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The *Schizosaccharomyces pombe mei4*$^+$ Gene Encodes a Meiosis-Specific Transcription Factor Containing a forkhead DNA-Binding Domain

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The *mei4*$^+$ gene of the fission yeast *Schizosaccharomyces pombe* was cloned by functional complementation. The *mei4* disruptant failed to complete meiosis-I but could proliferate normally. *mei4*$^+$ was transcribed only in meiosis-proficient diploid cells after premeiotic DNA replication. The *mei4*$^+$ open reading frame encodes a 57-kDa serine-rich protein comprised of 517 amino acids with a forkhead/HNF3 DNA-binding domain in the amino-terminal region. Transcription of *spo6*$^+$, a gene required for sporulation, was dependent on the *mei4*$^+$ function. Two copies of the GTAAAYA consensus sequence, proposed as the binding site for human forkhead proteins, were found in the promoter region of *spo6*$^+$. A gel mobility shift assay demonstrated the sequence-dependent binding of the GST-Mei4 forkhead domain fusion protein to DNA fragments with one of the consensus elements. Deletion of this consensus element from the *spo6* promoter abolished the transcription of *spo6*$^+$ and resulted in a sporulation deficiency. One-hybrid assay of Mei4 which was fused to the Gal4 DNA-binding domain localized the transcriptional activation domain in the C-terminal 140 amino acids of Mei4. These results indicate that Mei4 functions as a meiosis-specific transcription factor of *S. pombe.*

Meiosis is required for the formation of germ cells which transmit genetic information from generation to generation. This specialized nuclear division is characterized by a reduction in the chromosome number and frequent genetic recombination, both of which have contributed to the evolution of eukaryotes. Although meiosis has basically the same machinery, including spindles, centrosomes, and kinetochores, as mitosis in somatic cells, these two nuclear divisions are different in many aspects. Meiosis-specific gene products must be responsible for the various different features of meiosis, especially those of meiosis-I, such as the synapsis of homologous chromosomes, nondisjunction of sister chromatids, crossing over, and chiasmata formation.

Yeasts are simple eukaryotic organisms which undergo meiosis linked to ascospore formation. Meiosis is induced in diploid cells under conditions of nitrogen starvation, and the haploid tetrads culminate in ascospores. Genetic and cytological studies with the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have revealed that yeast meiosis consists of a reductional first division and an equational second division with no intervening S phase between them (7, 8, 20, 56), as meiosis in most eukaryotes. Many mutants defective in meiotic events have been isolated and analyzed to identify the meiosis-specific genes that are responsible for the differences between meiosis and mitosis (3, 8).

The meiosis-specific genes are expressed in the germ cells of higher eukaryotes and in the sporulating cells of yeasts. The transcriptional regulation of meiotic genes has been extensively studied with budding yeast (26) and fission yeast (56, 57). The investigation of transcription during early sexual processes of *S. pombe* has identified some DNA-binding proteins, such as those containing HMG boxes (42), homeobox domains (18), and CREB-like motifs (49).

The *mei4*$^+$ gene of the fission yeast *S. pombe* is indispensable for meiosis-I (3, 32, 38). In *mei4* mutants, elongated "horsetail" nuclei are at least transiently accumulated (32). This morphology is characteristic of prophase-I nuclei (5, 35). These results strongly suggest that the *mei4*$^+$ gene products are essential for meiotic prophase-I. Because morphological events unique to meiosis occur mainly during prophase-I, the activity of *mei4*$^+$ is particularly interesting.

In the present study, we cloned and analyzed *mei4*$^+$. Nucleotide sequencing suggests that the *mei4*$^+$ product contains a forkhead DNA-binding domain composed of approximately 120 amino acids which was originally identified as the DNA-binding domain of the hepatocyte-specific transcription factor of rodents (21, 52). More than 60 proteins with this motif in a wide variety of organisms have been compiled in the protein databases. Most of the family members function as tissue-specific transcription factors. Here we present evidence that Mei4 is a meiosis-specific transcription factor in fission yeast.

MATERIALS AND METHODS

Yeast strains and culture conditions. The *S. pombe* strains used in this study are listed in Table 1. Cells were grown on YEA complete medium or minimal medium (SD, PM, or EMM2) (9, 25, 29). Mating and sporulation were induced on a malt extract agar or synthetic sporulation medium (SSA or SPA) (9, 29). These media were supplemented with the required nutrients (50 to 100 mg/liter).

Synchronous meiosis was attained basically as described by McLeod and Beach

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TABLE 1. Yeast strains used in this study

Strain	Genotype	Source
S. pombe haploid strains		
L972	h^- prototroph	U. Leupold
L968	h^{90} prototroph	U. Leupold
C133-1D	h^{90} *mei4-P572 ade6-M216 leu1 ura1*	C. Shimoda
C133-4B	h^{90} *mei4-P572 ade6-M216 leu1*	C. Shimoda
C206-2A	h^{90} *mei1-B102 ade6-M210 leu1*	C. Shimoda
YW917	h^{90} *mei4::ura4*$^+$ ($\Delta 2$) *ura4-D18 ade6-M216 leu1*	Y. Watanabe
JZ878	h^{90} *ura4-D18 ade6-M216 leu1*	Y. Watanabe
S. pombe diploid strains		
CD16-1	h^+/h^- *ade6-M210/ade6-M216 lys5-391/+ +/cyh1*	C. Shimoda
C525	h^{90}/h^{90} *ura4-D18/ura4-D18 ade6-M216/ade6-M210 leu1/leu1*	C. Shimoda
C537	h^{90}/h^{90} *mei4::ura4*$^+$($\Delta 1$)/*mei4::ura4*$^+$ ($\Delta 1$)	C. Shimoda
NT-4A	h^{90}/h^{90} *ade6-M216/ade6-M210 ura4-D18/ura4-D18 leu1/leu1 spo6::ura4*$^+$/*spo6::ura4*$^+$	T. Nakamura
JY362	h^+/h^- *ade6-M210/ade6-M216 leu1/leu1*	Y. Watanabe
JZ807	h^+/h^- *mei4::ura4*$^+$ ($\Delta 2$)/*mei4::ura4*$^+$ ($\Delta 2$) *ura4-D18/ura4-D18 leu1/leu1 ade6-M210/ade6-M216*	Y. Watanabe
S. cerevisiae haploid strain SFY526	*MAT***a** *ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3,112 can*^{r} *gal4-542 gal80-538 URA3::GAL1-lacZ*	Clontech

(25). Diploid cells cultured in PM minimal medium to mid-log phase were suspended in a starvation medium, PM lacking ammonium chloride and glucose, and then shaken at 28°C for 12 to 15 h. Meiosis was initiated by adding glucose and glycerol to the starvation culture at final concentrations of 0.1 and 1%, respectively.

Yeast transformation was carried out by means of a highly efficient lithium acetate method (31).

Cloning of *mei4*$^+$. *mei4*$^+$ was cloned by complementation of the *mei4-P572* mutation. A homothallic strain, C133-4B (h90 *mei4-P572*) was transformed by an *S. pombe* genomic library containing partially digested *Sau*3A DNA fragments constructed in a multicopy plasmid, pDB248′ (2). The transformants on a sporulation medium (SSA) were stained with iodine vapor, which turned sporulated colonies brown (9). Several brown colonies were microscopically inspected for sporulation ability. A few sporulation-proficient transformants whose suppression activity proved to be plasmid borne were analyzed further.

The plasmid DNA carried by one transformant was rescued in *Escherichia coli* DH5. This plasmid, named pDB(*mei4*)1, carried a 13-kb insert (Fig. 1A). The complementation activity was localized on the 2.8-kb *Kpn*I/*Hin*dIII fragment by subcloning. pDB(*mei4*)2, carrying this fragment, complemented *mei4-P572* (Fig. 1A).

Gene disruption of *mei4*$^+$. The *mei4::ura4*$^+$ null allele was produced by a one-step gene disruption method (36). A *Bgl*II/*Nru*I fragment of 470 bp was replaced by a 1.6-kb *ura4*$^+$ cassette; this allele was designated *mei4::ura4*$^+$($\Delta 1$). A diploid strain (C525) was transformed with the *Kpn*I/*Hin*dIII fragment having this disrupted *mei4* allele, and stable Ura$^+$ transformants were isolated. Disruption was confirmed by genomic Southern hybridization (data not shown) and tetrad analysis. A Ura$^+$ segregant which was defective in meiosis was used as a haploid *mei4* null mutant. We also constructed another disrupted allele, *mei4::ura4*$^+$($\Delta 2$), in which the 1.5-kb *Nco*I/*Acc*I fragment was replaced by the *ura4*$^+$ cassette. The phenotypes of these disruptants were identical.

DNA sequencing. The 2.8-kb *Hin*dIII/*Kpn*I fragment containing *mei4*$^+$ was recloned into pUC118/119. A series of nested deletions were produced with exonuclease III and mung bean nuclease. Nucleotide sequences of both strands were determined by the dideoxy termination method (37, 58) with a commercial T7 DNA polymerase sequencing kit (Stratagene). The nucleotide sequence was analyzed with a Genetyx software package (SDC Co.). A similarity search for the amino acid sequence of Mei4 was carried out with proteins in the databases by using the BLAST algorithm.

Southern and Northern analysis. Genomic DNA was prepared from *S. pombe* strains basically as described by Hereford et al. (10). Restriction fragments were fractionated on a 0.8% agarose gel and transferred onto a nylon membrane (Biodyne A; Pall Co.). For Northern analysis, total RNA was prepared from *S. pombe* cultures by the method of Jensen et al. (16). The ^{32}P-labeled riboprobes were prepared by in vitro transcription with T7 RNA polymerase by using a 0.35-kb *Bgl*II/*Xho*I fragment on a pBluescript vector as a template. Hybridization was performed in 50% formaldehyde at 42°C (45). Ethidium bromide staining of rRNAs was used for a loading control. Hybridization with the *S. pombe* calmodulin gene (*cam1*) probe was used as an internal reference.

Site-directed mutagenesis. Oligonucleotide-directed mutagenesis of three forkhead consensus regions was carried out by heteroduplex-PCR protocols according to the instructions of the manufacturer (Takara Shuzo Co.). The oligonucleotides used for generating three different *mei4* mutant alleles (*mei4-K81Q*, *-F115D*, and *-W125S*) are as follows: *mei4-K81Q*, GGTGAAAAT(A)G(C)CAT(C)CGTGTTCTTA; *mei4-F115D*, AACAAAGCCG(T)A(T)TATCAAAGT; and *mei4-W125S*, ATGGTGGTTC(G)GCG(A)AAATAGC. The substituted nucleotides are underlined, and the corresponding wild-type nucleotides are in parentheses. The mutagenized sequences were designed to generate the new restriction sites *Eco*T22I (for *mei4-K81Q*), *Eco*RV (for *mei4-F115D*), and *Nru*I (for *mei4-W125S*). The amino acid sequences should be changed as follows: KPP (amino acids [aa] 81 to 83) to QAS in *mei4-K81Q*, F (aa 115) to D in *mei4-F115D*, and WQ (aa 125 and 126) to SR in *mei4-W125S*. Correct mutated nucleotides were confirmed by DNA sequencing. The mutated *mei4* DNA fragments were inserted into a multicopy plasmid, pAU-KS, and introduced into the *mei4* disruptant YW917 and a wild-type strain (JY878).

Construction of the GST-Mei4 fusion gene. An approximately 360-bp DNA segment containing the forkhead domain (aa 71 to 182) of *mei4*$^+$ was amplified by PCR and cloned into the pCRII vector (Invitrogen Co.). The *Eco*RV site which was derived from the forward primer was cut and ligated to *Bam*HI linkers. The *Bam*HI/*Eco*RI fragment carrying the *mei4* forkhead domain was inserted into the *Bam*HI and *Eco*RI sites located at the 3′ terminus of the glutathione *S*-transferase (GST) gene of pGEX-2T (Pharmacia Biotech) to construct pGEX(*mei4*).

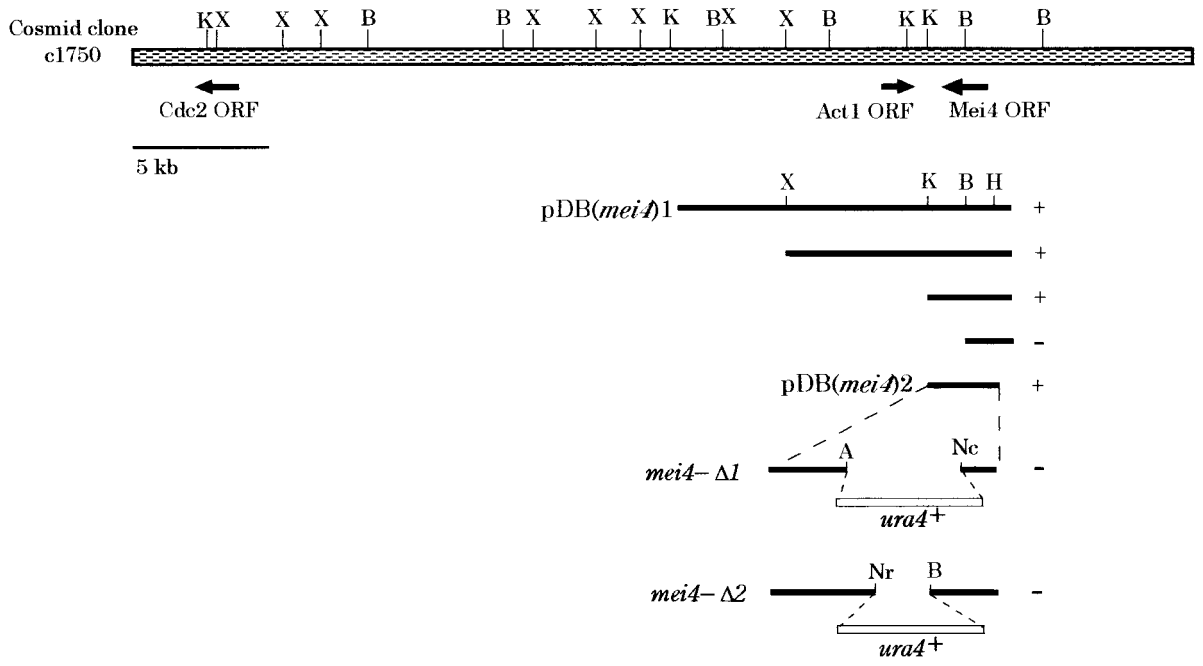
An *E. coli* strain, XLI-Blue, was transformed with pGEX(*mei4*). Expression of the GST-Mei4 fusion protein was induced by IPTG (isopropyl-β-D-thiogalactopyranoside) in Luria-Bertani medium. Cells were homogenized in buffer containing 30 mM Tris-HCl (pH 7.5) and 30 mM NaCl at 0°C. After centrifugation at 16,000 × *g* for 15 min at 4°C, most of the fusion protein was recovered in the supernatant fraction (data not shown).

Gel mobility shift assay. FLEX-U (CTTGAATCAAGTAAATATATATTTTCT), FLEX-D (AAATATTTGAGTAAACAAACAAAATCA), and a mock oligonucleotide (CCCTCTTTCTTTGTTCCTTAT) were labeled with [α-^{32}P]dATP by use of Klenow enzymes with random primers. A standard reaction mixture (20 μl) contained 24 ng of radiolabeled double-stranded oligonucleotide probe, an *E. coli* crude extract containing 9 ng of protein, and 2 μg of poly(dI-dC) 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). In some assays, 4.2 μg of salmon sperm DNA per ml was included in the reaction mixture. The reaction mixture was placed on ice for 60 min and then immediately loaded onto 4% native polyacrylamide gels in TGE buffer, containing 0.6% Tris-HCl (pH 8), 0.078% EDTA, and 2.9% glycine. Polyacrylamide gels were electrophoresed at 15 mA in TGE buffer at 4°C until free probes reached the bottom of the gel. They were fixed with 7% acetic acid and then exposed to X-ray film (Fuji NIF-RX film) for 12 to 18 h at −80°C.

Deletion of the FLEX-D from the *spo6* promoter. The *spo6* promoter sequence containing FLEX-D was amplified by PCR. Two different forward primers containing the *Xho*I site were used: *spo6*-X (GAGCTCGAGAAAATATTTGAGTAAACAAACAAAA) and *spo6*-DF (GAGCTCGAGAAAATATTTGAAACAAATC). The latter sequence lacked the FLEX core heptamer, GTAAACA. The wild-type *spo6*$^+$ gene was cloned into the multicopy plasmid pAL-KS to give pAL(*spo6*+). The amplified DNA was digested with *Xho*I and *Sal*I, and the fragment was then inserted into pAL(*spo6*+) to replace the corresponding region. The plasmids were designated pAL(*spo6*)X and pAL(*spo6*)DF, respectively.

One-hybrid analysis. A putative transcriptional activation domain of Mei4 was determined by a one-hybrid assay. The full-length Mei4 open reading frame

A



B

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MVENQGNVEAHGKPKKVILSLSLKESSKINDSQNVSNVSSKEKCETEALLREENKENLSS   60
DSIRQMIFGDEMAGFVDTGEKPPCSYATLIGLAILQSHNKQLTLSGIYTWIRNTFRYYLN  120
HDGGWQNSIRHNLSLNKAFIKVEKPKGKTLKGHYWTIDPDHMQNFVSVRLHRSHSTDSNS  180
KKRPSSKCHEIKPLTTREIPLARKRSRLNSFNSSTSTSGSSSNVAAEVSNDASQPSNQDS  240
SLNSNIVKPPLPPSNVQSNSSSSENVPKPNAETQEDLPTIDAHESSLYENVNDSRLYEVP  300
ACRNMALNTGYSDADPGYLRTSFRSNSHNSLPYSANEEEDVLQADFLVSQQSSMVSSYVS  360
SRDPHSMPYYRREPIPLRPSSRFYEYTRPTYGRTDTSCSAPGAFCSTQINSPSSYINYSK  420
CAPSSPTLSLQKHREHVKSLLYVPDLTPSFDGSDPWNPSSQLLSEPLFDQHSFQSSLDDL  480
MSVTCFRDSPELNHESSGYSSAPLMPSNRAFINDFSL                          517
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FIG. 1. Structure of *mei4*$^+$ and gene disruption. (A) Restriction map, subcloning, and gene disruption of *mei4*$^+$. The shaded box indicates the *S. pombe* cosmid clone c1750, which contained the *mei4*$^+$ ORF. Solid bars represent *S. pombe* genomic DNA fragments isolated. pDB(*mei4*)1 is the *mei4*$^+$-carrying plasmid which was cloned by complementation from a genomic library. Arrows show the directions and sites of the ORFs for Cdc2, Act1, and Mei4. Complementation of *mei4-P572* by each subclone: +, complements; −, does not complement. Restriction enzymes: K, *Kpn*I; X, *Xba*I; B, *Bgl*II; A, *Acc*I; Nc, *Nco*I; Nr, *Nru*I. (B) Amino acid sequence of Mei4 deduced from the nucleotide sequence, shown in one-letter notation. The forkhead DNA-binding domain is underlined. Possible phosphorylation sites of protein kinase A are double underlined. Serine duplexes, triplexes, and tetraplexes are boxed.

(ORF) was inserted into pGBT9 (Clontech) so that Mei4 was fused to the carboxyl terminus of the *S. cerevisiae* Gal4 DNA-binding domain (see Fig. 10). Similar constructs having several truncated *mei4* fragments were also made (see Fig. 10). These plasmids were transformed into *S. cerevisiae* SFY526.

β-Galactosidase activity was assayed as follows (1). A single colony of yeast transformants was grown in SD-Trp liquid medium at 28°C to the early stationary phase. Cells were washed with and resuspended in Z buffer. The optical density at 600 nm (OD_{600}) was determined as a measure of cell density. The cells were permeabilized by being vortexed in 0.8 ml of Z buffer containing 0.04 ml of 0.1% sodium dodecyl sulfate and 0.04 ml of chloroform, and then 0.16 ml of a 4-mg/ml *o*-nitrophenyl-β-D-galactoside solution was added. The reaction mixture was incubated at 30°C. The reaction was terminated by adding 3 volumes of 1 M Na_2CO_3. The OD_{420} of the supernatant was measured. One unit of β-galactosidase activity was defined as $(OD_{420} \times 1{,}000)/(OD_{600} \times T$ [minutes] $\times V$ [milliliters]), where T is the reaction time and V is the volume of cell suspension used in the assay.

Fluorescence-activated cell sorter (FACS) analysis. Cellular DNA content was determined by flow cytometry basically as described by Watanabe and Yamamoto (48). Cells were fixed with 70% ethanol and stained with propidium iodide, and then the fluorescence intensity was measured with a flow cytometer (model EPICS-C; Coulter).

DAPI staining. *S. pombe* cells were fixed with 3.7% formaldehyde at 28°C for 30 min. The nuclear chromatin region was stained with 4′,6-diamidino-2-phenylindole (DAPI) at 1 μg/ml. Stained cells were observed under a fluorescence microscope (Olympus BHS-RFK).

RESULTS

Cloning and sequencing of *mei4*$^+$. *mei4*$^+$ is essential for the progression through meiotic prophase-I. For further analysis of *mei4*-mediated steps, we isolated a 13-kb genomic DNA

TABLE 2. Mating and sporulation of *mei4*-defective and *mei4*-overexpressing strains

Expt[a]	Strain	Relevant genotype	Zygotes (%)	Asci (%)[b]		Nuclear type of zygotes (%)[c]			
				4 spore	2 spore	1N(r)	1N(ht)	2N	3-4N
1	JZ878	h^{90} *mei4*$^+$	10	60	0	4	5	1	90
	YW917	h^{90} *mei4*Δ	65	0	0	76	24	0	0
	YW917	h^{90} *mei4*Δ [pREP(*mei4*$^+$)]	13	47	8	13	5	2	80
2	YW917	h^{90} *mei4*Δ [pAL19]	69	0	0	28	71	1	0
	YW917	h^{90} *mei4*Δ [pAL(*mei4*$^+$)]	25	29	5	16	32	20	32
	YW917	h^{90} *mei4*Δ [pAL(*mei4-K85Q*)]	56	0	0	38	61	1	0
	YW917	h^{90} *mei4*Δ [pAL(*mei4-W115S*)]	63	0	0	40	60	0	0
	YW917	h^{90} *mei4*Δ [pAL(*mei4-F125D*)]	55	0	0	37	62	1	0

[a] In experiment 1 cells were cultured on SSA medium for 2 days; in experiment 2 cells were incubated in PM liquid medium lacking N for 12 h.
[b] Two- and four-spore asci were differentially counted.
[c] 1N(r), mononucleate zygotes containing a round nucleus; 1N(ht), mononucleate zygotes with a horsetail nucleus; 2N, binucleate zygotes; 3-4N, tri- or tetranucleate zygotes.

fragment which complemented the *mei4-P572* mutation. Subcloning localized the complementation activity on a 2.8-kb *Kpn*I/*Hin*dIII fragment (Fig. 1A).

Sequencing of this fragment (2,788 bp) identified an uninterrupted ORF composed of 1,551 nucleotides. Our sequence was identical to the sequence in the cosmid clone c1750, the nucleotide sequence of which was determined recently in the *S. pombe* genome sequence project. The *mei4*$^+$ gene has been mapped in the vicinity of *cdc2*$^+$ (0.6 centimorgan) on chromosome II (38). This cosmid clone (38 kb) also contained the ORF for Cdc2, indicating that our cloned gene was likely *mei4*$^+$ itself (Fig. 1A). This assumption was further verified by genetic crosses between the disruptant strain and the original *mei4-P572* mutant. The deduced *mei4*$^+$ gene product is a 57-kDa serine- and threonine-rich protein composed of 517 amino acids (Fig. 1B).

Phenotypes brought about by the disruption and overexpression of *mei4*$^+$. The chromosomal *mei4*$^+$ gene in a diploid strain (C525) was disrupted. The disrupted diploid strain was then sporulated, and the tetrads were dissected. Most asci produced four viable spore clones, indicating that the *mei4* null mutation did not confer lethality to the cells. There were no differences in growth and cell size between the wild-type strain and the *mei4*Δ mutant (data not shown).

A homothallic haploid strain harboring *mei4*Δ was able to mate, but the resultant diploid zygotes were asporogenic. DAPI staining revealed that many zygotes contained one horsetail or a rounded nucleus (Table 2 and Fig. 2A), indicating that both mutants were arrested before meiosis-I. There were no significant differences in meiotic phenotypes between the disrupted null mutant and the *mei4-P572* mutant.

We show below that *mei4*$^+$ is transcribed only in meiotic cells (see Fig. 3). Overexpression of *mei4*$^+$ in nutrient and sporulation media was examined. Ectopic expression of *mei4*$^+$ was attained by placing it under control of the inducible *nmt1* promoter on the pREP1 plasmid. This construct, named pREP(*mei4*), was introduced into *mei4*Δ null mutants. When *mei4*$^+$ was ectopically expressed by transferring cells to SSA medium without thiamine, it complemented the *mei4* mutation completely (Table 2). We noticed that a small fraction (at most 10%) of the population formed two-spored asci. Untimely and/or excess expression of *mei4*$^+$ may disorder the meiotic process. Overproduction of *mei4*$^+$ in vegetative cells in rich growth medium, however, did not cause meiosis and sporulation.

Premeiotic DNA replication in the *mei4*Δ strain. To see whether the premeiotic S phase was completed, nitrogen-starved *mei4*Δ cells were subjected to FACS analysis (Fig. 2B). At 2 h

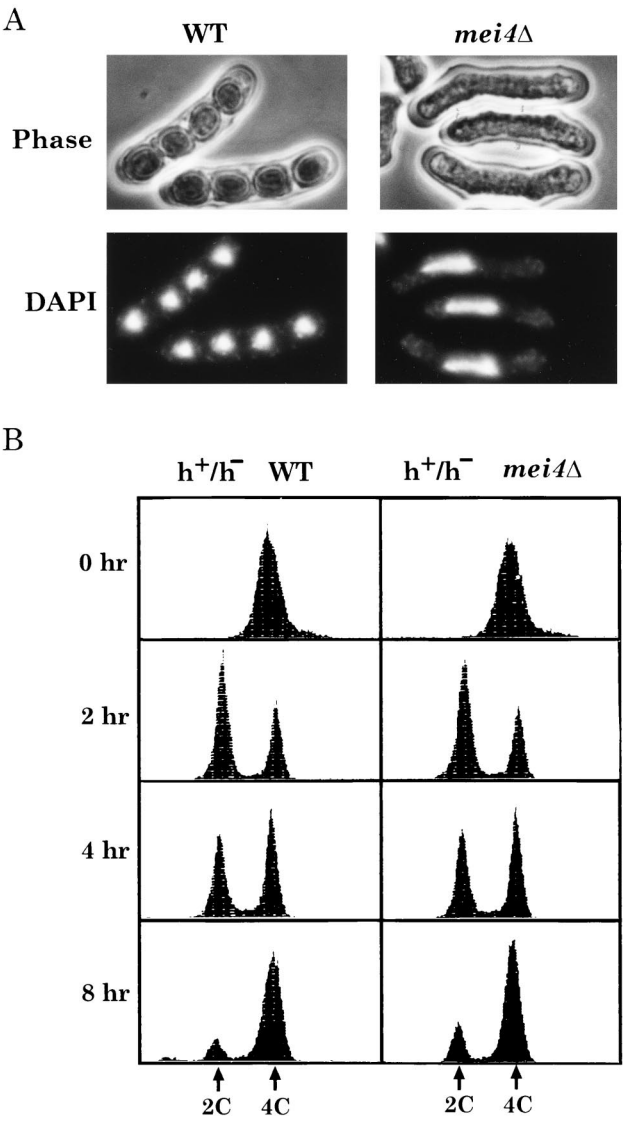

FIG. 2. Phenotypes of the *mei4*Δ strain. (A) Nuclear morphology. The wild-type (WT) strain (L968) and the *mei4* disruptant (YW917) were cultured on malt extract agar for 2 days. (B) Premeiotic DNA synthesis complete in a diploid *mei4*Δ strain. A wild-type diploid strain (JY362) and a homozygous *mei4*Δ mutant (JZ807) were cultured in nitrogen-free medium, PM lacking N, for the indicated times, and samples were processed for FACS analysis. Fluorescence intensities corresponding to 2C and 4C DNA contents are indicated.

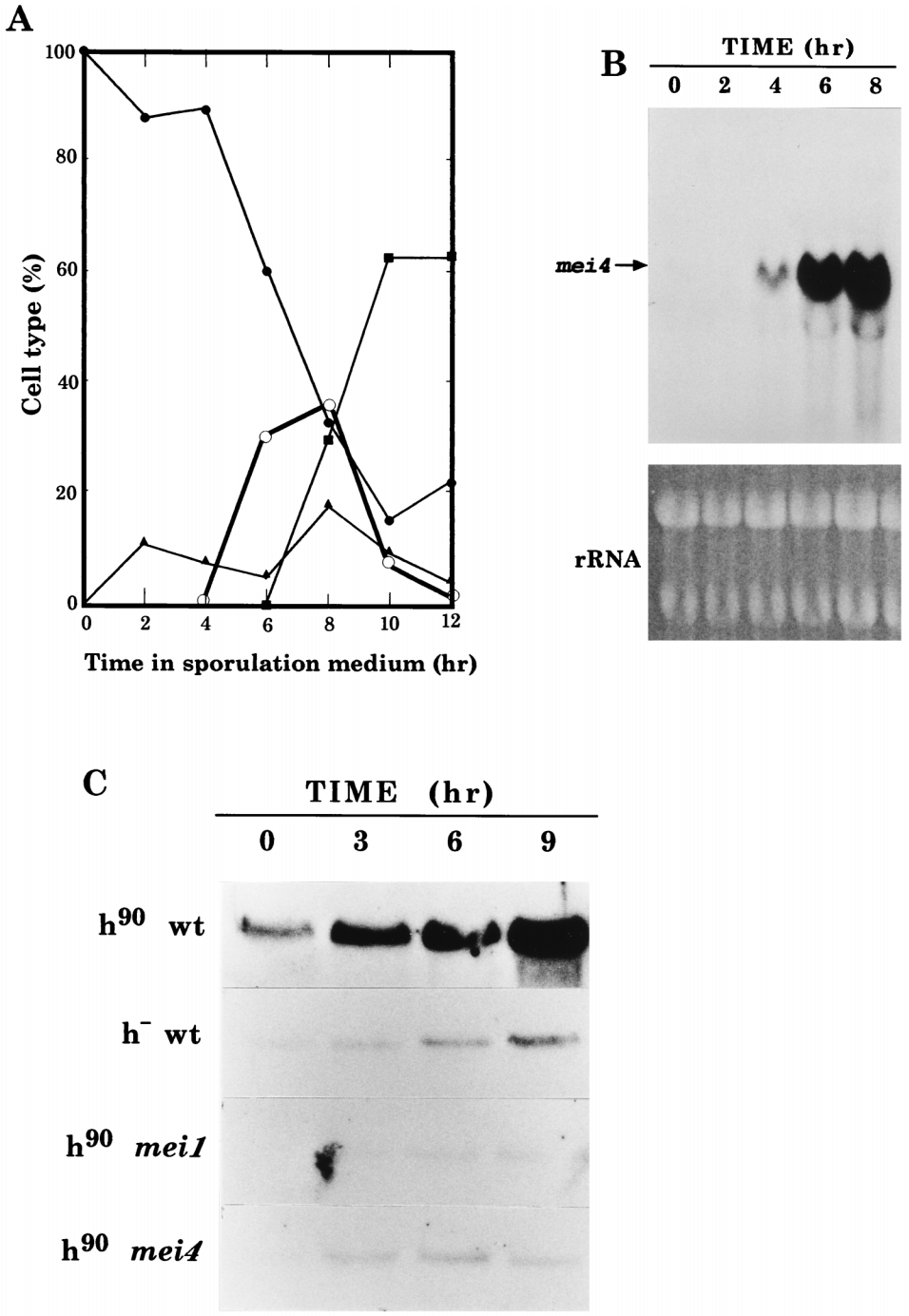

FIG. 3. Transcriptional regulation of *mei4*$^+$. (A) Kinetics of meiosis in a heterothallic diploid strain, CD16-1. Cells were cultured for 15 h in a modified minimal medium lacking both nitrogen and carbon sources (PM lacking N and C), and then glucose and glycerol were added to induce meiosis. A portion of this synchronously sporulating culture was withdrawn, fixed, and stained with DAPI. Frequencies of mononucleate cells with a rounded nucleus (closed circles), mononucleate cells with a horsetail nucleus (open circles), binucleate cells (triangles), and tetranucleate cells (squares) were determined. The other portion of the synchronous culture was subjected to RNA extraction. (B) Northern analysis. An autoradiogram of Northern analysis for *mei4* mRNA is shown. Gel staining with ethidium bromide to assess loading abundance is presented. (C) Northern analysis of *mei4*$^+$ in meiosis-deficient strains. Log-phase cultures grown in a minimal medium (PM with N) were incubated in PM lacking N for the indicated times. Samples were subjected to Northern analysis with a *mei4*-specific probe. To assess the loading abundance, ethidium bromide staining of the gels was done (data not shown). Strains: L968 (*h*^{90} wild type [wt]), L972 (*h*$^-$ wild-type), C206-2A (*h*^{90} *mei1-B102*), and C133-1D (*h*^{90} *mei4-P572*).

after the nutritional shift-down, a discrete G_1 (2C) peak appeared, which then shifted to a G_2 (4C) peak between 4 and 8 h, indicating normal execution of premeiotic DNA replication in *mei4*Δ cells. Changes in the DNA content of *mei4*Δ cells were very similar to those of the wild-type strain. We conclude that *mei4*Δ cells undergo premeiotic DNA replication normally.

Transcriptional regulation of *mei4*$^+$. The fact that *mei4*$^+$ is required only for meiosis-I prompted us to examine whether its

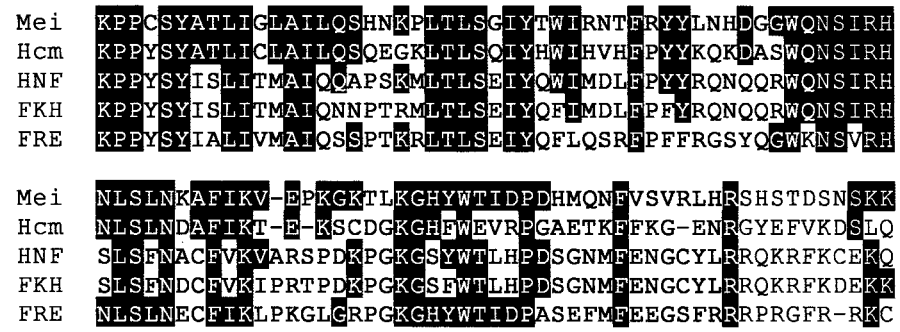

FIG. 4. Mei4 contains a forkhead DNA-binding motif. A comparison of the amino acid sequences of forkhead domains in a few typical forkhead proteins is shown. Amino acids identical in Mei4 and the others are shown in white against black. Mei, Mei4 (*S. pombe*); Hcm, Hcm1 (*S. cerevisiae*), HNF, HNF3α (rat); FKH, forkhead (fly); FRE, FREAC-1 (human).

expression is restricted to the meiotic process. Synchronous meiosis was induced in a wild-type diploid strain, CD16-1, by a shift-down to a starvation medium as has been reported previously (25). As shown in Fig. 3A, after 4 h of incubation in a nitrogen-free medium, cells entered prophase-I and so-called horsetail nuclei began to accumulate. Cells which finished meiosis-II appeared at 6 h, and mature asci were observable at 10 h.

Cells were harvested at 2-h intervals, and total RNA was subjected to Northern blot analysis with a *mei4*-specific probe. As Fig. 3B shows, the *mei4* transcripts (2.3 kb) were hardly detected in vegetative cells (at 0 h). Remarkable induction of *mei4*$^+$ mRNA was observed after 4 h of incubation. These results indicate that *mei4*$^+$ was transcriptionally regulated, and abrupt induction occurred before or at the onset of prophase-I. Similar observations have been reported by Iino et al. (14) based on the *pat1*-driven synchronous meiosis.

The *mei4*$^+$ transcript level in nitrogen-free sporulation me-

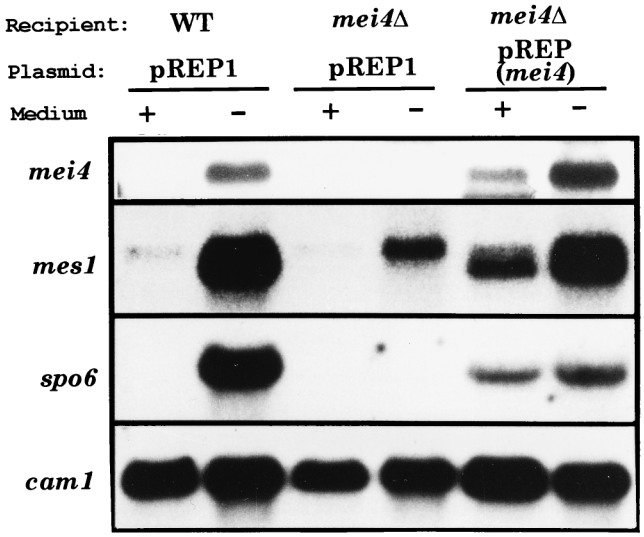

FIG. 5. Northern blot analysis indicating that transcription of *spo6*$^+$ is dependent on *mei4*$^+$ function. Strains: WT, a wild-type diploid strain (JY362); *mei4*Δ, a diploid strain harboring the *mei4*Δ null allele homozygously (JZ878). These strains were transformed by either pREP1 vector plasmid or pREP1(*mei4*). Transformants were incubated in PM with (+) or without (−) 10 mM NH_4Cl. Specific radioactive probes were for *mei4*, *mes1*, *spo6*, and *cam1*. Although the *spo6* probe hybridizes with two mRNA species (2.1 and 1.4 kb), only the meiosis-specific 1.4-kb mRNA is shown.

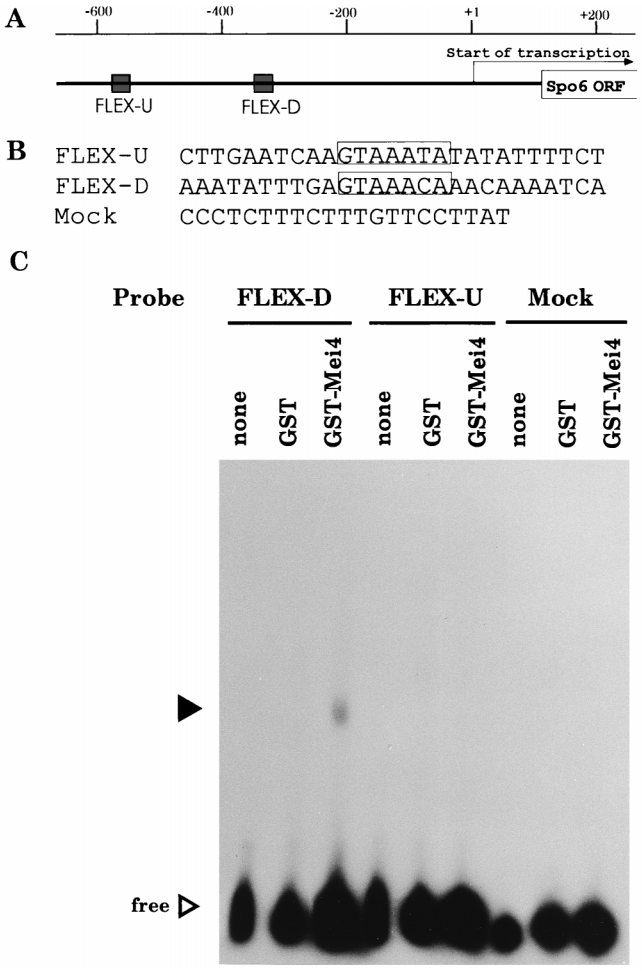

FIG. 6. Gel mobility shift assay with a recombinant Mei4 protein. (A) Map of the 5′ promoter sequence of *spo6*$^+$. Shaded boxes called FLEX-U and FLEX-D indicate the putative recognition sequences containing the core heptamer motif. An arrow represents the direction of transcription and the site of the start point. (B) Sequences of oligonucleotides used for probes. The mock oligonucleotide is the recognition site for Ste11. (C) Gel shift analysis. A whole-cell extract from *E. coli* expressing the GST-Mei4 forkhead fusion protein was incubated with labeled oligonucleotide probes. Closed and open triangles indicate shifted bands and free probes, respectively.

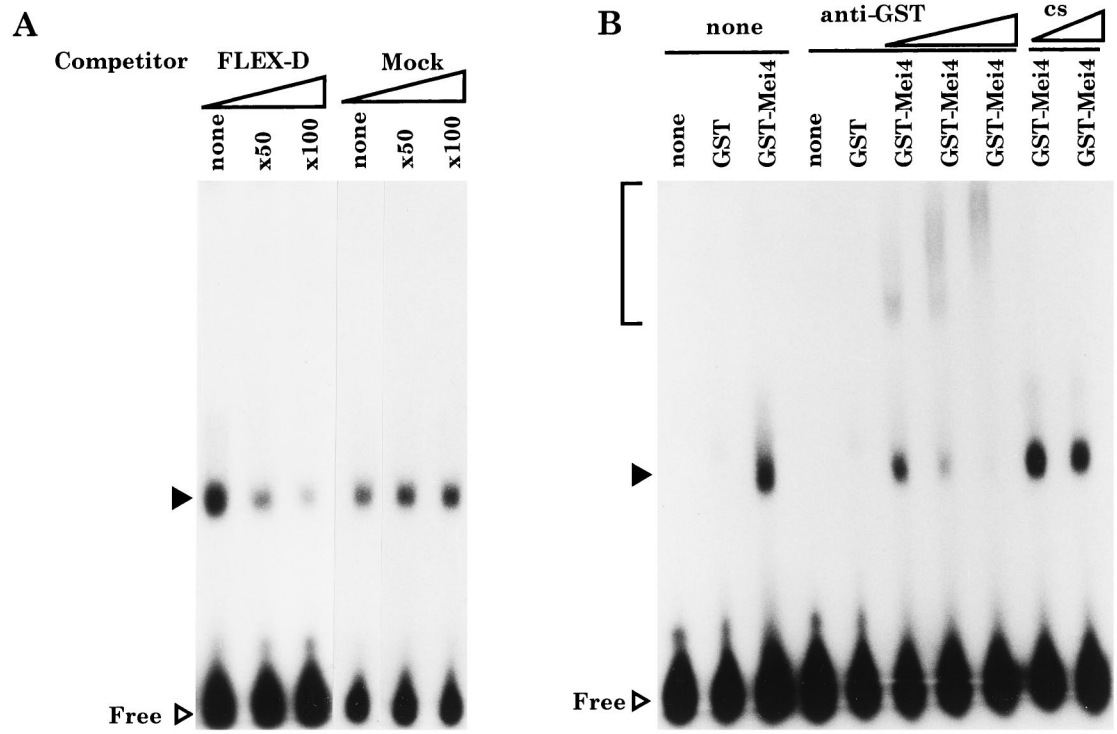

FIG. 7. Gel mobility shift analysis indicating the specificity of binding between FLEX-D and the recombinant Mei4 protein. (A) Competition assay. FLEX-D probes were incubated with GST-Mei4. Closed and open triangles indicate shifted bands and free probes, respectively. (B) Supershift experiment using the anti-GST antibody. A different amount of antibody was included in the reaction mixture. The probe used was FLEX-D. The supershifted bands due to anti-GST are indicated by a bracket. cs, control serum.

dium was severely reduced in both a heterothallic haploid strain and the mutant harboring *mei1-B102*, which is allelic to *mat2-Pm* (Fig. 3C). Because meiosis is blocked before the premeiotic S phase in these strains, the *mei4*$^+$ transcription is dependent upon meiosis rather than upon nitrogen starvation. Interestingly, the transcription level was also reduced in *mei4-P572* cells, suggesting a positive autoregulatory mechanism (Fig. 3C). These results indicate that transcription of *mei4*$^+$ is induced during early meiosis and probably before prophase-I.

Characteristics of the predicted Mei4 protein. The predicted amino acid sequence of Mei4 was examined for similarity to known proteins in databases by using the BLAST program. The amino-terminal domain of Mei4 (aa 81 to 172) shows prominent sequence homology with an array of proteins of the forkhead/HNF3 family, also called the winged helix family (6), including the *Drosophila* nuclear protein forkhead (53) and the murine HNF3, which is the hepatocyte-specific transcription factor (21) (Fig. 4). This domain may function as the sequence-specific DNA-binding domain. The third α-helix of HNF3, corresponding to aa 122 to 135 of Mei4, makes major-groove contact with DNA (6). The primary structure of this domain, composed of approximately 100 amino acids, was compared for Mei4 and 67 forkhead proteins in the databases. Its phylogenetic tree suggested that *S. cerevisiae* Hcm1 is the closest member and that Mei4 and Hcm1 seem to constitute a subfamily (data not shown). The *HCM1* gene was cloned as a high-copy suppressor of calmodulin mutants (59). The molecular function of Hcm1 has not yet been elucidated.

Another prominent feature of Mei4 was the abundance of serine and threonine residues, which constitute 25 and 8%, respectively. Serine duplex, triplex, and tetraplex motifs appeared 19 times (Fig. 1B), although their meaning is unclear. Two possible sites, KRPS and RKRS, for phosphorylation by the cyclic AMP-dependent protein kinase were found following the C terminus of the forkhead domain (aa 182 to 185 and aa 203 to 206).

Mutations in the forkhead domain abolish Mei4 function. In order to examine whether the forkhead domain is essential for the function of Mei4, highly conserved amino acids in this domain were altered by site-directed mutagenesis. Mutated *mei4* alleles on a multicopy plasmid were transformed into a *mei4* disruptant. As Table 2 shows, none of the three different mutant alleles could complement the *mei4* null mutation, indicating that these amino acid substitutions severely affected the *mei4* gene function. They did not interfere with meiosis or sporulation in a wild-type strain (data not shown), indicating that the mutations in the forkhead domain represented a dominant-negative phenotype.

Transcription of the meiotic *spo6*$^+$ gene depends on Mei4. We next searched for meiosis and sporulation genes whose transcription was dependent on the putative transcription factor Mei4. Transcription of genes known to be responsible for meiosis, sporulation, or recombination was examined in the *mei4*Δ strain. The *spo6*$^+$ gene is essential for meiosis-II and sporulation (3, 11, 12) and is transcribed to generate two mRNA species that are different in size and expression pattern (30). The 2.1-kb species was constitutively expressed at a low level in both vegetative and meiotic cells, while the smaller, 1.4-kb species is absent in vegetative cells and highly induced in meiotic cells (30). These two transcripts have different transcriptional start points (30). We found that the 1.4-kb meiosis-specific *spo6*$^+$ mRNA was almost absent in the *mei4*Δ mutant (Fig. 5). In addition, overexpression of *mei4*$^+$ stimulated the

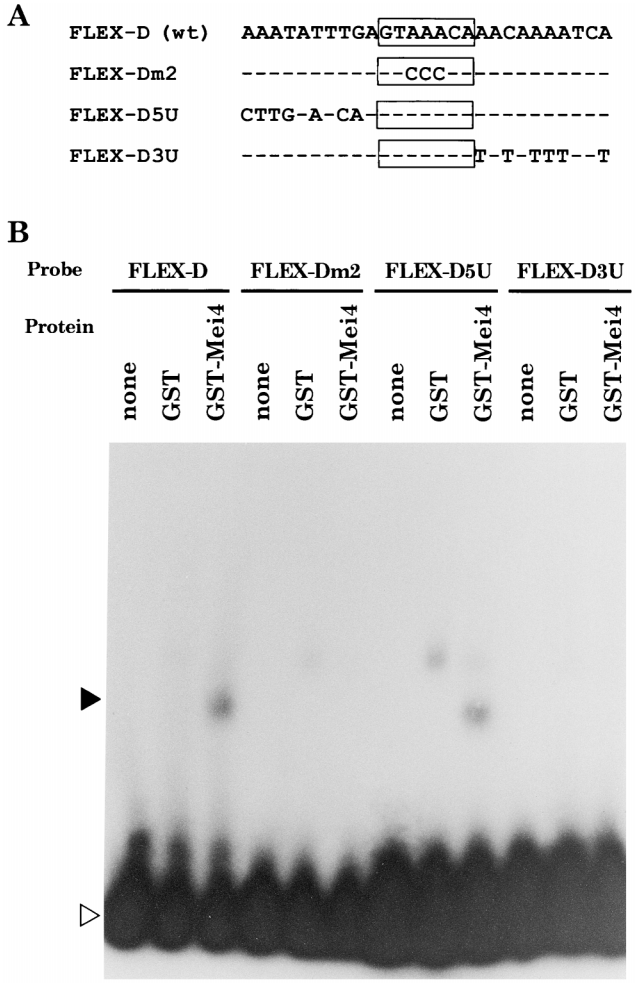

FIG. 8. Gel mobility shift analysis with mutated FLEX-D oligonucleotides. (A) Sequences of oligonucleotides used. The core heptamer is boxed, and nucleotides identical to those in FLEX-D are indicated by dashes. wt, wild type. (B) Results of gel shift assay. Closed and open triangles indicate shifted bands and free probes, respectively.

transcription of *spo6* even in a nutrient medium, indicating that *spo6*$^+$ may be a target gene of Mei4.

The *mes1*$^+$ gene, responsible for meiosis-II, carries one short intron, and splicing of this intron is accomplished only during meiosis (19). As shown in Fig. 5, both transcription and splicing of *mes1*$^+$ occurred in the wild-type strain cultured in nitrogen-free medium. However, transcription of *mes1*$^+$ was reduced in *mei4*Δ cells. In contrast, *mes1*$^+$ was induced in a *mei4*Δ strain overexpressing *mei4*$^+$, even when cells were cultured in nitrogen-rich medium. In addition, splicing of the *mes1* intron was highly dependent on the *mei4* function. These observations suggest that transcription of *mes1* and splicing of its intron are regulated by *mei4*$^+$.

The Mei4 forkhead domain binds to the FLEX sequence. We addressed the question of whether Mei4 could bind to a specific sequence. In the case of the human forkhead proteins called FREAC, the recognition sequence was GTAAAYA, which seemed to be a consensus core sequence for forkhead proteins in general (34). In this article, this heptamer sequence will be designated the core heptamer. Interestingly, two sequences which are identical to the FREAC core heptamer were found in the possible 5′ regulatory region of *spo6*$^+$ (Fig. 6A). Therefore, we examined the binding of Mei4 to two kinds of core heptamer-containing sequences of 27 nucleotides, as presented in Fig. 6B. These sequences were designated FLEX-U and FLEX-D (for FREAC-like consensus element of spo six), where the suffixes U and D represent the upstream and downstream elements, respectively.

To demonstrate that Mei4 can bind to the FLEX sequences, a fusion protein composed of GST and the forkhead domain of Mei4 (aa 71 to 182) was subjected to a gel retardation assay with radioactive FLEX-U and FLEX-D as probes. As shown in Fig. 6C, the shifted band was observed only with FLEX-D. The fusion protein caused no mobility shift with either FLEX-U or the mock oligonucleotide. The GST protein without the forkhead region did not recognize any oligonucleotides examined. These gel shift assays indicated that the Mei4 fusion protein could bind to the FLEX-D sequence by the amino-terminal forkhead domain.

We then tested competition of binding with nonlabeled oligonucleotides to examine the sequence specificity. Binding of GST-Mei4 fusion protein to the labeled FLEX-D oligonucleotide was severely inhibited by adding cold competitor of the same sequence, but no such competition was observed with unrelated oligonucleotides (Fig. 7A). Thus, we conclude that the binding was sequence specific.

To confirm that the GST-Mei4 fusion protein itself bound to FLEX-D, a supershift analysis with an anti-GST antibody (Pharmacia Biotech) was carried out. When the antiserum was included in the reaction mixture, the shifted band was further retarded (Fig. 7B), clearly indicating that the GST-Mei4 fusion protein was responsible for the mobility shift.

The GST-Mei4 fusion protein specifically recognizes FLEX-D. To corroborate that the core consensus sequence of FLEX-D was important for recognition by Mei4, we tested binding of Mei4 to the mutated oligonucleotide in which the central AAA of the core heptamer was replaced by CCC (Fig. 8A). The fusion protein could not bind to this mutated oligonucleotide (FLEX-Dm2) (Fig. 8B).

The GST-Mei4 fusion protein recognized FLEX-D but not FLEX-U (Fig. 6C). Only the sixth base was different in the core heptamers of FLEX-U (GTAAATA) and FLEX-D (GT AAACA). This difference, however, did not affect the binding affinity, as the replacement of T by C or vice versa only slightly influenced the band intensity (data not shown). This suggests that the variation in the FLEX core heptamer itself is not the cause for the binding affinity. Thus, we examined the significance of the flanking sequences. The 5′ and 3′ flanking sequences of FLEX-D were replaced with those of FLEX-U as shown in Fig. 8A. The gel shift assay (Fig. 8B) indicated that oligonucleotides in which the 3′ flanking sequence was replaced (FLEX-D3U) could hardly be recognized by the GST-Mei4 fusion protein. A FLEX-D5U probe gave an additional band when mixed with the GST-Mei4 fusion protein. This slower-migrating band might be a nonspecific one, because a band of the same mobility was also found with GST alone. We conclude that the 3′ flanking sequence, in addition to the core heptamer, is important for recognition by Mei4.

FLEX-D is essential for the transcription of *spo6*$^+$. The forkhead domain of Mei4 could bind to FLEX-D in vitro. We addressed the question of whether this sequence could function as a transcriptional *cis* element. The FLEX-D core heptamer was deleted from plasmid-borne *spo6*$^+$. The plasmid carrying this deleted allele of *spo6* (*spo6-DF*) was transformed into a diploid strain (NT-4A) homozygous for *spo6*Δ to test its ability to complement the sporulation defect. As shown in Fig. 9A, the *spo6-DF* allele complemented the *spo6* null mutation only very weakly, while the *spo6*$^+$ and *spo6-X* genes carrying

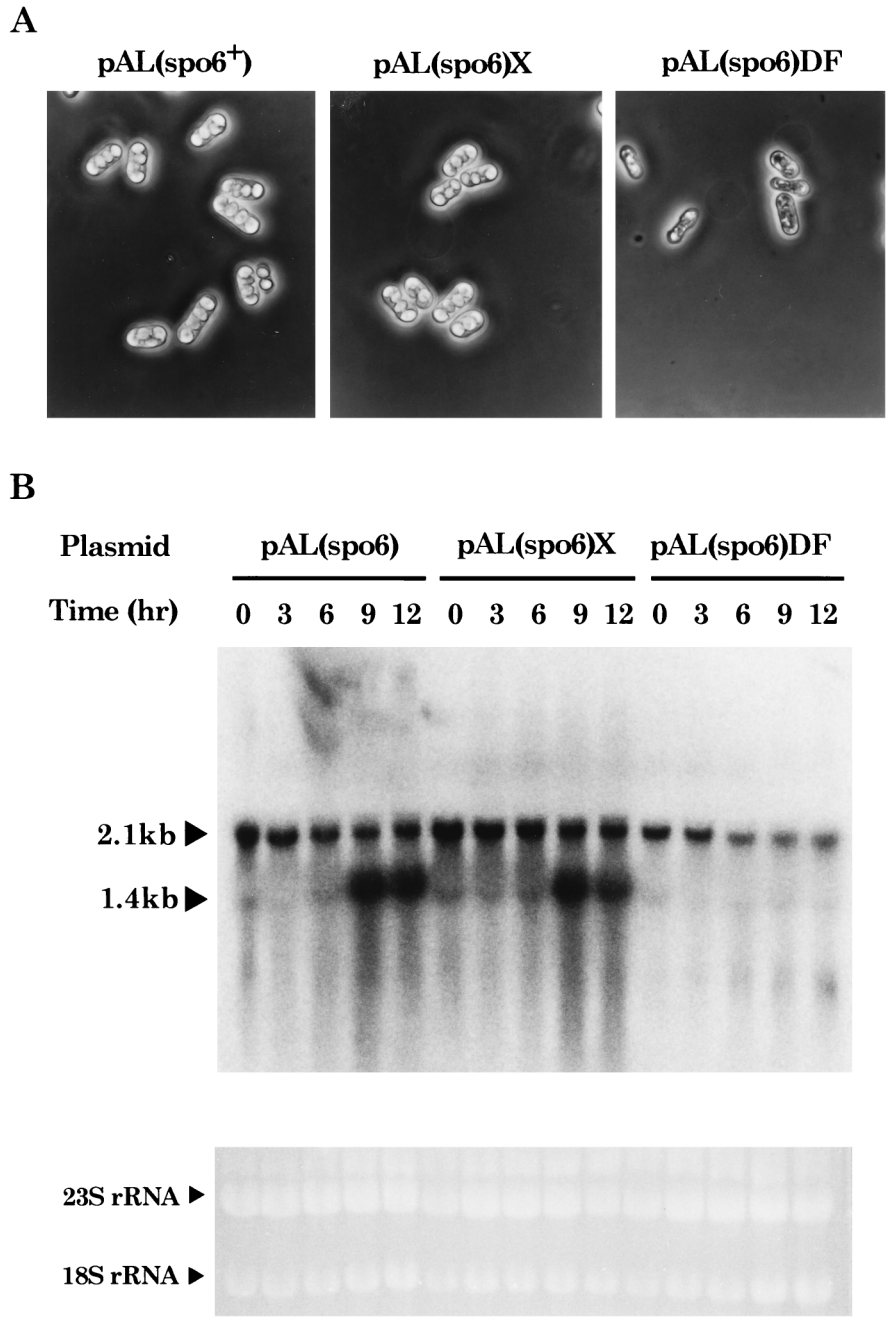

FIG. 9. Deletion of the core heptamer in the FLEX-D element from the *spo6* promoter reduces sporulation ability and *spo6* transcription. (A) Complementation of the sporulation defect of *spo6*Δ. Plasmid pAL(*spo6*) is pAL-KS carrying *spo6*$^+$. pAL(*spo6*)X and pAL(*spo6*)DF are pAL-KS containing the *spo6* gene with or without the core heptamer of FLEX-D, respectively. A diploid *spo6* disruptant, NT-4A, was transformed with the pAL-KS-based plasmids, and then the transformants were sporulated on SSA for 2 days at 28°C. (B) Northern blot analysis showing the reduced expression of *spo6* caused by the deletion of the promoter element. A diploid *spo6* disruptant strain (NT-4A) transformed with the indicated plasmids was grown in nitrogen-rich medium (EMM2 with N) and shifted to nitrogen-free medium (EMM2 without N). Cells were harvested at the indicated times after the transfer to EMM2 without N. Total RNA was extracted and subjected to Northern analysis with the *spo6*-specific probe. Two different mRNA species, of 2.1 and 1.4 kb, are indicated. Gel staining with ethidium bromide indicates roughly equal amounts of RNA in each lane.

the core heptamer in the promoter were able to rescue the sporulation defect.

Transcription of *spo6* in these transformants was examined by Northern analysis (Fig. 9B). Two classes of the *spo6* transcripts were detected. The meiosis-specific 1.4-kb band was prominent in the *spo6*$^+$ and *spo6-X* strains but was almost completely missing in the *spo6-DF* strain. On the other hand, the 2.1-kb mRNA species was not affected by the mutation. These observations support that FLEX-D serves as the *cis*-acting element for the Mei4 transcription factor in *spo6*$^+$ cells.

The activation domain resides in the C-terminal region of Mei4. To dissect the transcriptional activation activity of Mei4, we used an *S. cerevisiae* one-hybrid analysis. The full-length Mei4 protein was fused to the *S. cerevisiae* Gal4

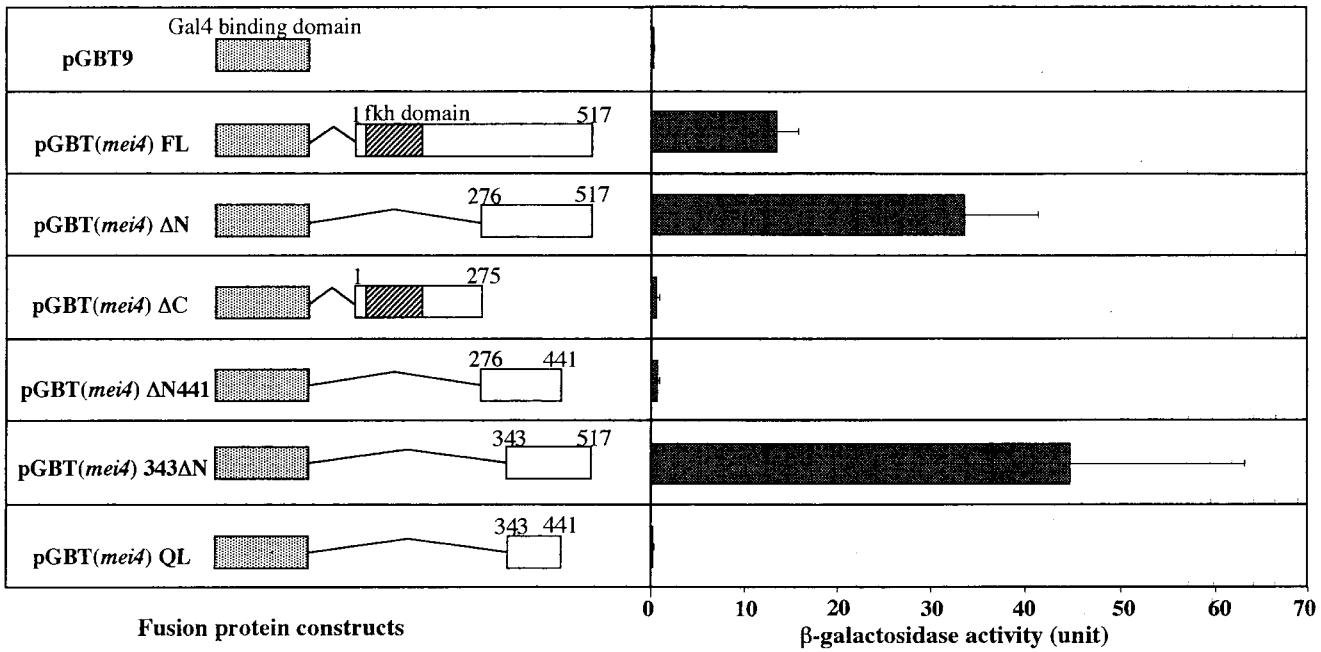

FIG. 10. One-hybrid analysis to localize the activation domain of Mei4. (Left panel) Construction of fusion proteins between a Gal4 DNA-binding domain and Mei4 proteins. Numerals represent the positions of amino acid residues from the N terminus. *S. cerevisiae* SFY526 was transformed with the indicated plasmids. fkh, forkhead. (Right panel) β-Galactosidase activities, with standard deviations, for three independent transformants.

DNA-binding domain on plasmid pGBT9. The resulting plasmid, pGBT(*mei4*)FL, was transformed into *S. cerevisiae* SF526, which carried the *GAL1* promoter upstream of the *lacZ* reporter gene. The β-galactosidase activity in the transformants with pGBT(*mei4*)FL was significantly higher than that in transformants with the control plasmid pGBT9 (Fig. 10), confirming that Mei4 is able to activate transcription.

Deletion analysis localized the activation domain of Mei4 in the C-terminal region from aa 343 to 517. As expected, the N-terminal half containing the forkhead domain did not stimulate transcription. Deletion of the region between aa 441 and 517 completely eliminated the activation activity.

We conclude that Mei4 is a meiosis-specific transcription factor, in which the N-terminal 120 aa (aa 71 to 190) constitute a DNA-binding domain and the C-terminal 175 aa (aa 343 to 517) constitute a transcriptional activation domain (Fig. 4C).

DISCUSSION

Mei4 is a meiosis-specific transcription factor. The following observations support that Mei4 is a meiosis-specific transcription factor of *S. pombe*. (i) *mei4*$^+$ is transcribed only in meiotic cells. (ii) Mei4 contains a forkhead DNA-binding domain in the N-terminal region. Mutations introduced into this domain abolish the *mei4* function. (iii) A meiosis-specific gene, *spo6*$^+$, is not transcribed in *mei4* null mutants. Ectopic expression of *mei4*$^+$ in rich growth medium causes transcription of *spo6*$^+$. (iv) A recombinant Mei4 protein could bind specifically to the FLEX-D DNA fragment, a putative *cis* element on *spo6*$^+$. Deletion of this element totally eliminated the transcription of *spo6*$^+$. (v) A one-hybrid assay proved the ability of Mei4 to activate transcription. The activation domain was localized in the C terminus.

Consensus *cis* element recognized by forkhead proteins. We demonstrated that Mei4 is able to bind to the 27-mer DNA fragment called FLEX-D. It contains the core heptamer GTA AACA, which is identical to the elements required for human forkhead proteins (34). Furthermore, the core sequence of FLEX-D meets the requirement for the binding of HNF3β, (G/A)(T/C)(C/A)AA(C/T)A (33). Our mutational analysis demonstrated that this core heptamer is indispensable for the DNA-protein recognition between FLEX-D and Mei4. The fact that mammalian and yeast forkhead proteins recognize the common *cis* element suggests that DNA-binding properties have been conserved rather tightly among the forkhead family proteins.

A transcriptional cascade operates to drive meiosis in *S. pombe*. Recent studies revealed that several putative transcription factors are integrated into a regulatory cascade leading to the initiation and progression of meiosis in *S. pombe*. A part of this cascade culminating in the expression of *spo6*$^+$ and *mes1*$^+$, which are essential for meiosis and sporulation, is illustrated in Fig. 11.

Sexual differentiation in fission yeast is initiated on nutritionally poor media. Starvation signals are mediated through both the adenylate cyclase-protein kinase A pathway (15, 23, 24, 28) and the Wik1 (Wak1)-Wis1-Sty1 (Spc1) mitogen-activated protein kinase cascade (40, 41, 46). The latter pathway activates the Atf1 (Gad7) transcription factor, which is phylogenetically related to the mammalian transcription factor Atf (17, 54). Those signal transduction pathways may join at the expression of *ste11*$^+$, which encodes a key transcription factor responsible for a wide variety of genes required for sexual development. The transcription of *ste11*$^+$ requires Atf1 and is repressed by A kinase (Pka1). Ste11 recognizes the promoter element called the TR box, which is found in the 5′ upstream region of all of the target genes, including *mei2*$^+$, *mat1*$^+$, *ste6*$^+$, and others (13, 18, 42, 47, 55). Most of the target genes are transcribed in response to a nitrogen starvation signal (1, 13, 39, 42, 55). The *mat1*$^+$ products, Mat1-Pm and Mat1-Mm, cooperatively activate transcription of *mei3*$^+$ (1, 18), which encodes the inhibitor of the Pat1 protein kinase (25). Ste11

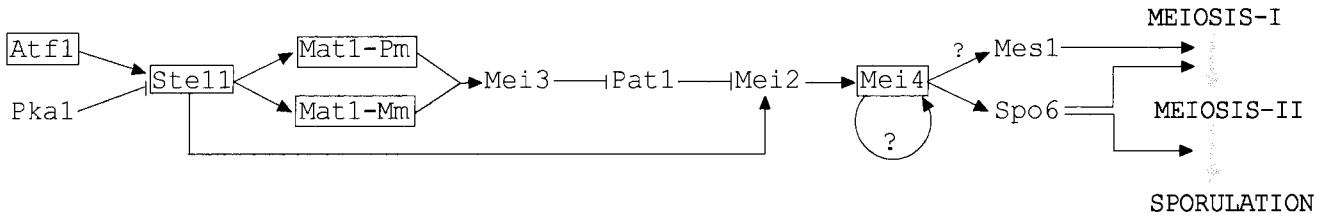

FIG. 11. A regulatory cascade including some putative transcription factors, culminating in the expression of *spo6*$^+$ and *mes1*$^+$, which are necessary for meiosis and sporulation. Arrowheads and vertical bars indicate stimulatory and inhibitory actions, respectively. Putative transcription factors are boxed. Other gene products: Pka1, a catalytic subunit of A kinase; Pat1, a serine/threonine protein kinase; Mei3, a Pat1 inhibitor; Mei2, an RNA-binding protein.

thus promotes meiosis by indirectly enhancing *mei3*$^+$ transcription (Fig. 11).

The mitotic G_1-to-S transition is controlled by a complex of transcription factors, Cdc10-Res1 or Cdc10-Res2, which recognizes the MCB box (4, 22, 27, 43, 44). Two genes necessary for DNA replication, *cdc18*$^+$ and *cdc22*$^+$, are the targets of these complexes (22). Another transcription activator, called Rep1, is also included in the complex (43). The complex composed of Rep1, Cdc10, and Res2 (or Res1) primarily supports premeiotic DNA synthesis rather than mitotic DNA synthesis (27, 60). Interestingly, transcription of *res2*$^+$ requires *rep1*$^+$, and that of *rep1*$^+$ requires Ste11 (43). Therefore, the premeiotic S phase is dependent on Ste11.

As mentioned above, transcription of *mei4*$^+$ is stimulated by nitrogen starvation. Because the transcriptional activation is observed neither in haploid strains nor in an *mei1* mutant in which meiosis is blocked before the premeiotic DNA synthesis, the *mei4* expression is not a simple response to starvation signals. It is important to identify the factor regulating the transcription of *mei4*$^+$. One likely candidate is Mei2, which is a crucial inducer of meiosis (48). As shown in Fig. 11, Mei2 is positively regulated by Ste11 and negatively controlled by the Pat1 protein kinase which blocks the initiation of meiosis in haploid cells under growth conditions (42, 50). Heterothallic haploid cells carrying the activated *mei2* allele (*mei2-SATA*) undergo meiosis even in a rich growth medium (50). *mei4*$^+$ transcription is totally abolished in *mei2*Δ cells, and full transcription of *mei4*$^+$ was observed in the *mei2-SATA* mutant in nitrogen-rich medium (51). These facts indicate that *mei4* transcription is dependent upon *mei2*$^+$ function. From the fact that the *mei4* mRNA level decreases in *mei4-P572* cells (Fig. 3C), we speculate that there is a positive feedback mechanism for *mei4*$^+$ transcription by its product. However, the GTAAAYA motif for Mei4-binding sites is not present in the promoter region of *mei4*$^+$.

Targets of Mei4. We demonstrated that Mei4 regulates expression of *spo6*$^+$ and *mes1*$^+$ at the transcriptional level. Two copies of the consensus core motif GTAAAYA were found in the promoters of both genes. However, the phenotypes of *spo6*Δ and *mes1*Δ mutants are not identical to that of the *mei4*Δ mutant, which is blocked in prophase-I, suggesting that there are certain targets of Mei4 other than *spo6*$^+$ and *mes1*$^+$. It is noteworthy that the spliced mature *mes1*$^+$ mRNA could not be detected in *mei4*Δ cells. Splicing of *mes1* mRNA appears to be regulated by some hypothetical meiosis-specific splicing factor(s). We speculate that the expression of these putative splicing factors may depend on the Mei4 transcription factor.

It is known that *cdc13*$^+$ and *cdc25*$^+$ are not fully transcribed in *mei4* disruptant strains (14). The reduction of the mRNA levels of these *cdc* genes may be responsible for the arrest of *mei4* null mutants in prophase-I. Genes that are essential for the progression through prophase-I have not yet been reported for *S. pombe*. It is highly probable that some of these genes are possible targets of Mei4.

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