6-D1 leu1-32 lys1-131 mei3::LEU2⁺ ura4-(595 or 294)::mei3::LUC::ura4⁺</i>	This work
WP367	<i>h⁻/h⁺ diploid (WP356 × WP151)</i>	This work
WP434	<i>h⁹⁰ ade6-210 leu1-32 mei3::LEU2⁺ ura4-595::mei3-d1::ura4⁺</i>	This work
WP495	<i>h⁹⁰ ade6-210 leu1-32 mei3::LEU2⁺ ura4-595::mei3-d2::ura4⁺</i>	This work
WP496	<i>h⁹⁰ ade6-210 leu1-32 mei3::LEU2⁺ ura4-595::mei3-d3::ura4⁺</i>	This work
WP499	<i>h⁹⁰ ade6-210 leu1-32 mei3::LEU2⁺ ura4-595::mei3-d4::ura4⁺</i>	This work

To understand the molecular mechanisms of *mei3* transcriptional regulation, we performed a detailed mutational analysis of the *mei3* promoter. We define five positive regulatory elements that synergistically activate and correctly regulate *mei3* transcription but obtain no evidence for negative control elements. We demonstrate that one of the critical *mei3* promoter elements is a binding site for P_m and that P_m functions as a direct activator of *mei3* transcription in vivo. In addition, we suggest that another *mei3* promoter element may contain multiple binding sites for M_c. These observations indicate that the underlying logic of meiotic transcriptional regulation in *S. pombe* is very different from that found in *S. cerevisiae*. In addition, the analysis here represents the first detailed dissection of a promoter from *S. pombe*.

MATERIALS AND METHODS

DNA molecules. The starting molecule, pWH4, is a derivative of pBSKS+ (Stratagene) that has a mutated *SacI* site and contains the 1.78-kb *HindIII* *ura4* fragment cloned between the *PstI* and *SmaI* sites and a 1.95-kb *HindIII-EcoRI* *mei3* fragment with a translationally silent mutation of the *KpnI* site in the coding region. pWH6 is a derivative of pWH4 in which the coding and 3' untranslated regions of *mei3* are replaced by a 1.9-kb *SacI-SpeI* *ade6* fragment containing the coding region (with an engineered *SacI* site at the ATG initiation codon) and 3' untranslated region. pWH8 is a derivative of pWH4 in which the *mei3* coding region was replaced by a 1.69-kb *SacI-PstI* fragment containing the firefly luciferase coding region (7) with an engineered *SacI* site at the ATG initiation codon.

mei3 deletion mutant DNAs were generated by standard methods with exonuclease III or *Bal31* nuclease digestion or by PCR mutagenesis. In general, promoter fragments contain *KpnI* sites at the 5' end, *SacI* sites at the 3' end, and *Sall* (in most cases) or *MluI* sites (in some cases) at the junction point. Linker-scanning and base pair substitution mutations were created by PCR-mediated site-directed mutagenesis with appropriate oligonucleotides. Insertion-scanning mutations were created by linearizing linker-scanning derivatives with the linker restriction site, blunting the ends, and religating or by inserting oligonucleotide linkers of appropriate size into the linker restriction site. All mutations were created in pWH4 and were confirmed by DNA sequencing. To generate *ade6* and luciferase fusions, *Asp718-SacI* *mei3* promoter fragments were transferred into pWH6 and pWH8, respectively.

To generate altered-specificity derivatives of P_m, an 830-bp DNA fragment that encodes P_m and extends from the endogenous *BamHI* site 140 bp upstream of the transcription start site to an engineered *XbaI* site 140 bp downstream of the translational stop codon was generated by PCR amplification of *S. pombe* genomic DNA and cloned between the *BamHI* and *XbaI* sites of pBS-KS. Derivatives of this plasmid containing desired mutations in the putative DNA recognition helix of P_m were generated by site-directed two-step PCR mutagenesis. Wild-type and mutant P_m fragments (generated by cleavage with *PstI* and *Ecl136II*) were subcloned between the *PstI* and *SmaI* polylinker sites of pAD2 (1), a high-copy-number plasmid with an *ade6* selectable marker.

Construction of yeast strains. *S. pombe* strains used in this study are listed in Table 1. DNA molecules containing the mutant *mei3* promoter derivatives were linearized with *StuI* and introduced into appropriate *ura4* mutant strains, as

described previously (4). To confirm proper integration of these DNAs into the *ura4* locus, *Ura⁺* strains were analyzed on Southern blots in which *SmaI*-digested genomic DNA (10 μg) was hybridized with a *ura4* probe (³²P-labeled 1.78-kb *HindIII* fragment). The parental strain (or gene convertants) yields a 5.3-kb fragment, whereas correct integrants yield a single fragment of 12 kb (size varies slightly depending on whether the DNA was derived from pWH4, pWH6, or pWH8). Therefore, proper integration could be assayed on a Southern blot by observing a shift of a single 5.3-kb *SmaI ura4* fragment to a single *SmaI ura4* fragment of approximately 12 kb. Incorrect or multiple integrants contain multiple *ura4* fragments of various sizes.

Phenotypic assays. To assay for sporulation, WP16 (*h⁹⁰ leu1-32 ade6-210 ura4-595 mei3::LEU2⁺*) cells containing pWH4 derivatives were grown overnight at 30°C in 2 ml of YEL plus Ade medium, washed once in H₂O and once in 150 mM NaCl, and resuspended in the 20-μl residual volume of 150 mM NaCl. Twenty microliters of this cell suspension was spotted on SPA sporulation plates, incubated for 3 days at room temperature, and exposed to iodine vapors for 3 min. Iodine stains the polysaccharides that are found in the ascus walls and in the cell walls of ascospores but not in vegetative cells. Wild-type cells appear as a dark ring at the edges of the spots, representing cells that are in contact with the surface of the plate and which have mated and sporulated. Cells that are not in contact with the plate surface do not receive enough nutrients to mate and sporulate; hence, they are unstained. Sporulation phenotypes are defined as follows: ++, sporulation indistinguishable from the wild type; +, noticeably less sporulation than the wild type; ±, barely detectable sporulation; −, no detectable sporulation (see Fig. 1). Sporulation phenotypes are consistent among independent *Ura⁺* transformants containing the same *mei3* promoter construct.

The activity of *mei3* promoter mutants in nitrogen-sated haploids was assayed by using WP17, WP45, or WP162 (heterothallic *ura4 ade6Δ*) strains containing pWH6 derivatives (*mei3-ade6* fusions). *Ade⁺* phenotypes were tested by the ability of these strains to grow on glucose minimal medium lacking adenine and by their color on rich medium (*Ade⁺* strains are white, whereas *Ade⁻* strains are red).

For altered-specificity experiments, pAD2 plasmids expressing wild-type and mutant derivatives of P_m were introduced into WP16 derivatives carrying wild-type or mutant D alleles by selecting for *Ade⁺* colonies. Plasmid-borne P_m derivatives were expressed from the natural promoter and hence presumably only under conditions in which the endogenous P_m was expressed.

Quantitation of *mei3* promoter activity by luciferase assays. pWH8 derivatives integrated into the *ura4* locus of WP162 (*h⁻ mei3 ura4 lys1*) were mated to WP151 (*h⁺ mei3 ura4 pro2*) on a sporulation plate overnight, and diploids were subsequently selected on minimal NBA plus 0.01% Ade plates. Diploid (or haploid control) cultures (50 ml) were grown at 30°C in EMM-2 minimal medium lacking lysine and proline to mid-log phase (*A*₆₀₀ = 0.7) and then divided into two portions. One portion (35 ml) was washed twice with water, resuspended in 30 ml of EMM-2 containing amino acids but lacking NH₄Cl, and incubated at 22°C for 4 h with shaking. The remaining portion was diluted into 45 ml of EMM-2 lacking lysine and proline and grown for 3.5 h at 30°C. After measuring the *A*₆₀₀, cultures were washed once with ice-cold H₂O, resuspended in 0.9 ml of buffer (100 mM KPO₄ [pH 7.8], 1 mM dithiothreitol) on ice, and divided into three samples (approximately 0.4 ml) to which 0.2 ml of acid-washed glass beads were added. Cells were disrupted by vortexing in a multitube vortexer on high at 4°C for 12 min, and insoluble material was removed by microcentrifugation for 5 min at 4°C and placed on ice. Luciferase activity was measured by adding 100 μl of each sample to a glass vial containing 200 μl of assay buffer (25 mM Tricine, 15 mM MgCl₂, 5 mM ATP, 500 μg of bovine serum albumin per ml) and 100 μl

of 0.5 μ M luciferin that had been placed in a luminometer. Photon emission was detected for 10 s, and luciferase activity was normalized to the number of cells. To minimize experimental variation, luciferase activities of the various strains were normalized to that of the strain containing the intact *mei3* promoter (502 nucleotides of *mei3* promoter sequence) by using samples that were prepared and analyzed in parallel, and assays were carried out on independent occasions. The experimental error is $\pm 30\%$ (except for strains that express very low luciferase activity, where the error is higher).

RESULTS

Defining the minimal *mei3* promoter by sequential 5' deletions. The *mei3* promoter region (defined here as sequences 502 bp upstream of the mRNA initiation site) contains a putative TATA element (TATAAG) located between -33 and -28 with respect to the mapped transcription start site (19). This location is in excellent accord with *in vitro* transcription reactions reconstituted with *S. pombe* components (9, 18), and TATAAG is a moderately functional TATA element (27). In the vicinity of the mRNA initiation site, the *mei3* promoter contains a sequence (GCATCCA, located between -2 and $+5$) that weakly resembles a eukaryotic initiator element (consensus PyPyAN[T/A]PyPy) (14).

To assess whether the isolated *mei3* promoter (to -502) is sufficient to confer nitrogen-starvation-dependent transcriptional induction, a *mei3* segment (-502 to $+94$) was fused translationally to the luciferase structural gene. A stable diploid strain containing this DNA integrated at the *ura4* locus was grown to mid-log phase in nitrogen-rich medium and shifted to medium lacking nitrogen. A peak of luciferase activity was observed 3 h after nitrogen starvation (data not shown), a result in accord with the observation that *mei3* mRNA levels are maximal between 2.5 and 4 h after nitrogen starvation (19). Thus, the information necessary for proper *mei3* transcription resides in the 502 nucleotides of *mei3* promoter and 5' untranslated region.

To define the minimal *mei3* promoter region, *mei3* DNAs successively deleted for sequences upstream of the mRNA initiation site were integrated in single copy at the *ura4* locus of a *mei3* deletion strain (WP16), and the resulting transformants were tested for sporulation efficiency. Deletions that contain 502 and 327 bp upstream of the mRNA initiation site ($\Delta 1$ and $\Delta 2$, respectively) have sporulation phenotypes indistinguishable from a wild-type strain, whereas a deletion retaining 278 bp ($\Delta 3$) has markedly reduced sporulation efficiency (Fig. 1). These results suggest that sequences downstream of -328 are necessary and sufficient to confer proper *mei3* expression and that the region between -502 and -327 is not essential.

Crude internal deletions define two positive regulatory regions. To define elements necessary for *mei3* transcription, we analyzed the sporulation phenotypes conferred by a set of internal deletions within the *mei3* promoter region. Two separable promoter regions are identified from the crude deletion analysis shown in Fig. 1. Region I (-327 to -259) is defined by the observation that strains containing $\Delta 7$ sporulate normally whereas strains containing the more extensive $\Delta 5$ fail to sporulate. A deletion that removes much of region I ($\Delta 6$) is partially defective for sporulation. For region II (-158 to -66), the distal end is defined by $\Delta 7$, which sporulates normally, and the proximal end is loosely defined by $\Delta 15$, which sporulates with a reduced efficiency. Most internal deletions within region II are completely unable to sporulate. However, a small deletion at the distal end, $\Delta 9$, sporulates with reduced efficiency, suggesting that this derivative only partially disrupts a positive element in region II.

Dissection of region I into elements A and B. Region I was dissected by smaller internal deletions and linker-scanning mu-

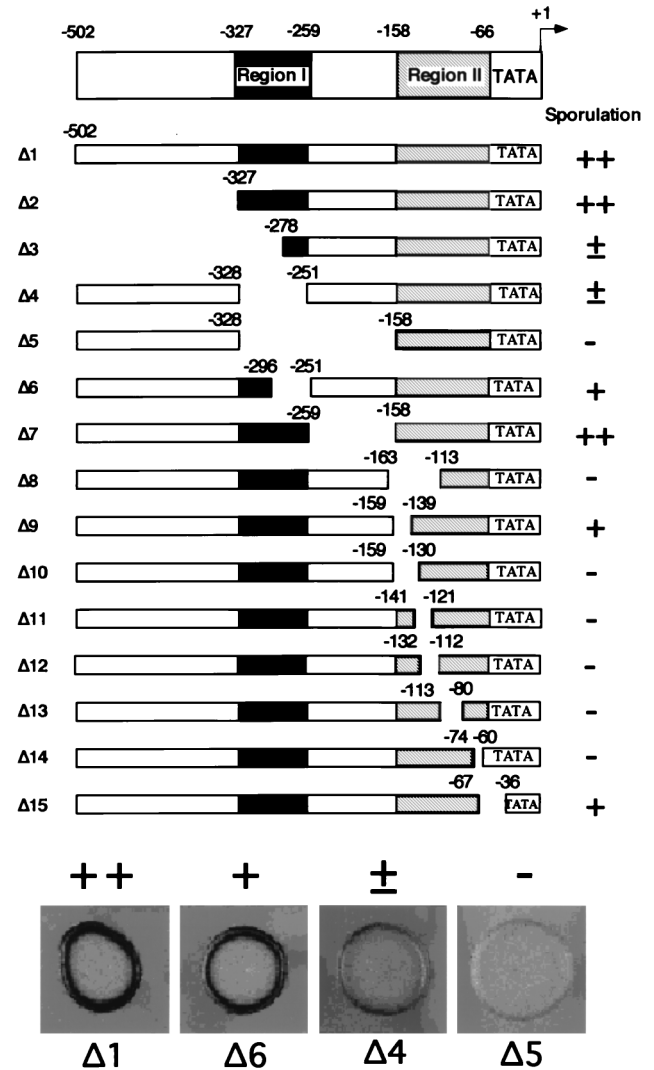


FIG. 1. Definition of two positive regulatory regions by deletion analysis of the *mei3* promoter. For each *mei3* deletion mutant, solid bars indicate regions that are present and blank spaces indicate regions that are deleted; nucleotide positions at the boundaries indicate the last residue that is present and are defined with respect to the $+1$ transcriptional initiation site. Sporulation phenotypes (assessed by iodine staining) conferred by these *mei3* derivatives are defined as follows: ++, indistinguishable from a wild-type strain; +, noticeably reduced from wild-type levels; \pm , barely detectable; -, no detectable sporulation. Sporulation assays that indicate the phenotypic range are shown for selected *mei3* derivatives ($\Delta 1$, $\Delta 6$, $\Delta 4$, and $\Delta 5$). The approximate locations of regions I and II are indicated (see text).

tations (Fig. 2). Elements A and B are defined by nonoverlapping portions of region I that confer increased sporulation over that conferred by $\Delta 4$, which is completely deleted for region I. Element A (-327 to -295) is defined by $\Delta 6$ and $\Delta 19$, which confer partial sporulation activity in the absence of element B. This location is consistent with the phenotypic distinction between $\Delta 2$ and $\Delta 3$ (Fig. 1). However, deletion of element A has no significant phenotypic effect if element B is present (e.g., $\Delta 16$ and $\Delta 17$). Element B (-273 to -256) is defined by the comparison of $\Delta 4$, which has an extremely weak sporulation phenotype, and $\Delta 17$, which confers wild-type sporulation. Sequences between -262 and -273 are important for element B, because their removal leads to reduced promoter activity (compare $\Delta 18$ and $\Delta 19$). Consistent with this observation, link-

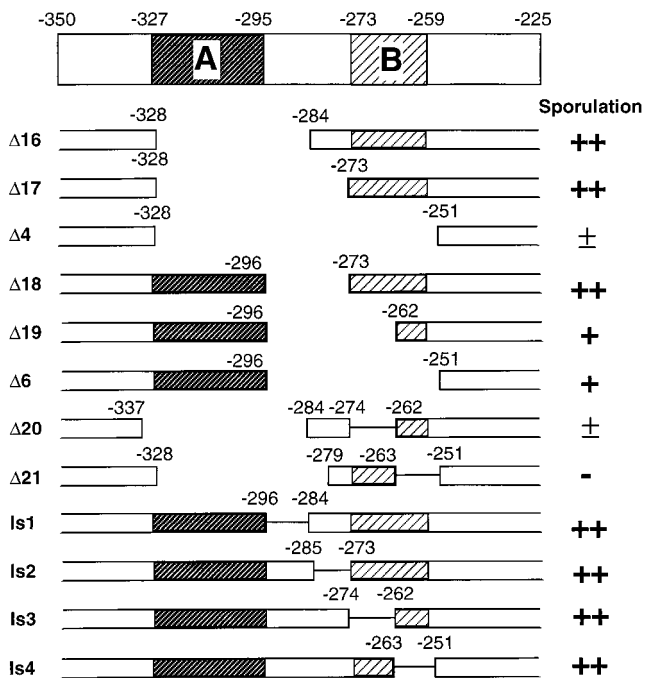


FIG. 2. Definition of elements A and B by deletion analysis of region I. The structures and sporulation phenotypes of the indicated *mei3* derivatives are shown as described in the legend to Fig. 1, with the addition that solid lines indicate the location of linker-scanning mutations. The approximate locations of elements A and B are indicated (see text).

er-scanning mutations in element B significantly reduce ($\Delta 20$) or eliminate ($\Delta 21$) sporulation under conditions where element A is deleted. However, linker-scanning mutations in element B (ls1 to ls4) do not affect sporulation in the context of the intact *mei3* promoter region, presumably due to compensation by element A and perhaps by sequences between -273 and -295. In this regard, the downstream endpoint of element A is poorly defined and may extend beyond -295. Thus, region I contains at least two partially redundant positive elements that contribute to *mei3* transcription.

Identification of elements D and E in region II. Region II was initially dissected by insertion-scanning mutations that replace 10 bp of the *mei3* promoter region with 12 or 14 bp of heterologous sequence (Table 2). At least one positive element is located between -158 and -86, because all insertion-scanning mutations in this region are severely or completely defective for sporulation whereas $\Delta 22$, $\Delta 51$, and $\Delta 52$ are fully functional. In addition, there is a distinct element (termed E) that is defined by a small internal deletion ($\Delta 14$ [Fig. 1]) which removes residues -73 to -60. Element E is clearly separable from the further upstream elements by virtue of insertion-scanning mutations $\Delta 51$ and $\Delta 52$, which do not detectably affect sporulation; these derivatives also establish the upstream boundary of element E at -72.

Because insertion-scanning mutations affect both nucleotide sequence and spacing, we further dissected region II with 10-bp linker-scanning mutations covering sequences from -167 to -27 (Fig. 3). In striking contrast to the result with the comparable insertion-scanning mutations, nearly all of the linker-scanning mutations confer wild-type sporulation. The two exceptions, ls13 (-92 to -83) and ls15 (-74 to -65), are completely defective for *mei3* promoter function, respectively defining two positive elements, D and E. Sporulation is re-

TABLE 2. Insertion-scanning mutations

<i>mei3</i> allele	Endpoint		Spacing change	Sporulation phenotype
	Upstream	Downstream		
$\Delta 22$	-167	-158	+4	++
$\Delta 23$	-158	-149	+4	+
$\Delta 24$	-158	-149	+20	\pm
$\Delta 25$	-149	-140	+2	-
$\Delta 26$	-149	-140	+4	-
$\Delta 27$	-149	-140	+8	-
$\Delta 28$	-149	-140	+20	-
$\Delta 29$	-140	-131	+4	-
$\Delta 30$	-140	-131	+10	-
$\Delta 31$	-140	-131	+12	-
$\Delta 32$	-140	-131	+14	-
$\Delta 33$	-140	-122	+1	\pm
$\Delta 34$	-131	-122	+4	-
$\Delta 35$	-131	-122	+10	-
$\Delta 36$	-131	-122	+12	-
$\Delta 37$	-131	-122	+14	-
$\Delta 38$	-131	-113	+1	-
$\Delta 39$	-122	-113	+4	-
$\Delta 40$	-122	-113	+4	-
$\Delta 41$	-122	-113	+10	-
$\Delta 42$	-122	-113	+12	-
$\Delta 43$	-122	-113	+14	-
$\Delta 44$	-112	-113	+8	-
$\Delta 45$	-112	-103	+4	-
$\Delta 46$	-112	-103	-1	-
$\Delta 47$	-102	-93	+4	\pm
$\Delta 48$	-102	-93	+10	-
$\Delta 49$	-102	-93	+12	-
$\Delta 50$	-102	-93	+14	-
$\Delta 51$	-86	-83	+2	++
$\Delta 52$	-82	-73	+4	++

duced, but not eliminated, by ls19, a linker-scanning mutation that destroys the putative TATA element. The fact that the ls15 mutation eliminates sporulation indicates that element E is defined by a specific sequence, not by an effect on spacing. Further, residues -72 to -67 (TCCGTG) are particularly important for element E function because linker-scanning mutations on either side of this region, ls14 (-82 to -73) and ls16 (-66 to -57), confer normal sporulation (Fig. 4).

Element D is defined by the -92 to -83 linker-scanning mutation (ls13), and residues -92 to -87 (TTACAC) are particularly important because a disruption of sequences further downstream ($\Delta 51$) does not affect sporulation (Fig. 4). The spacing between elements D and E is not critical because insertion-scanning mutations ($\Delta 51$ and $\Delta 52$) between them have minimal phenotypic effects. Element D coincides with a sequence (CTTTACACG, located between -86 and -94) that has eight of nine matches with the consensus half-site, A(A/T)NTACAPyPu, recognized by the *S. cerevisiae* $\alpha 2$ homeodomain (28).

Mating-type protein P_m interacts with element D in vivo. Given the element D sequence and the strong similarity between the $\alpha 2$ and P_m homeodomains (28), we hypothesized that P_m might activate *mei3* transcription in vivo by directly binding to element D of the promoter. This hypothesis predicts the existence of altered-specificity derivatives of P_m that function at *mei3* promoters containing specifically mutated versions of element D (Fig. 5A). Based on DNA-binding specificity experiments involving the bicoid and antennapedia homeodomains (11, 12), we generated a bicoid-like derivative of P_m (S155K) and the following four alleles of element D: a bicoid-

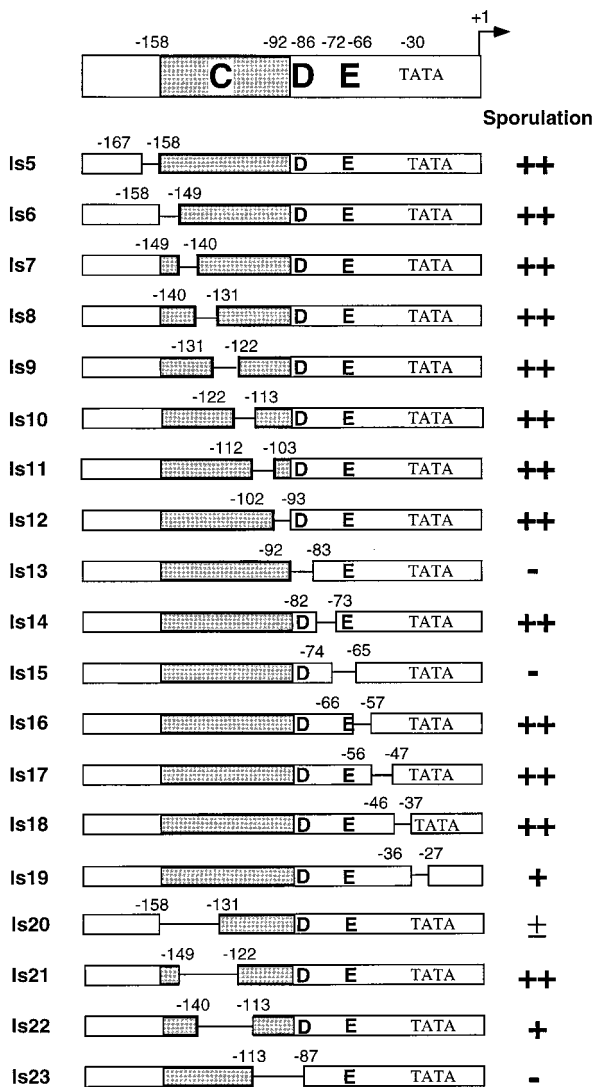


FIG. 3. Definition of elements D and E by deletion analysis of linker-scanning mutations. The structures and sporulation phenotypes of the indicated *mei3* derivatives are shown as described in the legend to Fig. 1, except that linker-scanning mutations are defined by the regions that are mutated; e.g., Is13 substitutes 10 bp for nucleotides -92 to -83 inclusive. The locations of elements D, E, and C, which is defined primarily by insertion-scanning mutations (see Table 3), are also indicated.

like site (TAATCCC [d3]), antennapedia-like binding sites (TTATTAG [d2] and TAATTAG [d4]), and a mutant site (TTGAGCG [d1]) that presumptively would not interact with homeodomains (underlining indicates differences from wild-type *mei3* element D).

Multipcopy plasmids expressing the P_m derivatives from the natural promoter were introduced into wild-type strains containing single integrated copies of the *mei3* alleles containing element D mutations (Fig. 5B). As expected, promoters containing the element D mutations are extremely defective in the ability to support sporulation in the presence of normal amounts of P_m (vector only). However, the bicoid-like S155K derivative confers efficient (and perhaps greater than wild-type) levels of sporulation (and hence *mei3* expression) in combination with the bicoid-like d3 allele but not with any of the other mutations of element D. In addition, sporulation is

observed when the S155K derivative is introduced into a strain containing the wild-type promoter but lacking P_m (data not shown). Thus, the S155K derivative has relaxed DNA-binding specificity in that it interacts with wild-type and bicoid-like versions of element D but not with antennapedia-like or mutated forms of this element. The ability of the S155K derivative of P_m to suppress a specific mutation of element D in a manner consistent with the known DNA-binding specificity of bicoid (11, 12) indicates that P_m interacts with element D in vivo. Consistent with this conclusion, overexpression of wild-type P_m weakly suppresses the sporulation defect conferred by the d2, d3, and d4 alleles.

Unusual properties of element C. Element C is defined by the observation that sporulation is severely or completely defective in strains containing insertion-scanning mutations that locally disrupt any small region between -158 and -92 (Table 2). Although the boundaries of elements C and D are not precisely defined (and may overlap), element C is distinct from element D because its function cannot be eliminated by linker scanning. Mutations that disrupt the distal ($\Delta 9$, $\Delta 23$, $\Delta 24$) or proximal ($\Delta 47$) end of element C confer some *mei3* promoter function, whereas centrally located mutations are generally nonfunctional. This observation might reflect disruption of redundant subelements at the ends of element C or the importance of centrally located residues for element C function.

The unusual feature of element C is that 10-bp linker-scanning mutations anywhere within this large region do not significantly affect sporulation (Fig. 3), whereas insertion-scanning mutations at the same positions drastically reduce *mei3* promoter function (Table 2). It is unlikely that the linker sequences themselves encode positive elements that compensate for the disruption of element C. In all cases tested, changing the linker sequence does not affect the sporulation phenotype. In addition, several of the internal deletions that disrupt element C function have the same junction sequences as the linker-scanning mutations that have no phenotypic effect. Finally, in all cases tested, *mei3* function is disrupted by small deletions within element C that decrease spacing by 1, 5, 9, and 18 bp or by insertion mutations that increase spacing by 1, 2, 4, 8, 9, 10, 12, 14, and 20 bp (Table 2). In the most dramatic example, Is11 and $\Delta 46$ differ only by the deletion of a single base pair yet have completely different sporulation phenotypes. Taken together, these observations suggest that any change in spacing drastically affects element C function whereas a change in the nucleotide sequence has much less effect.

We also analyzed very long linker-scanning mutations in which 27- or 28-bp regions were replaced with heterologous DNA (Fig. 3). Two such mutations (Is20 and Is22) significantly reduce, but do not completely eliminate, *mei3* expression. However, the -149 to -121 mutation (Is21) has substantial overlap with the above mutations yet confers wild-type levels of sporulation. These apparently contradictory observations are suggestive of redundancy in element C (see Discussion). As expected, a large linker-scanning mutation that also disrupts element D (Is23) completely eliminates *mei3* promoter function.

Quantitation of *mei3* promoter activity in mutant strains. To quantitate *mei3* promoter activity, *mei3* derivatives with a range of sporulation phenotypes were fused to the luciferase reporter gene and integrated into the *ura4* locus. Luciferase activity was assayed in uninduced (nitrogen sated) and induced (nitrogen starved for 4 h) *mei3* diploids (Table 3). Interestingly, all derivatives with noticeable defects in sporulation (i.e., +, ±, and - phenotypes) show <10% of the wild-type levels of luciferase activity. This observation suggests that a limited amount of *mei3* transcription is sufficient to confer significant

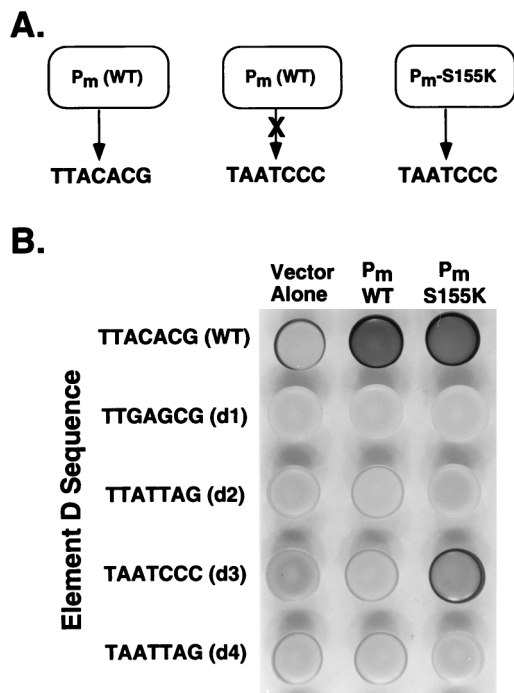


FIG. 5. P_m interacts with element D in vivo. (A) Wild-type P_m interacts with wild-type element D (TTACACG) but not with the bicoid-like d3 allele (TAAATCCC), indicating that the P_m -element D interaction is critical for sporulation. An altered-specificity derivative of P_m (S155K) that interacts with the d3 allele should effectively recreate the P_m -element D interaction and permit sporulation. (B) Sporulation phenotypes (assayed by iodine staining) for strains containing the indicated alleles of element D (WP20, WP434, WP495, WP496, and WP499) and plasmids overexpressing the wild type or S155K derivative of P_m . All strains, including the control containing the pAD2 vector, contain the chromosomal copy of P_m .

wide variety of mutations throughout the *mei3* promoter region. This result is significant because promoter analyses of the type described here have uncovered negative regulatory elements in a wide variety of eukaryotic promoters. Indeed, in the case of meiotic regulation in *S. cerevisiae*, negative elements were easily identified in the promoters of *IME1* (6, 22) and *IME2*, a gene that acts downstream of *IME1* (3).

It is impossible to prove that a given promoter does not contain a negative element. It is always possible that a negative element in a promoter might be obscured by redundant negative elements or by overlapping positive elements that are required for expression. Thus, the validity of the argument that a given promoter lacks a negative element depends on the number and variety of mutations tested; in this regard, our analysis is extensive. Furthermore, negative elements are generally easy to uncover, because even a partial loss of repression can lead to significant levels of transcription (e.g., 50% repression results in transcription only twofold below wild-type levels). Because our assays for inappropriate transcription in haploid cells (iodine staining and *Ade*⁺ phenotype) are very sensitive, it is likely that the various *mei3* promoter derivatives are inactive in haploid cells and that our analysis would uncover mutants that only partially relieve repression.

The *mei3* promoter is complex and contains at least five positive elements. The *mei3* region between -327 and the ATG initiation codon is necessary and sufficient to mediate proper *mei3* expression. Within this region, we have defined five promoter elements by the following criteria. Element E is defined by a linker-scanning mutation (ls15) and a small dele-

TABLE 3. Luciferase assays

<i>mei3</i> allele	Endpoint		Phenotype	
	Upstream	Downstream	Sporulation	Luciferase activity (%)
$\Delta 1$	None	-502	++	100
$\Delta 7$	-258	-159	++	32 \pm 8
$\Delta 16$	-327	-285	++	62 \pm 8
$\Delta 18$	-295	-274	++	37 \pm 5
$\Delta 22$	-167	-158	++	90 \pm 30
$\Delta 51$	-86	-83	++	40 \pm 7
$\Delta 52$	-82	-73	++	180 \pm 51
ls1	-295	-285	++	63 \pm 25
ls3	-273	-263	++	109 \pm 24
ls5	-167	-158	++	53 \pm 6
ls6	-158	-149	++	47 \pm 7
ls7	-149	-140	++	30 \pm 6
ls8	-140	-131	++	34 \pm 6
ls9	-131	-122	++	44 \pm 11
ls10	-122	-113	++	41 \pm 5
ls11	-112	-103	++	32 \pm 3
ls12	-102	-93	++	47 \pm 14
ls14	-82	-73	++	109 \pm 26
ls16	-66	-57	++	138 \pm 50
ls17	-56	-47	++	81 \pm 10
ls18	-46	-37	++	97 \pm 24
$\Delta 9$	-158	-140	+	5 \pm 2
ls22	-140	-113	+	2 \pm 1
$\Delta 11$	-140	-122	\pm	2 \pm 1
$\Delta 5$	-327	-159	-	2 \pm 1
$\Delta 10$	-158	-131	-	3 \pm 1
$\Delta 34$	-131	-122	-	1 \pm 1
$\Delta 45$	-112	-103	-	4 \pm 1
$\Delta 46$	-112	-103	-	3 \pm 1
ls13	-92	-83	-	6 \pm 1
dl	-90	-88	-	9 \pm 4

tion ($\Delta 14$) that eliminate sporulation. Element D is defined by linker-scanning mutations and multiple base pair substitutions that eliminate sporulation. Element C is defined by insertion-scanning mutations that map to a large region (-158 to -93) and greatly reduce or eliminate *mei3* expression. Element B is defined by $\Delta 19$, a moderately sized deletion (-295 to -262) that significantly reduces *mei3* expression, and by two linker-scanning mutations that eliminate sporulation when element A is deleted (derivatives $\Delta 21$ and $\Delta 22$). However, linker-scanning mutations in element B (ls3 and ls4) have no phenotypic effects in an otherwise intact *mei3* promoter. Element A is defined loosely as a region (-327 to -295) that is important for *mei3* expression when element B is deleted.

The five promoter elements are separable because regions between them can be heavily mutated or deleted with no detectable effect on sporulation and modest or no reduction of *mei3* expression (as assayed by luciferase fusions). Thus, elements A and B are separated by $\Delta 18$ (-295 to -273), elements B and C are separated by $\Delta 7$ (-256 to -158) and the insertion-scanning mutation $\Delta 22$, and elements D and E are separated by linker-scanning (ls14) and insertion-scanning ($\Delta 51$ and $\Delta 52$) mutations. Elements C and D have not been physically separated, but they are functionally distinct because element D can be inactivated by linker-scanning and base pair substitution mutations whereas element C can be inactivated only by insertion-scanning mutations. With the notable exception of element C (see below), the *mei3* promoter elements do not require precise spacing relationships with each other.

Several promoter derivatives are modestly affected in *mei3* expression, despite displaying a wild-type sporulation pheno-

type (Table 3). Such partially defective *mei3* derivatives can be interpreted in a variety of ways. First, these derivatives could inactivate a promoter element whose function is partially redundant with another element; e.g., the modest effects of linker-scanning mutations in element C might reflect redundant functions within this element (see below). Second, these derivatives might partially affect the function of one of the five defined elements by altering residues at the element boundaries. This explanation is likely for $\Delta 51$, which affects the downstream edge of element D, and for $\Delta 18$, which affects the poorly defined boundary between elements A and B. Third, in the case of deletion mutants (particularly the large $\Delta 7$ between regions I and II), transcriptional defects might arise from altered spacing between elements. While recognizing that these partially defective mutants affect our description of the *mei3* promoter, we have defined the five positive elements based on the properties of mutants with severe phenotypic and transcriptional effects.

It is important to note that our view of the *mei3* promoter region represents a formal description that does not presuppose a particular molecular mechanism. Furthermore, our description represents the simplest interpretation that is consistent with all the data. As is always the case with promoter dissections, we cannot exclude more complex interpretations. Finally, although we have identified and localized five elements within the *mei3* promoter region, the precise boundaries of some of these elements (particularly A, B, and C) have not been defined and the existence of additional positive elements has not been excluded.

Evidence that M_c binds to element C and acts as an architectural transcription factor. Element C is very large (60 bp) and has the unusual property of being inactivated by insertion-scanning mutations but not by linker-scanning mutations. We doubt that element C is simply a spacer sequence that maintains the correct distance between other promoter elements, because insertion-scanning or deletion mutations that change the spacing relationship of elements A, B, and E to element C do not have phenotypic effects. Instead, we suggest that element C contains subelements that have a precise spacing relationship to each other and/or to element D. However, the failure of 10-bp linker-scanning mutations to inactivate element C implies that such putative subelements would be functionally redundant. Consistent with this possibility, some linker-scanning mutations within element C reduce *mei3* expression (Table 3), and a large linker-scanning mutation (ls20) can virtually inactivate element C.

Interestingly, element C contains six sequences (ATTGTA, ATTATT, TTTGTT, ATAGTT, TTTGTT, and TTTGAT) that resemble the consensus recognition sequence, (A/T)TTGTT, for DNA-binding proteins with an HMG structural motif (10). One of the mating-type proteins, M_c (15), contains a region that strongly resembles (32% identity, 64% similarity over 76 amino acids) the HMG domain of SRY, a protein that determines male sex in mammals. Furthermore, M_c behaves like a typical HMG protein; it binds the HMG consensus sequence with high affinity primarily through interactions with the minor groove, and it severely bends DNA (8). For these reasons, we speculate that element C consists of multiple M_c interaction sites and that M_c binds and bends DNA to form a highly specific nucleoprotein structure that is important for *mei3* transcription (Fig. 6). This model, in which M_c functions as an "architectural transcription factor" (26), could account for the apparent paradox of functional redundancy and severe spacing constraints, particularly if protein-protein interactions between M_c molecules energetically compensate for weak protein-DNA interactions due to linker-scanning mutations.

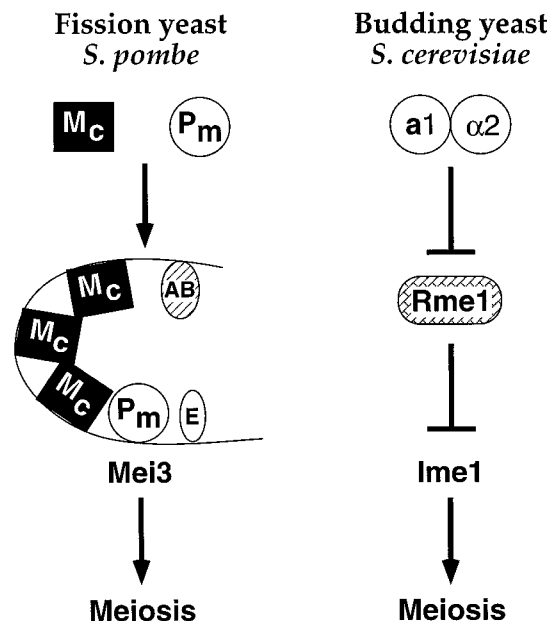


FIG. 6. Different regulatory logic for cell type control of the key meiosis-inducing genes in fission and budding yeasts. In *S. pombe*, diploids express two distinct DNA-binding proteins, M_c , which contains an HMG domain, and P_m , which contains a homeodomain homologous to $\alpha 2$. P_m binds to element D of the *mei3* promoter, and M_c is likely (but not directly shown) to bind multiple sites within element C. These proteins, together with putative proteins interacting with elements E and A or B, stimulate transcription of *mei3*, the trigger for meiosis. Haploids do not undergo meiosis because P_m and perhaps these other proteins are not present or active. Given the unusual properties of element C, we speculate that *mei3* transcription requires the formation of an enhanosome that depends on DNA-binding and bending properties of the HMG protein M_c (see text). In *S. cerevisiae*, haploids do not undergo meiosis because they express Rme1, a repressor that binds the promoter of *IME1*, the key meiosis-inducing gene. Diploids can undergo meiosis because they specifically express the $\alpha 1$ and $\alpha 2$ homeodomain proteins, which form a heterodimeric repressor that directly binds the *RME1* promoter and blocks production of Rme1. Hence, $\alpha 1$ - $\alpha 2$ indirectly cause the expression of Ime1 by repressing a repressor, whereas P_m and presumably M_c directly bind the *mei3* promoter and activate transcription. Furthermore, $\alpha 2$ functions as a repressor whereas the homologous P_m functions as an activator. The key meiosis-inducing proteins, Meic and Ime1, have unrelated molecular functions, and the pathways downstream of these proteins are completely different.

P_m interacts with element D and directly activates *mei3* transcription. Several lines of evidence indicate that element D interacts with P_m in vivo. First, element D strongly resembles an $\alpha 2$ binding site, and the P_m homeodomain is 41% identical (70% similar) to the $\alpha 2$ homeodomain. Although the recognition helix of P_m is five residues shorter than that of $\alpha 2$, all of the residues that contact bases or phosphates (28) are conserved between $\alpha 2$ and P_m , including homeodomain residue 50 (S155 in P_m), which plays an important role in DNA-binding specificity (11). Second, mutational analysis of element D indicates that the critical base pairs for sequence recognition by $\alpha 2$ are essential for *mei3* expression. Third, overexpression of P_m partially suppresses the sporulation-defective phenotype conferred by several element D mutations. Fourth, and most convincing, the S155K derivative of P_m strongly suppresses a specific mutation of element D; it permits *mei3* expression from the d3 allele but not from other mutant forms of element D. Furthermore, this allele-specific suppression reflects a direct protein-DNA interaction between P_m and element D because the combination of compensating mutations is consistent with the known DNA-binding specificity of bicoid (11, 12). Given that P_m interacts with element D in vivo and that ele-

ment D is positively required for *mei3* transcription, we conclude that P_m functions as a transcriptional activator at the *mei3* promoter.

The *mei3* promoter acts as an on-off developmental switch because transcription requires synergistic activation by multiple elements. It is commonly observed that efficient transcription of eukaryotic genes requires the combinatorial and synergistic action of distinct elements in the promoter region. Wild-type levels of *mei3* transcription require the synergistic activation of elements C, D, and E and region I (which contains elements A and B). Mutations in any of these four components drastically reduces *mei3* expression.

In the context of wild-type *S. pombe* cells, the requirement for at least four independent elements restricts *mei3* expression to circumstances when the proteins bound to these elements are functionally active. At a minimum, these circumstances include the presence of the M and P mating-type loci, the pheromone signal, and the environmental condition of nitrogen starvation. Given that the *mei3* promoter elements appear to be unrelated in DNA sequence, we presume that P_m , M_c , and two or three other proteins must be present and active under the specific conditions that are appropriate for *mei3* transcription (Fig. 6). Under other conditions, we presume that one or more of these proteins are absent or inactive. Thus, the *mei3* promoter constitutes an on-off developmental switch that is responsible for the tight regulation of *mei3* expression that is critical in the *S. pombe* life cycle.

In several respects, the complex *mei3* promoter resembles the human beta interferon enhancer. Transcription of the beta interferon gene is restricted to the specific circumstance of virus infection, and this restriction reflects the requirement for the binding of multiple and distinct proteins to their cognate elements in the enhancer (16, 24). In this sense, the beta interferon enhancer functions as an on-off environmental switch. The function of the beta interferon enhancer depends on the architectural transcription factor HMG-I, and it is exquisitely sensitive to spacing changes between individual elements. Thus, transcription of the beta interferon gene requires the assembly of a precisely structured multiprotein-DNA complex termed an "enhancesome" (24). By analogy, we speculate that developmentally regulated expression of *mei3* might involve the assembly of an enhancesome (Fig. 6), a structure not previously described for unicellular eukaryotes. However, the observation that elements A, B, E, and perhaps D do not require precise spacing relationships suggests that a putative *mei3* enhancesome might be restricted to element C and M_c . In contrast, the beta interferon enhancesome is composed of distinct elements that interact with different proteins.

The regulatory logic for cell type control of the key meiosis-inducing gene differs between fission and budding yeasts. In many respects, mating-type control of sporulation is similar in *S. cerevisiae* and *S. pombe*. Both organisms contain one active and two silent mating-type cassettes, each of the two mating-type alleles expresses two proteins, and *S. cerevisiae* $\alpha 2$ and *S. pombe* P_m contain homologous homeodomains. Nevertheless, in contrast to the *S. cerevisiae* mating-type proteins, which directly repress the Rme1 repressor of the key meiotic control gene (*IME1*), the *S. pombe* mating-type proteins (P_m and probably M_c) directly activate *mei3*, the gene that triggers meiosis (Fig. 6). Furthermore, the homologous homeodomain proteins regulate transcription of the direct target genes in opposite manners; i.e., $\alpha 2$ functions as a repressor whereas P_m functions as an activator. Finally, to deal with the critical issue of preventing expression of meiosis-inducing genes in haploid cells, *S. cerevisiae* uses an active repression mechanism whereas *S. pombe* does not appear to do so. Thus, our results demonstrate

that the underlying logic of cell type control of the key meiosis-inducing gene in fission yeast is fundamentally different from that in budding yeast.

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