Autoregulated Expression of *Schizosaccharomyces pombe* Meiosis-Specific Transcription Factor Mei4 and a Genome-Wide Search for Its Target Genes

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ABSTRACT

The *Schizosaccharomyces pombe mei4*$^+$ gene encoding a forkhead transcription factor is necessary for the progression of meiosis and sporulation. We searched for novel meiotic genes, the expression of which is dependent on Mei4p, since only the *spo6*$^+$ gene has been assigned to its targets. Six known genes responsible for meiotic recombination were examined by Northern blotting, but none were Mei4 dependent for transcription. We determined the important *cis*-acting element, designated FLEX, to which Mei4p can bind. The *S. pombe* genome sequence database (The Sanger Centre, UK) was scanned for the central core heptamer and its flanking 3′ sequence of FLEX composed of 17 nucleotides, and 10 candidate targets of Mei4 were selected. These contained a FLEX-like sequence in the 5′ upstream nontranslatable region within 1 kb of the initiation codon. Northern blotting confirmed that 9 of them, named *mde1*$^+$ to *mde9*$^+$, were transcriptionally induced during meiosis and were dependent on *mei4*$^+$. Most *mde* genes have not been genetically defined yet, except for *mde9*$^+$, which is identical to *spn5*$^+$, which encodes one of the septin family of proteins. *mde3*$^+$ and a related gene *pit1*$^+$ encode proteins related to *Saccharomyces cerevisiae* Ime2. The double disruptant frequently produced asci having an abnormal number and size of spores, although it completed meiosis. We also found that the forkhead DNA-binding domain of Mei4p binds to the FLEX-like element in the putative promoter region of *mei4* and that the maximum induction level of *mei4* mRNA required functional *mei4* activity. Furthermore, expression of a reporter gene driven by the authentic *mei4* promoter was induced in vegetative cells by ectopic overproduction of Mei4p. These results suggest that *mei4* transcription is positively autoregulated.

IN multicellular organisms, gametes differentiate into morphologically and functionally specialized cells. Sporulation in single-celled eukaryotes such as yeasts is a morphogenetic process equivalent to gametogenesis, because an ascospore is a highly specialized cell and its formation is preceded by meiotic nuclear division. Programmed gene expression guarantees an accurate progression of ordered events during cellular morphogenesis. Accordingly, a number of specific transcription factors might be involved in gametogenesis of higher eukaryotes and sporulation of yeasts.

Transcriptional control in the course of sporulation has been studied extensively in the budding yeast *Saccharomyces cerevisiae* (Kupiec *et al.* 1997). To understand genome-wide alterations in the expression level of individual genes during sporulation, DNA microarray technology has been applied (Chu *et al.* 1998). Among ~6200 protein-encoding genes in the *S. cerevisiae* genome, ~500 are transcriptionally upregulated during sporulation. Such a shift in gene expression might be attained by transcriptional cascades. Ndt80 is supposed to be one of the most important sporulation-specific transcription factors, because it can affect the expression of >200 genes (Chu *et al.* 1998). Ndt80 is also a key mediator of the meiotic recombination checkpoint, which represses the activity of Ndt80, resulting in a meiosis blockade at the pachytene stage (Chu and Herskowitz 1998; Hepworth *et al.* 1998).

To date, only Mei4 has been found as the meiosis-specific transcription factor in the fission yeast *Schizosaccharomyces pombe* (Horie *et al.* 1998). Mei4p is required primarily for progression through prophase-I, because *mei4* mutants arrest in this stage. The only defined target of Mei4p is *spo6*$^+$ (Horie *et al.* 1998). The arrest phenotype of *mei4* mutants (Bresch *et al.* 1968; Olson *et al.* 1978; Shimoda *et al.* 1985) cannot be explained by the failure of *spo6* expression, because *spo6*$^+$ is required only for meiosis-II and sporulation (T. Nakamura, personal communication). Therefore, more meiosis-specific genes are probably expressed under Mei4 control, and some of them must be indispensable for prophase-I. Mei4 protein contains a forkhead DNA-binding domain at its N-terminal region (Horie *et al.* 1998). The domain is composed of ~120 amino acids and is evolutionarily conserved (Weigel and Jackle 1990). The *cis*-acting element located in the 5′ upstream region of *spo6* was determined by gel mobility shift assay (Horie *et al.* 1998). The Mei4 forkhead domain binds 27-bp oligonucleotides containing the heptamer core, GTAAACA,

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TABLE 1

Strains used in this study

Stain	Genotype	Source
JZ670	*h⁻ pat1-114 leu1 ade6-M210* *h⁻ pat1-114 leu1 ade6-M216*	M. Yamamoto
JY741	*h⁻ ura4-D18 ade6-M216 leu1*	M. Yamamoto
C525	*h⁹⁰ ura4-D18 ade6-M210 leu1* *h⁹⁰ ura4-D18 ade6-M216 leu1*	C. Shimoda
C525C-1A	*h⁹⁰ mei4::ura4⁺ ura4-D18 ade6-M210 leu1*	C. Shimoda
C650-7C	*h⁹⁰ ade6-M210 leu1*	C. Shimoda
AB1-2C	*h⁻ pat1-114 ura4-D18 ade6-M210 leu1*	This study
AB4	*h⁻ mei4::ura4⁺ ura4-D18 pat1-114 leu1 ade6-M210* *h⁻ mei4::ura4⁺ ura4-D18 pat1-114 leu1 ade6-M216*	This study
AB11-15C	*h⁻ mei4-KA42 pat1-114 ura4-D18 ade6-M216 leu1*	This study
AB12-11B	*h⁻ mei4-KA5 pat1-114 ura1 ade6-M216 leu1*	This study
AB13-1D	*h⁻ mei4-KE76 pat1-114 ura4-D18 ade6-M210 leu1*	This study
AB16-8B	*h⁹⁰ mde3::ura4⁺ ura4-D18 ade6-M210 leu1*	This study
AB19-7D	*h⁹⁰ pit1::LEU2 leu1 ade6-M210 ura-D18*	This study
AB20-24A	*h⁹⁰ mde3::ura4⁺ pit1::LEU2 ura4-D18 leu1 ade6-M210*	This study

which shares an identical binding motif for the human forkhead protein, FREAC (Pierrou *et al.* 1994). This nucleotide sequence was designated FLEX (FREAC-like element of *spo6*). Mutational analysis revealed that the core heptamer is essential and that its 3′ flanking sequence of 10 nucleotides is important for recognition by Mei4p (Horie *et al.* 1998).

The environmental cue for meiosis is nutrient depletion, especially nitrogen starvation. Most of the *S. pombe* genes responsible for sexual reproduction are transcriptionally induced by a nitrogen starvation signal mediated by the HMG family transcription factor, Ste11 (Sugimoto *et al.* 1991). Since *mei4⁺* is not transcribed in haploid cells starved for a nitrogen source, Ste11 is not sufficient to induce *mei4* transcription. The Mei2 RNA-binding protein is a crucial inducer of meiosis in *S. pombe* (Watanabe and Yamamoto 1994). The transcription of *mei4* requires *mei2* function and proceeds rapidly in diploid cells shortly after commitment to meiosis (Horie *et al.* 1998). Little is known about the transcriptional regulation of the *mei4⁺* gene, in spite of its importance. The aims of this study were to identify novel genes, the expression of which is governed by Mei4, and to confirm positive autoregulation of *mei4* gene expression.

MATERIALS AND METHODS

Strains, media, and genetic techniques: The *S. pombe* strains listed in Table 1 were cultured in media as described (Egel and Egel-Mitani 1974; Gutz *et al.* 1974; Moreno *et al.* 1990). Cells were grown on YEA complete or SD minimal media at 30°. Mating and sporulation were induced at 28° on the malt-extract agar medium (MEA). For liquid sporulation culture, cells were incubated in PM, shifted to PM-N, a nitrogen-free version of PM, and incubated at 28° with shaking.

Standard procedures for *S. pombe* genetics followed those of Gutz *et al.* (1974). *S. pombe* was transformed using lithium acetate (Okazaki *et al.* 1990).

Synchronous meiosis in *pat1* temperature-sensitive mutants: The temperature-sensitive *pat1-114* mutant (Iino and Yamamoto 1985; Nurse 1985) was cultured on YEA medium at 24° for 2 days. The cells were transferred to PM-N at a density of OD_{530} = 0.65 and were shaken at 24° for 15 hr to arrest the cell cycle at the G1 phase. Shifting the incubation temperature to 34° induced meiosis, which proceeded in a synchronous fashion (see Figure 1).

Northern blotting: Total RNA was prepared from *S. pombe* cultures according to the method of Jensen *et al.* (1983). DNA probes were labeled with ^{32}P by random priming. The templates for random priming of all the *mde* genes, as well as *rec6*, *rec12*, and *aro3*, were gel-purified polymerase chain reaction (PCR) products. The synthetic oligonucleotides used in the PCR reaction are listed in Table 2. Other templates consisted of the following fragments: *mei4*, a 2.3-kb *Hin*dIII-*Hpa*I fragment (Horie *et al.* 1998); *rec7*, a 1.1-kb *Bgl*II fragment; *rec8*, a 0.6-kb *Bam*HI-*Hae*II fragment (Lin *et al.* 1992); *rec10*, a 2.2-kb *Sac*I fragment (Lin and Smith 1995); and *dmc1*, a 0.3-kb *Eco*RI fragment (A. Shinohara, unpublished data). Northern hybridization proceeded under standard conditions (Thomas 1980). Ribosomal RNA stained by ethidium bromide was used as the loading control. Hybridization with the *S. pombe aro3* probe was the internal reference (Nakanishi and Yamamoto 1984; Iino *et al.* 1995). The intensity of the hybridization bands was quantified with a Fuji BAS1000 Bio-Imaging analyzer.

Gene disruption of *mde3* and *pit1*: A 2970-bp DNA fragment containing the *mde3⁺* open reading frame (ORF) was amplified by PCR, with the forward primer GGCACGCTTGATACC and reverse primer CTTTCACTCATGGCG. The *Hin*dIII fragment of the amplified fragment was cloned into pBluescript-II SK⁻ (Stratagene, La Jolla, CA). The *mde3::ura4⁺* null allele was produced by a one-step gene disruption method (Rothstein 1983). A *Bgl*II fragment of 965 bp was replaced by a 1.8-kb *ura4⁺* cassette (Grimm *et al.* 1988). A diploid strain (C525) was transformed with the *Hin*dIII fragment having this disrupted *mde3* allele, and stable Ura⁺ transformants were isolated. Disruption was confirmed by the size of the PCR products using several different pairs of primers and also by tetrad dissection of the diploid disruptants.

TABLE 2

Sequences of the synthetic oligonucleotides used for PCR amplification

Gene	Forward primer	Reverse primer
mde1	5′-GGGGTCGAC(*Sal*I)CGTTGAATGATAGCTCTATAG-3′	5′-CCCGTCGAC(*Sal*I)TATCATCTTCCTCCCTCTTCC-3′
mde2	5′-CAAGGGGTGCGAAGG-3′	5′-CTCAGCTGACGCAGG-3′
mde3	5′-CCCGTCGAC(*Sal*I)ATGAGTAATGAAAGTATTTACTCAGTG-3′	5′-CCCGGATCC(*Bam*HI)TGGCGAACTTAGATGTAAGC-3′
mde4	5′-CCCCTCGAG(*Xho*I)GATGCTAAAGGTGGGCGATC-3′	5′-CCCGCGGCCGC(*Not*I)GTAACACGATCAGGATGTGC-3′
mde5	5′-GGTGGTAATCCCGGC-3′	5′-CATGGATGTTATTCAACGC-3′
mde6	5′-ACGCATCAGAGGCGC-3′	5′-CAGCCAACTGTTCCC-3′
mde7	5′-CCCCTCGAG(*Xho*I)GGAGCATTTAATAACCCCC-3′	5′-CCCGCGGCCGC(*Not*I)GAAAGCAACAGCCTTCGCTCG-3′
mde8	5′-CCCCTCCAGATACAG-3′	5′-CCCAAGACATCGTCC-3′
mde9	5′-CAGGCCCGATTTCC-3′	5′-CGCAGGACGGCCAGC-3′
rec6	5′-CGTCTCGGTACATCATAAAGAGCTC-3′	5′-CCTTACTCAGTCTGCAGCCGAAAGC-3′
rec12	5′-CTAATTGCTTGTAACAGGCTAAAGG-3′	5′-TCCAAGAACTTAACGTACTGAGTGC-3′

A 2720-bp DNA fragment containing the *pit1*$^+$ ORF was amplified by PCR, with the forward primer CCCCTCGAG(*Xho* I)CACGGTTGG CTTACAATTCAA and reverse primer CCCGCGGCCGC(*Not*I)AAGGCGAACAAAATTCCGG. The PCR product was digested with *Xho*I and *Not*I and cloned into pBluescript-II SK$^-$ (Stratagene). A 0.3-kb *Sal*I/*Bam*HI fragment was replaced by a 2.2-kb *LEU2* cassette containing the *S. cerevisiae LEU2* gene. A diploid strain (C525) was transformed with the *Xho*I/*Not*I fragment having the disrupted *pit1* allele, and stable Leu$^+$ transformants were isolated. Disruption was confirmed by the methods mentioned above.

Construction of a *mei4-lacZ* fusion plasmid: The 3.3-kb *Bam*HI fragment containing the *Escherichia coli lacZ* gene cut from pMC1871 (Casadaban *et al.* 1983) was fused to the *mei4*$^+$ ORF on pAU(*mei4*)KHL, yielding pAU(*mei4*)NL. This plasmid thus contained the authentic *mei4* promoter, part of the 5′-terminal Mei4p-coding region (1–275 amino acids) and LacZ (8–1021 amino acids; see Figure 7A).

β-Galactosidase assay: The heterothallic haploid strain JY741 was transformed with pAU(*mei4*)NL and cultured in PM liquid medium at 30° to the early stationary phase. β-Galactosidase activity was assayed according to Horie *et al.* (1998).

Preparation of GST-Mei4 fusion protein: Plasmid pGEX(*mei4*) contains a forkhead DNA-binding domain of Mei4p fused to glutathione-S-transferase (GST; Horie *et al.* 1998). The *E. coli* strain BL-21 (Studier and Moffatt 1986) was transformed with pGEX(*mei4*) and the GST-Mei4 fusion protein was expressed by adding isopropyl-β-d-thiogalactopyranoside in Luria-Bertani medium. Cells were homogenized by sonication (INSONATOR 201M, Kubota Manufacturing Co. Ltd.) in a buffer containing 30 mm Tris-HCl (pH 7.5) and 30 mm NaCl at 4°. The supernatant fraction obtained by centrifugation at 10,000 × *g* for 30 min at 0° was used as a crude preparation of the GST-Mei4 fusion protein.

Gel mobility shift assay: Three sets of complementary oligonucleotides, Fmei4-D, Fmei4-U, and FLEX-D, were synthesized and annealed to generate double-stranded DNA fragments with the following nucleotide sequences (only one strand is presented): Fmei4-D, 5′-ATACCGTAAATATGTAAACACAAG CAAGGA-3′; Fmei4-U, 5′-TATAAATTTAGTAAATAAATAAT ACAA-3′; FLEX-D, 5′-AAATATTTGTGTAAACAAACAAAA TCA-3′. These fragments were labeled with [γ-^{32}P]dATP using polynucleotide kinase (Takara Shuzo Co.). A standard reaction mixture (20 μl) contained 24 ng of radiolabeled double-stranded oligonucleotide probes, and an *E. coli* crude extract contained 9 ng of protein, 2 μg of poly(dI-dC), and 8.4 μg of salmon sperm DNA in binding buffer (100 mm Tris-HCl [pH 8.0], 10 mm $MgCl_2$, 60 mm KCl, 1 mm spermidine, 0.1% Nonidet P-40, 7 mm β-mercaptoethanol, and 10% glycerol). The reaction mixture was placed on ice for 60 min and then loaded onto 4% native polyacrylamide gels in TGE buffer. Electrophoresis proceeded at 15 mA in TGE buffer at 4° until free probes reached the bottom of the gel. Resolved bands were fixed with 7% acetic acid and then exposed to X-ray film (Fuji NIF-RX film) for 12–18 hr at −80°.

RESULTS

Mei4-independent transcription of early meiotic genes: To date, only *spo6*$^+$ has been recognized as a target gene for the Mei4 transcription factor (Horie *et al.* 1998). Although *mei4*Δ cells arrest in meiotic prophase-I (Bresch *et al.* 1968; Olson *et al.* 1978; Shimoda *et al.* 1985; Horie *et al.* 1998), *spo6*$^+$ is required for meiosis-II and sporulation (T. Nakamura, unpublished data). We surmised that Mei4p governs the transcription

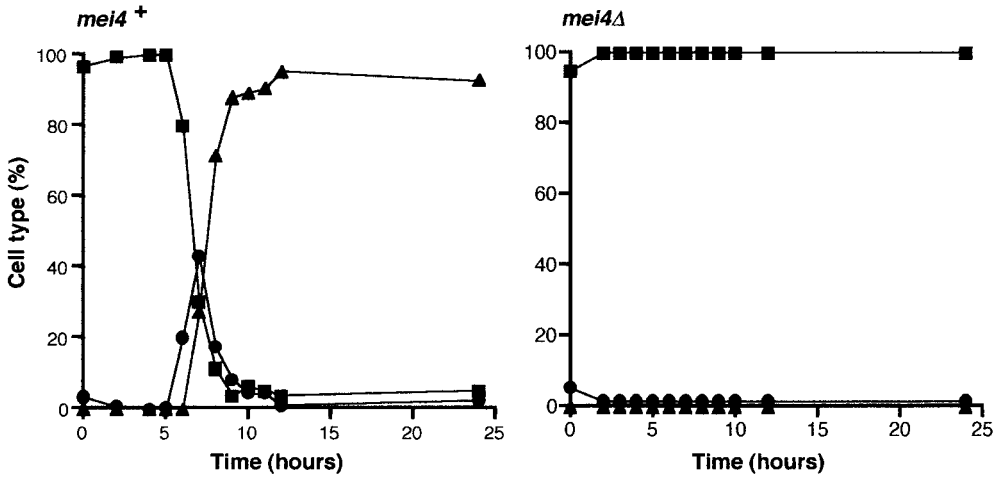

Figure 1.—Kinetics of synchronous meiosis. Diploid strains JZ670 (*mei4*$^+$) and AB4 (*mei4*Δ) harbored the homozygous *pat1-114* allele. Meiosis was synchronously induced by shifting the incubation temperature from 24° to 34°. Portions of the culture were sampled at intervals and were stained with 4′,6-diamidino-2-phenylindole (DAPI). About 300 cells for each point were differentially counted on the basis of the number of nuclei per cell. Squares, mononucleate cells; circles, binucleate cells; triangles, tri- or tetranucleate cells.

of some of the genes that function during the early stages of meiosis. Other meiosis-deficient *S. pombe* mutants with a phenotype similar to that of *mei4*Δ have not yet been identified. In the search for novel target genes of Mei4p, the dependence on *mei4*$^+$ activity of several typical early meiotic genes, such as *rec6*$^+$, *rec7*$^+$, *rec8*$^+$, *rec10*$^+$, *rec12*$^+$ (Lin *et al.* 1992; Lin and Smith 1994, 1995), and *dmc1*$^+$ (A. Shinohara, personal communication), has been examined. Some of these genes are transcribed exclusively during the early meiotic process (Lin *et al.* 1992; Lin and Smith 1994, 1995). In addition, *spo5*$^+$ was tested as a representative late meiotic gene (H. Asakawa, personal communication). In the following experiments, *pat1*-driven meiosis was adopted to achieve fairly good synchrony in meiotic divisions (see materials and methods; Iino *et al.* 1995). The level of synchrony attained is shown in Figure 1. *mei4*Δ cells were arrested at the mononucleate stage in *pat1*-driven meiosis (Figure 1). Portions of the synchronous culture were removed, and RNA was purified and Northern blotted (Figure 2). Hybridization of *mei4*$^+$ cultures with specific probes did not reveal signals for the *rec*/*dmc* genes at 0 hr. Signals were detected at 2 hr, the intensity of which peaked 4 hr after the temperature shift, and then rapidly declined. These genes were also transcribed in *mei4*Δ cultures (Figure 2), indicating that these early genes do not rely on Mei4p for transcription.

In *mei4*Δ cells, the elevated transcript level persisted, in contrast to wild-type cells in which such elevation was only transient (Figure 2). This finding raises the notion that the transcripts of meiosis-specific genes are stable in *mei4*Δ cells. To test this, the turnover rates of specific RNA molecules were determined according to Surosky and Esposito (1992). The turnover rates of the *spo5* and *dmc1* mRNA molecules were not significantly different between *mei4*$^+$ and *mei4*Δ cells (data not shown). Therefore, it seems less likely that the persistence of these mRNA molecules in *mei4*Δ cells is due to their increased stability. These results indicate that Mei4p is required for turning off the transcription of some meiosis-specific genes. Persistent transcription might be the secondary effect of arrest at prophase-I in *mei4*Δ cultures.

A genome-wide screen of target genes with Mei4p-dependent transcription: Since Mei4-dependent target genes were not identified among known early meiotic genes, we screened for novel genes that are dependent on Mei4p for transcription. A large volume of data is available in the *S. pombe* genome sequence database at The Sanger Centre (UK). In addition, our mutational analysis of the FLEX sequence of *spo6* revealed that the central core heptamer (GTAAACA) and its 3′ flanking sequence (AACAAAATCA) are very important for Mei4 binding (Horie *et al.* 1998). Using this 17-nucleotide sequence and its complementary sequence as a query, we conducted a computer-aided search of the *S. pombe* genome sequence database. Since no complete match was found except for *spo6*, up to four mismatch bases were allowed in the 3′ flanking sequence. Position of the FLEX-like sequence in the respective genes was also examined. We found 10 genes whose FLEX-related sequence resides in the 5′ upstream nontranslatable region within range of 1 kb from the initiation codon.

The transcription of these potential Mei4-dependent genes was examined by Northern blotting. The gene symbol *mde* was adopted to indicate *M*ei4-*d*ependent *e*xpression. We compared the expression of 10 putative *mde* genes in JZ670 (*mei4*$^+$) and AB4 (*mei4*Δ) strains. Nine genes, designated *mde1*$^+$ to *mde9*$^+$, were transcribed when meiosis was induced in wild type, but not in *mei4*Δ under the same conditions (Figure 3A). The transcript level of one gene, SPAC19A8.10, was too low to determine whether or not the expression is Mei4p dependent (data not shown).

As mentioned above, *mei4*$^+$ was not transcribed in vegetative cells. If Mei4p is involved directly in the activation of transcription, the ectopic expression of *mei4*$^+$ might cause the transcription of these genes. To test this theory, *mei4* was expressed by the thiamine-repressible *nmt1* promoter in mitotic cells. C525C-1A transformed with pREP(*mei4*$^+$) was incubated in PM medium with or without thiamine.

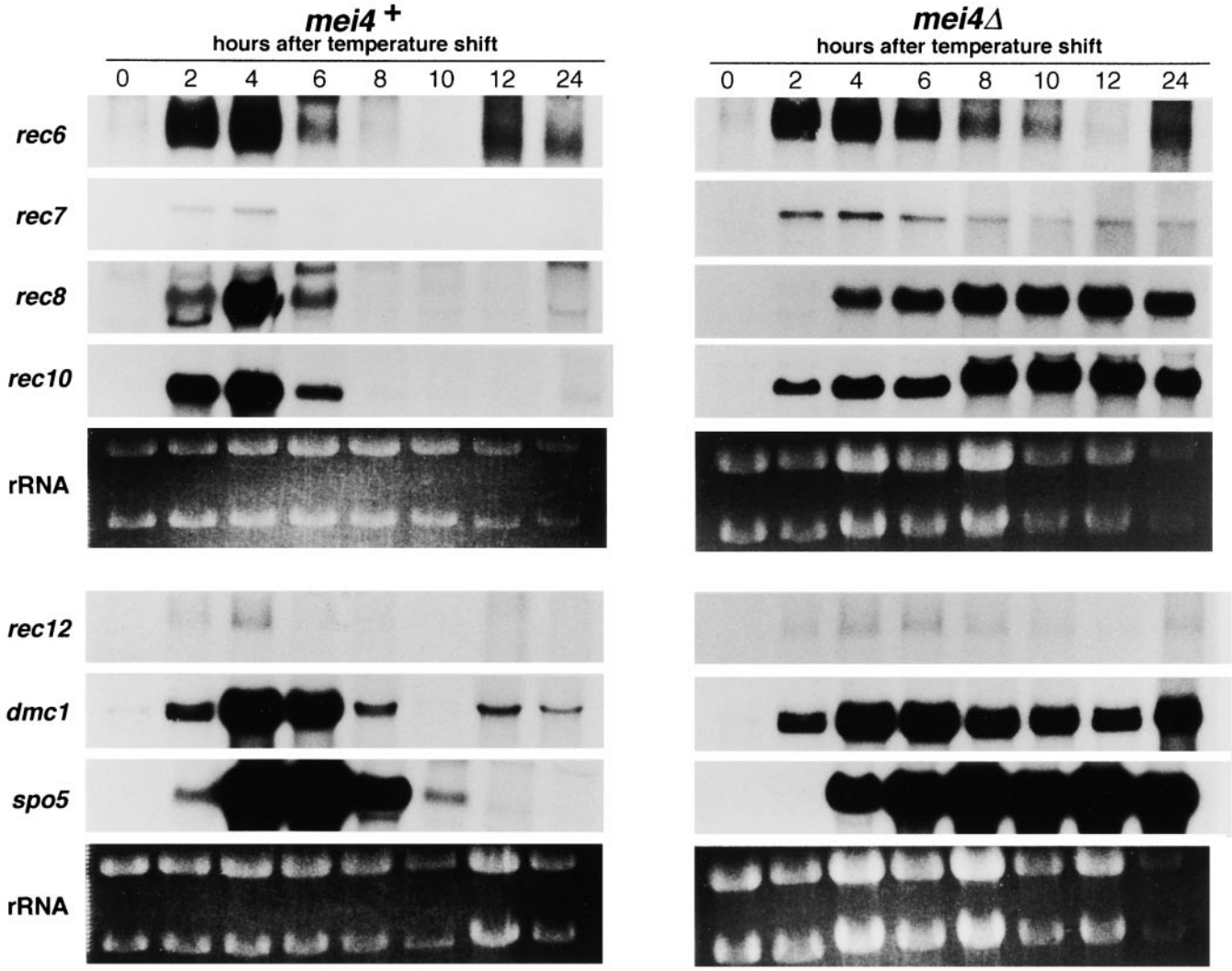

Figure 2.—Transcription profile of meiosis-specific genes in wild-type and *mei4*Δ cells. RNA was prepared from diploid strains JZ670 (*mei4*$^+$) and AB4 (*mei4*Δ) and was analyzed by Northern blot hybridization as described in materials and methods. Meiosis was synchronously induced as described in the legend to Figure 1. The approximate equality of RNA was checked by staining gels with ethidium bromide, which reveals the rRNA.

Seventeen hours after transfer to thiamine-free PM medium, *mei4* was induced in the cells. Figure 3B shows that the overexpression of *mei4* stimulated the transcription of these putative candidate *mde* genes even in growth medium. As *mde1* was expressed in the medium under repressed conditions (containing thiamine), this gene might be transcribed in response to the very low level of Mei4p present in the presence of thiamine. The *mde4* transcript was also detected when cells harboring pREP1 vector plasmid were incubated for 17–19 hr in PM with or without thiamine (data not shown), indicating that transcription of *mde4* was not due to overproduction of Mei4p. The transcript level of *mde4* was very low after incubation in nitrogen-free medium for 15 hr and was enhanced after *pat1*-driven meiosis dependent on *mei4*$^+$ (Figure 3A). We concluded that these nine genes, *mde1*$^+$ to *mde9*$^+$, are likely targets of Mei4p.

The position of the likely FLEX sequence in the *mde1*$^+$ to *mde9*$^+$ genes relative to the initiation codon and alignment of the FLEX sequence is shown in Table 3. As suggested in our previous study (Horie *et al.* 1998), the 3′ flanking sequence seemed important and in fact 5 of the 10 nucleotides were conserved in all of the 9 *mde*$^+$ genes. We concluded from these results that the consensus *cis*-element is GTAAACAAACA-A.

Mde3p is homologous to *S. cerevisiae* Ime2 kinase and is necessary for normal sporulation: Sequence data of these *mde* genes indicated that they are mostly novel genes with unknown biological functions (Table 3). The exception was *mde9*$^+$, which is identical to *spn5*$^+$ and encodes a putative septin protein (Longtine *et al.* 1996). In addition, Mde3p attracted our attention because it has sequence similarity to the *S. cerevisiae* meiosis-specific protein kinase, Ime2 (Kominami *et al.* 1993; Foiani *et al.* 1996). We studied the function of *mde3*$^+$ in meiosis and sporulation.

The *S. pombe* genome sequence project has revealed that another gene, SPAC3C7.06c, also encodes an Ime2-

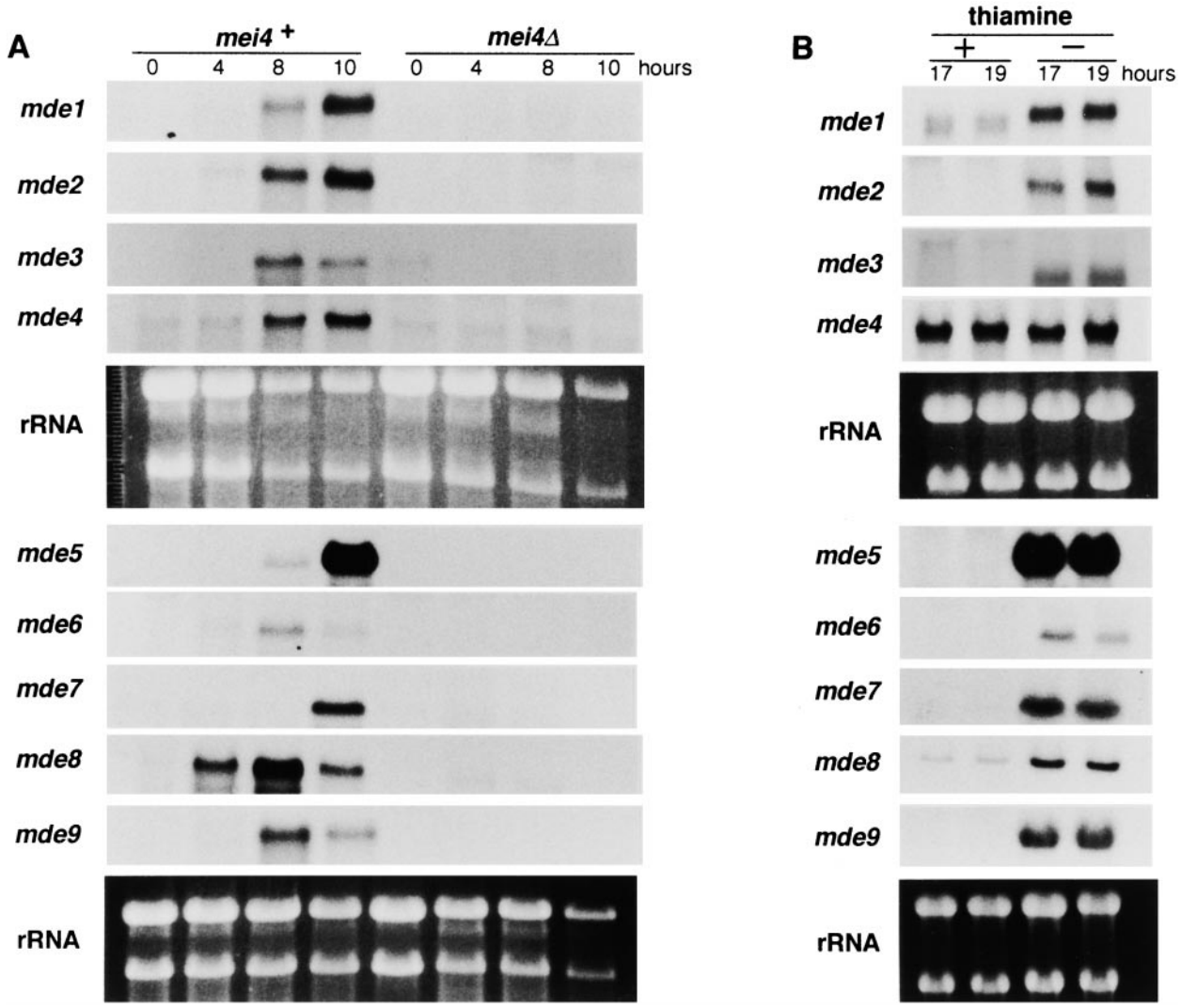

Figure 3.—Northern blots indicating Mei4-dependent transcription of the *mde*$^+$ genes. (A) Effect of *mei4* disruption on transcription of *mde*$^+$ genes in *pat1*-driven synchronous meiosis. Meiosis of the diploid strains harboring homozygous *pat1-114*, JZ670 (*mei4*$^+$), and AB4 (*mei4*Δ) was synchronized and at intervals total RNA was prepared and analyzed by Northern blot hybridization as described in materials and methods. (B) Effect of ectopic expression of *mei4*$^+$ on *mde* transcription. C525C-1A transformed with pREP(*mei4*$^+$) was incubated in PM with (+) or without (−) thiamine (20 μm). After 17 and 19 hr of incubation, samples from both cultures were examined by Northern blotting. The approximate equality of RNA was checked by staining gels with ethidium bromide.

related protein. Hereafter, this gene is designated *pit1* (*S. pombe* **I**me-**t**wo homolog). Figure 4A shows the sequence similarity, especially in the kinase subdomain I–X, among Ime2p, Mde3p, and Pit1p. In contrast to *mde3*$^+$, 3.3-kb *pit1*$^+$ mRNA was present in vegetative cells and its abundance was not enhanced after shift to nitrogen-free sporulation medium (data not shown).

To know the role of *mde3*$^+$ and *pit1*$^+$, both genes were disrupted (Figure 4B). These null mutants showed no growth defects at incubation temperatures ranging from 20° to 37°. The *S. cerevisiae IME2* gene plays an indispensable role in controlling timing of premeiotic DNA replication and meiosis (Foiani *et al.* 1996; Dirick *et al.* 1998). Thus, we studied whether the *mde3*Δ and *pit1*Δ mutants were defective in meiosis and sporulation. The homothallic haploid strain harboring *mde3*Δ could mate and could undergo meiosis and sporulation. The sporulating culture, however, contained nonsporulating zygotes and aberrant asci with less than four mature spores (Figure 5B). Apparently, small immature spores were also produced (Figure 5B). The frequency of these aberrant asci with abnormal size and number of spores is significantly higher than wild-type strain (Figure 5A). These defects were observed also with *pit1*Δ and were more remarkable in the *mde3*Δ *pit1*Δ double disruptant strain. Next, kinetics of meiotic nuclear division was monitored by DAPI staining. No differences in the progression of meiosis between wild-type and the mutant strains were observed (data not shown). These results indicate that *mde3*$^+$ and *pit1*$^+$ play an important role

TABLE 3

List of *mde* genes

Gene name[a]	FLEX-like elements: Position[b]	Direction[c]	Sequence[d]	Predicted gene product
spo6 (SPBC1778.04)	−73 −99	→	AAATATTTGA*ĠṪȦÀȦĊȦAAACAAAATCA*	Dbf4-related protein
mde1 (SPAC16E8.05c)	−158 −184	←	AATGACCAAT*GTAAACAAACAAAATCG*	—
mde2 (SPBC31F10.08)	−78 −104	←	GGTATGGAAT*GTAAACAAACAAACTCA*	—
mde3 (SPBC8D2.18c)	−60 −86	→	ATGTTGGAAT*GTAAACAAACA*TAAACA	Ime2-related protein
mde4 (SPBC6B1.04)	−253 −279	←	TTCAAGATAC*GTAAACAAACAAAATTC*	—
mde5 (SPAC4A8.01)	−66 −92	←	TTACTTCATT*GTAAACAAACAAAAATA*	α-Amylase precursor
mde6 (SPAC15A10.10)	−79 −105	←	AGCACCTAAT*GTAAACAAACAAAAGAG*	—
mde7 (SPCC320.07c)	−282 −308	→	TTTTATCGAG*GTAAACAAACAAAAAAA*	RNA-binding protein
mde8 (SPBC19F8.01c)	−26 −52	←	TTATTAAGCA*GTAAACAAACAAACCCA*	Septin-related protein
mde9/*spn5* (SPAC24C9.15c)	−251 −277	→	TGCGAGTCAT*GTAAACAAACAAACATT*	Septin-related protein

[a] The name of ORF assigned by the genome sequence project is given in parentheses.
[b] Numbered with the translational start point at +1.
[c] Arrows indicate the direction of FLEX on the coding strand of the respective ORF.
[d] Completely conserved nucleotides are shown in italic type. Dots represent a core heptamer.

in spore formation, but no indispensable role in the progression of meiosis.

Transcription of *mei4* is positively autoregulated: We found that the transcript level of *mei4* is reduced in the *mei4-P572* mutant (Horie *et al.* 1998). To confirm this observation, we tested the *mei4* mRNA level in three independently isolated *mei4* mutants. Figure 6 shows that *mei4* mRNA abundance in these mutants was one-half to one-third relative to the wild-type level. Therefore, full expression of *mei4* requires *mei4*$^+$ function. We next examined whether or not *mei4* transcription is activated by ectopically overproduced Mei4p. The plasmid pAU(*mei4*)NL (Figure 7A), containing the *mei4* gene with its promoter fused to the *E. coli lacZ* gene, was introduced into the *h*$^-$ haploid strain JY741. To ectopically overexpress *mei4*$^+$, the plasmid pREP(*mei4*$^+$) (Figure 7A) was introduced as well. β-Galactosidase activity due to *mei4-lacZ* was remarkably increased when Mei4p was overproduced by incubating cells in thiamine-free medium (Figure 7B). This activity was low in transformants bearing the empty vector pREP1. These results support the view that *mei4* transcription is positively upregulated by its own product. This positive autoregulation of *mei4* transcription might explain the rapid induction of *mei4* and may play a role in the transcription of downstream genes in response to environmental cues for the induction of meiosis.

We have previously reported that *mei4*$^+$ has no FLEX-like sequences (Horie *et al.* 1998). We could not find any potential FLEX sequences in the genome-wide search described above. As Mei4p can stimulate the transcription of *mei4* itself, we reexamined the nucleotide sequence carefully and found the FLEX-like sequence of reverse orientation in the 5′ upstream region of this gene. These potential FLEX-like Mei4p-binding sites, designated Fmei4-U and Fmei4-D (Figure 8, A and B), are highly diverged from the consensus query sequence. The downstream element (Fmei4-D) contains two tandem repeats of the core heptamer, GTAAAYA. Using bacterially produced recombinant GST-Mei4 protein (Horie *et al.* 1998), we tested binding to these potential FLEX sequences as probes with a gel mobility shift assay. Figure 8C shows shifted bands with the original *spo6*-FLEX-D and Fmei4-D, but not with Fmei4-U. The GST protein without the forkhead region of Mei4p gave no positive signals. The intensity of the shifted band of Fmei4-D was reproducibly weaker than that of *spo6*-FLEX-D. Such inefficient binding might be due to the divergence of the Fmei4-D sequence from the consensus. These gel shift assays indicated that the Mei4 fusion protein binds to the likely *cis*-element of *mei4*. These facts further support the notion that *mei4* transcription is positively autoregulated.

DISCUSSION

Identification of novel Mei4p targets: Mei4p is a forkhead family transcriptional regulator that is required for the progression of meiosis and sporulation in *S. pombe* (Horie *et al.* 1998). We selected novel target genes of the Mei4 transcriptional regulator by means of a genome-wide screen for its recognition sequence. This strategy necessitates at least two conditions: the identification of DNA-binding motifs and the availability of genome nucleotide sequences. Our previous study revealed that the forkhead DNA-binding domain of Mei4p recognizes a *cis*-element composed of 27 nucleotide pairs (Horie *et al.* 1998). The *S. pombe* genome sequence project is ~80% complete.

To find novel Mei4 target genes in the genome, we used a 17-bp stretch of the FLEX element containing the central core and its 3′ flanking region as a query.

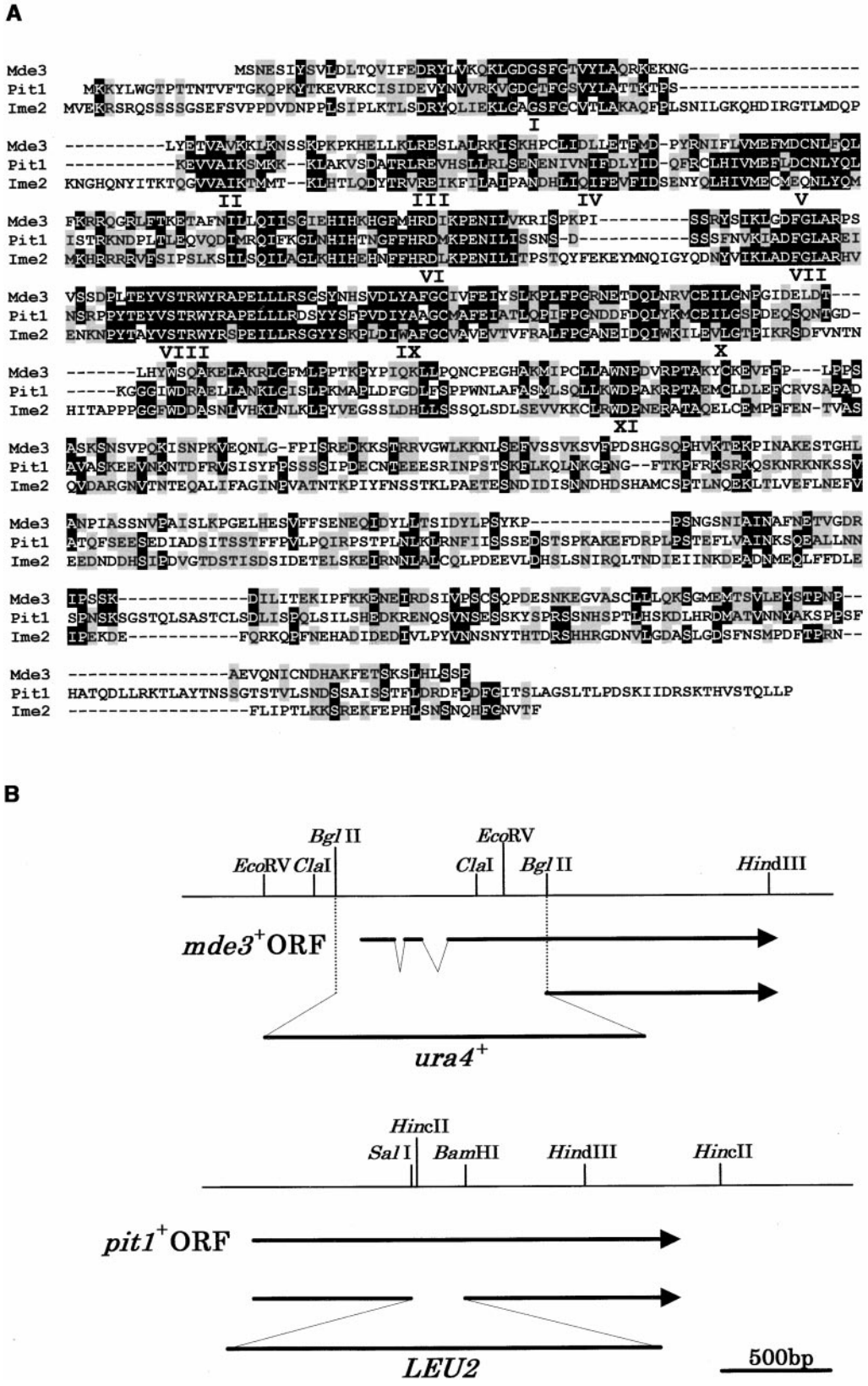

Figure 4.—Mde3p and Pit1p are homologous to Ime2 protein kinase. (A) Amino acid alignments of Mde3p, Pit1p of *S. pombe*, and Ime2p of *S. cerevisiae*. Identical amino acid residues are highlighted by reverse letters, and similar amino acids are shaded. Roman numerals above the amino acids show the kinase consensus subdomains, according to Hanks *et al.* (1988). (B) Restriction map and disruption constructs of *mde3*$^+$ and *pit1*$^+$.

This screen revealed nine genes, designated *mde1*$^+$ to *mde9*$^+$, the expression of which was strongly dependent on wild-type *mei4*$^+$ function. The FLEX-like nucleotide sequences of these novel *mde* genes are aligned in Table 3. Whereas mismatches of 1–4 nucleotides were allowed on screening, 12 nucleotides are completely conserved among these genes. The revised consensus FLEX sequence based on these data is GTAAACAAACA(A/T)

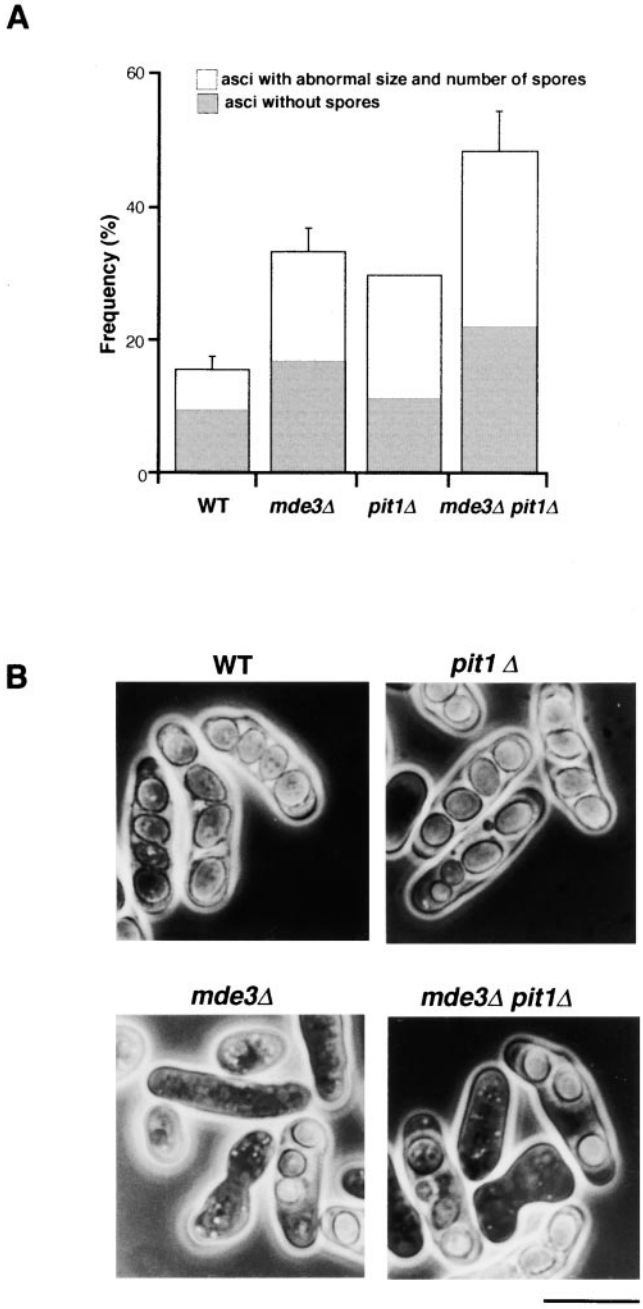

Figure 5.—Aberrant sporulation of *mde3*Δ, *pit1*Δ, and *mde3*Δ *pit1*Δ. (A) Frequency of aberrant asci and nonsporulating zygotes. Homothallic haploid strains, C650-7C (WT), AB16-8B (*mde3*Δ), AB19-7D (*pit1*Δ), and AB20-24A (*mde3*Δ *pit1*Δ), were streaked on YEA. Three independent colonies from each strain were transferred to MEA and incubated for 30 hr at 30°. At least 120 zygotes and zygotic asci were counted for each sample under a phase-contrast microscope. Aberrant asci having only one to three normal mature spores and zygotes having no visible spores were differentially counted. Averages of three independent clones with standard deviation (vertical bar) are presented. (B) Phase-contrast microphotographs of *mde3*Δ, *pit1*Δ, and *mde3*Δ *pit1*Δ cells on MEA sporulation medium. Bar, 10 μm.

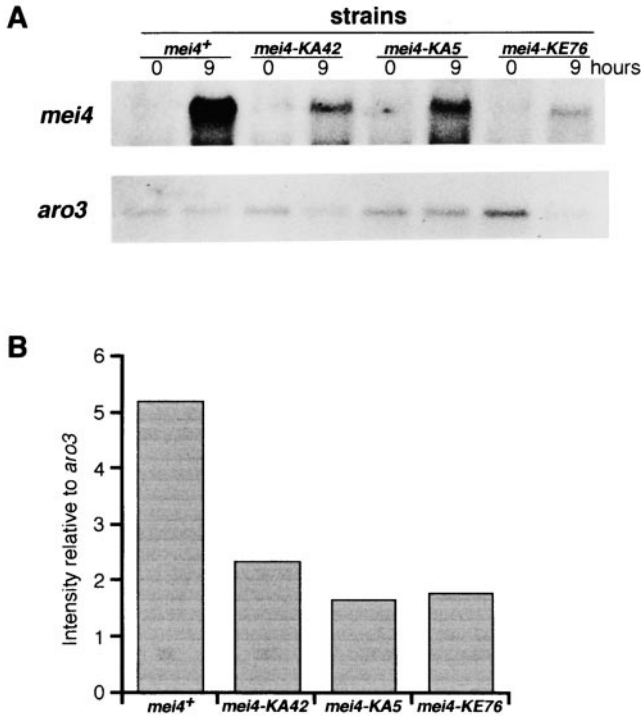

Figure 6.—Northern blots indicating that the level of *mei4* transcript was reduced in *mei4* mutants. (A) Autoradiogram. The level of *mei4* mRNA in three *mei4* mutants during meiosis. Haploid strains harboring *pat1-114* allele were used: AB1-2C (*mei4*$^+$), AB11-15C (*mei4-KA42*), AB12-11B (*mei4-KA5*), and AB13-1D *(mei4-KE76*). Meiosis was induced by shifting the incubation temperature from 24° to 34°. Cultures were sampled after 9 hr at 34°. RNA preparations were subjected to Northern analysis with *mei4*- and *aro3*-specific hybridization probes. (B) Relative intensity of the *mei4* transcripts to *aro3*. The autoradiograms shown in A were quantified using a Fuji bio-imaging analyzer (BAS1000).

A(A/C). This approach to identifying targets of a particular transcription factor might be applicable to other systems if the two prerequisites described above are fulfilled.

Sequence data predict that the *mde* gene products include the *S. cerevisiae* Ime2 homolog, two septin-like proteins, an α-amylase precursor, and a putative RNA-binding protein (Table 3). Four of the identified *mde* genes have neither significant homology with known proteins nor functional motifs. The *spn5* mutation allelic to *mde9* impaired spore formation in *S. pombe* (J. Baeler, personal communication). The essential role of septin proteins in sporulation was also reported in budding yeasts (Fares *et al.* 1996; Virgilio *et al.* 1996).

The *S. pombe mes1*$^+$ gene, which is essential for the meiotic second division (Bresch *et al.* 1968; Shimoda *et al.* 1985), contained one short intron (Kishida *et al.* 1994). Splicing of the *mes1* mRNA requires *mei4*$^+$ function probably in an indirect manner (Horie *et al.* 1998). In this context, interestingly, *mde7*$^+$ encodes a putative RNA-binding motif. An attractive hypothesis

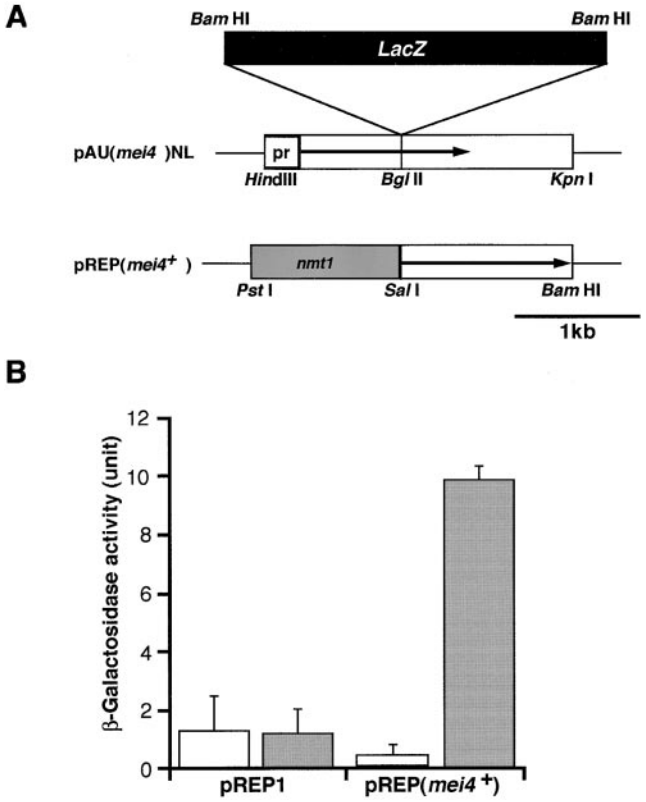

Figure 7.—Expression of the *mei4-LacZ* fusion construct in vegetative cells. (A) Structure of pAU(*mei4*)NL and pREP (*mei4*$^+$). For details of plasmid construction, see materials and methods. Arrow, *mei4* ORF; pr, authentic *mei4* promoter; shaded box, *nmt1* promoter. (B) Expression of *mei4-LacZ* fusion gene as revealed by β-galactosidase activity. The haploid strain, JY741, was cotransformed with pAU(*mei4*)NL and pREP(*mei4*$^+$). The control was pREP1 instead of pREP (*mei4*$^+$). The transformants were incubated in PM medium with (open) or without thiamine (shaded) for 17 hr. The β-galactosidase activity is expressed as the means of three independent transformants with standard deviations.

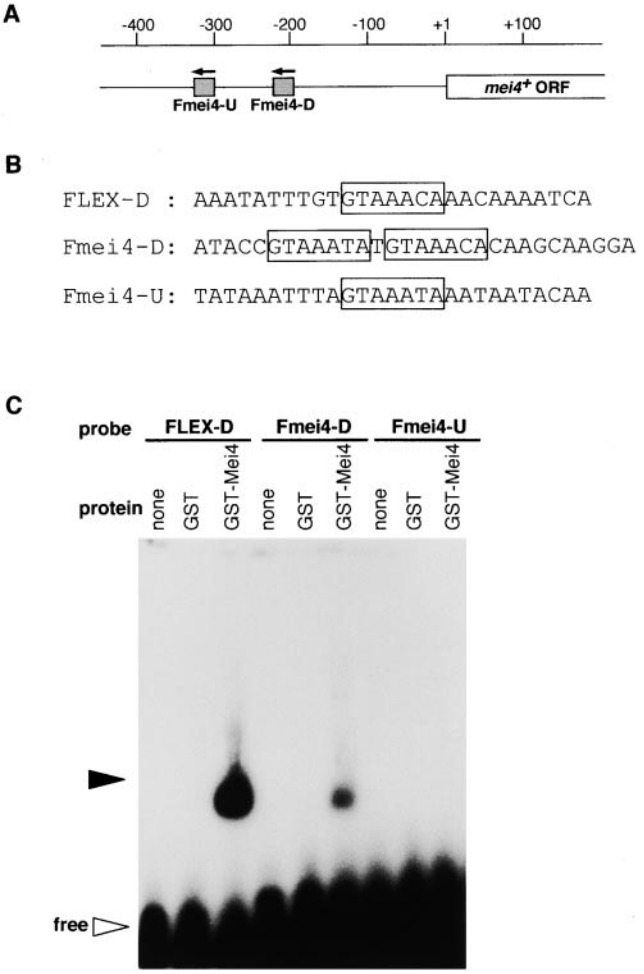

Figure 8.—Gel mobility shift assay with recombinant Mei4 protein. (A) Diagram of the 5′ promoter sequence of *mei4*. The putative recognition sequences in the 5′ upstream region of *mei4*, Fmei4-D and Fmei4-U, are indicated by shaded boxes. Nucleotides in upper scale are numbered with the translational initiation point as +1. (B) Nucleotide sequences of oligonucleotide probes. Conserved heptamer is boxed. (C) Gel shift analysis. Crude extracts from *E. coli* expressing the GST-Mei4 (forkhead) fusion protein were incubated with labeled oligonucleotide probes. Controls were labeled oligonucleotide probe without extract (none) and *E. coli* extracts expressing GST not fused with the Mei4 moiety (GST). Solid and open arrowheads indicate shifted bands and free probes, respectively.

that Mei4p regulates the *mes1* splicing through the *mde7*$^+$ gene product is worth examining.

Ime2p of the budding yeast is a serine/threonine protein kinase essential for the normal timing of premeiotic DNA replication and meiotic division and the completion of sporulation (Foiani *et al.* 1996; Dirick *et al.* 1998). The *mde3*Δ mutant frequently produced aberrant asci, which had only zero to three spores and immature spores. However, we could not observe any delay of meiosis, unlike *ime2* mutants (Foiani *et al.* 1996; Dirick *et al.* 1998). Since *S. pombe* has another gene, named *pit1*$^+$, encoding an Ime2-like protein, the weak meiotic phenotype of *mde3*Δ cells is possibly due to *pit1*$^+$, which is expressed constitutively. The *mde3*Δ *pit1*Δ double disruptant, however, displayed the normal progression of meiotic nuclear division. Our observation implies that *S. pombe* Ime2-like proteins regulate sporulation in a substantially different way than *S. cerevisiae* Ime2p. Of course, we could not exclude the possibility that *S. pombe* has a third Ime2-related protein that has not yet been identified.

Currently, we are performing gene knockout experiments with the other *mde* genes. Elucidation of the cellular function of these meiosis-specific genes expressed downstream of Mei4p could shed light on meiosis and sporulation in the fission yeast.

Positive autoregulation of *mei4* transcription: *mei4*$^+$ itself is also regulated primarily at the transcriptional level. The following facts suggest a positive autoregulation of *mei4* transcription. First, the *mei4* transcript level is greatly reduced in *mei4* mutant cells (Figure 6). Sec-

ond, the ectopic expression of Mei4p in vegetative cells induces a reporter gene that is transcribed under the control of the *mei4* promoter (Figure 7B). Finally, recombinant Mei4p binds to the FLEX-like *cis*-element of the *mei4* promoter (Figure 8). These observations imply that the low level of Mei4p that is initially produced enhances further transcription of *mei4*.

This type of positive autoregulation has been found in other yeast genes. For example, the *S. cerevisiae PDR3* gene encoding the zinc finger transcription factor implicated in drug resistance is positively autoregulated (Delahodde *et al.* 1995). Another example is found in the copper detoxification phenomenon in *Candida glabrata*. The Amt1 transcription factor regulates the expression of a family of metallothioneins and the transcription of *AMT1* is positively autoregulated (Zhou and Thiele 1993). This positive feedback mechanism plays a critical role in copper detoxification. Positive transcriptional autoregulation of *mei4*$^+$ may be required for a rapid response to some external cue for meiosis under poor nutrient conditions.

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