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Comprehensive isolation of meiosis-specific genes identifies novel proteins and unusual non-coding transcripts in *Schizosaccharomyces pombe*

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ABSTRACT

In order to isolate meiosis-specific genes in *Schizosaccharomyces pombe*, we have constructed a subtracted cDNA library enriched in clones whose expression is enhanced during meiosis induced by nitrogen starvation. Using northern blot analysis, we isolated 31 kinds of clones whose expression was induced in a meiosis/sporulation-specific manner. We comprehensively named them *meu* after meiotic expression upregulated. The transcription of 20 *meu* genes was found to be dependent on the *mei4*+ gene, which encodes a transcription factor required for the progression of meiosis. DNA sequencing indicated that most of the *meu* genes encode novel proteins. Notably, five of the *meu* genes harbor no apparent protein coding sequences, and the transcripts form stable hairpin structures, suggesting that they may generate non-coding RNAs or antisense RNAs. The results presented here imply that RNAs are also important for the comprehensive characterization of genomic expression.

INTRODUCTION

The fission yeast *Schizosaccharomyces pombe* is an ideal model system in which to study meiotic processes at the molecular level. Diploid cells of fission yeast produce haploid cells through a developmental program of sporulation, which consists of meiosis and spore morphogenesis. The process of meiosis includes DNA replication, recombination and chromosome segregation. Unlike the budding yeast *Saccharomyces cerevisiae*, fission yeast cells are most stable in the haploid state and are essentially asexual under rich nutritional conditions. The initiation of meiosis in fission yeast is known to be under the control of two independent, convergent regulatory pathways, i.e., the mating types and nutritional condition (1). Fission yeast displays two mating types, h^+ and h^-, and the haploid cells with distinct mating types form zygotes, undergo meiosis and generate haploid spores when cells are starved of nutrients, especially nitrogen. Unlike budding yeast, glucose starvation is not mandatory for mating and meiosis. When these spores are returned to rich nutritional conditions, they keep growing and do not undergo conjugation unless they are starved of nutrients. Zygotes can also grow as diploid cells if they are put on a rich medium immediately after conjugation. These diploid cells can also proceed to meiosis when they are starved of nitrogen.

A number of the genes that are required for these events have been cloned and their function during meiosis and/or sporulation analyzed. Many of the genes exhibit elevated levels of transcription only during the meiotic process, not during the vegetative growth phase (2). Compared to the mitotic cell cycle, however, only a limited amount of information is available on the regulatory mechanisms of meiosis or sporulation at a molecular level. This is partly because the number of meiosis- or sporulation-specific genes that have been isolated up until now remains small. To alleviate this problem, genes whose expression was specifically induced during meiosis and sporulation have been isolated on the assumption that many of them will be related to the regulation of these processes (3). However, primarily because of technical problems, only a limited number of such genes have been isolated so far. We have recently developed a novel protocol for the preparation of a subtracted cDNA library of high quality that permits comprehensive cloning from the library between two kinds of closely related cells (4–7). In this study, we prepared a subtracted cDNA library from *S.pombe* using this technique and have isolated 31 types of clones whose expression was induced during meiosis. We report here the characterization of their gene products.

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+AB016983, AB017033, AB017034, AB017617, AB20594, AB054299–AB054318, AB054529–AB054532

MATERIALS AND METHODS

Strains and media

We used CD16-1 (*h⁺/h⁻ ade6M-210/ade6-M216 cyh1/+ +/lys5-391*) and CD16-5 (*h⁻/h⁻ ade6-M210/ade6-M216 cyh1/+ +/lys5-391*) strains for RNA preparation for use in northern blot analysis, cDNA library construction and cDNA subtraction. To examine *mei4⁺* dependency, we used the temperature-sensitive mutant *pat-1-114* (8) and the *pat-1-114 mei4*-null double mutant strain for RNA preparation for use in northern blot analysis. For genomic Southern analysis, we used the TP4-1D (*h⁺ his2⁻ leu1-32 ura4-D18 ade6-M216*) strain for total DNA preparation. The yeast cells were grown in a standard rich medium (YPD or YEA) or in a synthetic medium (EMM2) (9). For induction of mating and meiosis, cells were cultured in EMM-N medium (9,10).

Preparation of the subtracted cDNA library

CD16-1 cells were directed to meiosis as described above and collected at 1 h intervals (1, 2, 3, 4, 5 and 6 h). For mRNA preparation, cells were mixed and disrupted by glass beads in the presence of 5.5 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% sodium lauryl sarcosinate and 0.2 M 2-mercaptoethanol. From this mixture, mRNA was prepared and the cDNA library was constructed by a linker-primer method using the pAP3neo vector as described previously (4). This cDNA library was changed to the single-stranded DNA form with the aid of the R408 helper phage. To isolate recombinant phage, the bacteria were removed by two rounds of centrifugation at 17 000 *g* at 4°C for 15 min, then cleared through a 0.22 µm sterile filter. To remove contaminating *Escherichia coli* DNA, 25 ml of the supernatant was incubated at room temperature for 3 h with 10 U/ml of DNase I. Subsequently, the phage was precipitated by adjusting the solution to 4% polyethylene glycol and 0.5 M NaCl. After incubation for 20 min at room temperature, the mixture was centrifuged at 17 000 *g* at 4°C, and the pellet was resuspended in 400 µl of TE (10 mM Tris–HCl pH 7.5, 1 mM EDTA). To obtain phage DNA, the phage protein was digested with 50 µg of proteinase K by incubation at 42°C for 1 h in the presence of 0.1% SDS. The single-stranded phage DNA was extracted by phenol/chloroform three times and the ethanol-precipitated DNA was pelleted and dissolved in 20 µl of TE and stored at –20°C before use.

CD16-5 cells were also directed to meiosis, and poly(A)⁺ RNA (mRNA) was extracted from cells collected at 1 h intervals (0, 1, 2, 3, 4, 5, 6, 7 and 8 h). mRNA (10 µg) was dissolved in 20 µl of H_2O and mixed with 30 µl of a 1 µg/µl solution of Photoprobe biotin (VECTOR Laboratories, Burlingame). To label the mRNA with biotin, the solution was irradiated on ice for 20 min using a sun lamp at a height of 10 cm. Then 50 µl of 0.1 M Tris–HCl pH9.5/1 mM EDTA was added, and the solution was extracted three times with water-saturated 2-butanol and twice with chloroform. After ethanol precipitation, the mRNA pellet was resuspended in 20 µl of H_2O. To increase the density of the biotin residues, the biotinylation step was repeated twice. This photobiotinylated RNA (5 µg) and 1 µg single-stranded DNA were mixed in a hybridization mixture containing 25 µl 40% formamide, 50 mM HEPES pH 7.5, 1 mM EDTA, 0.1% SDS and 0.2 M NaCl. To prevent non-specific hybridization between regions of poly(A) in the single-stranded DNA and the biotinylated mRNA, 1 µg of polyadenylic acid (Pharmacia) was also added. The reaction mixture was placed in a FUNA-PCR tube (Funakoshi, Tokyo), heated at 65°C for 10 min, then incubated at 42°C for 48 h.

After hybridization, the biotinylated RNA and DNA hybrids were removed using 10 µg of streptavidin (Gibco BRL) as described previously (4). To change the recovered single-stranded DNA to a double-stranded form, the single-stranded DNA dissolved in 20 µl of H_2O was mixed with 10 µl of the primer oligonucleotide (5′-GGAAGTGTTACTTCTGCTCT-3′; 20 ng/µl). The solution was heated at 65°C for 10 min and incubated at room temperature for 5 min to anneal the oligonucleotide. A primer extension reaction was then performed in 40 µl of extension buffer containing the annealing reaction product, 20 mM Tris–HCl pH 8.5, 10 mM $MgCl_2$, 250 µM dATP, dTTP, dGTP and dCTP, and 4 U of *Bca*BEST DNA polymerase (TaKaRa). The reaction was stopped by heating the mixture at 65°C for 1 h, and the products were subjected to electroporation into the MC1061A strain of *E.coli,* according to the protocol we reported previously, to attain a maximum transformation efficiency (11).

Northern blot and genomic Southern analyses

To obtain RNA from cells in meiosis, CD16-1 and CD16-5 cells were shaken at 30°C in EMM2 medium containing nitrogen until they reached log phase (1×10^7cells/ml) and then transferred into EMM2 medium without nitrogen and incubated under the same conditions. Cells were collected at 2 h intervals (for CD16-1, 0–12 h; for CD16-5, 0–10 h), mixed with 10% SDS, phenol/chloroform and RNA extraction buffer (9), and disrupted by glass beads (ϕ = 0.5 mm). The samples were then centrifuged and the supernatant was treated sequentially with phenol/chloroform and chloroform before precipitation with ethanol. The precipitate was dissolved in H_2O and again precipitated in the presence of 2 M LiCl to obtain RNA for northern blot analysis, which was performed as described (12). The DNA fragments containing the protein coding sequence (CDS) of *mei4⁺*(13), *rep1⁺* (14) and *aro3⁺* (15) to be used for probes were generated by PCR.

The *pat-1-114* mutant and *pat-1-114 mei4*-null double mutant were shaken at 24°C in YEA medium containing nitrogen until log phase (1×10^7cells/ml). The cells were then transferred into EMM2 medium without nitrogen at 24°C and incubated further under the same conditions to arrest the cell cycle at the G_1 phase. Shifting the incubation temperature to 34°C induced meiosis that proceeded in a synchronous fashion.

To prepare the genomic DNA, TP4-1D cells were shaken at 30°C in YEA medium until log phase. Cells were collected and mixed with 10% SDS, phenol/chloroform and DNA extraction buffer (9), and disrupted by glass beads. The DNA was digested with *Ban*III, *Hin*cII and *Msp*I restriction enzymes to perform Southern blot analysis (12).

RESULTS

Preparation of a subtracted cDNA library enriched in meiosis- or sporulation-specific cDNA species

In order to perform a large-scale isolation of meiosis- or sporulation-specific genes of *S.pombe*, we employed a strategy to

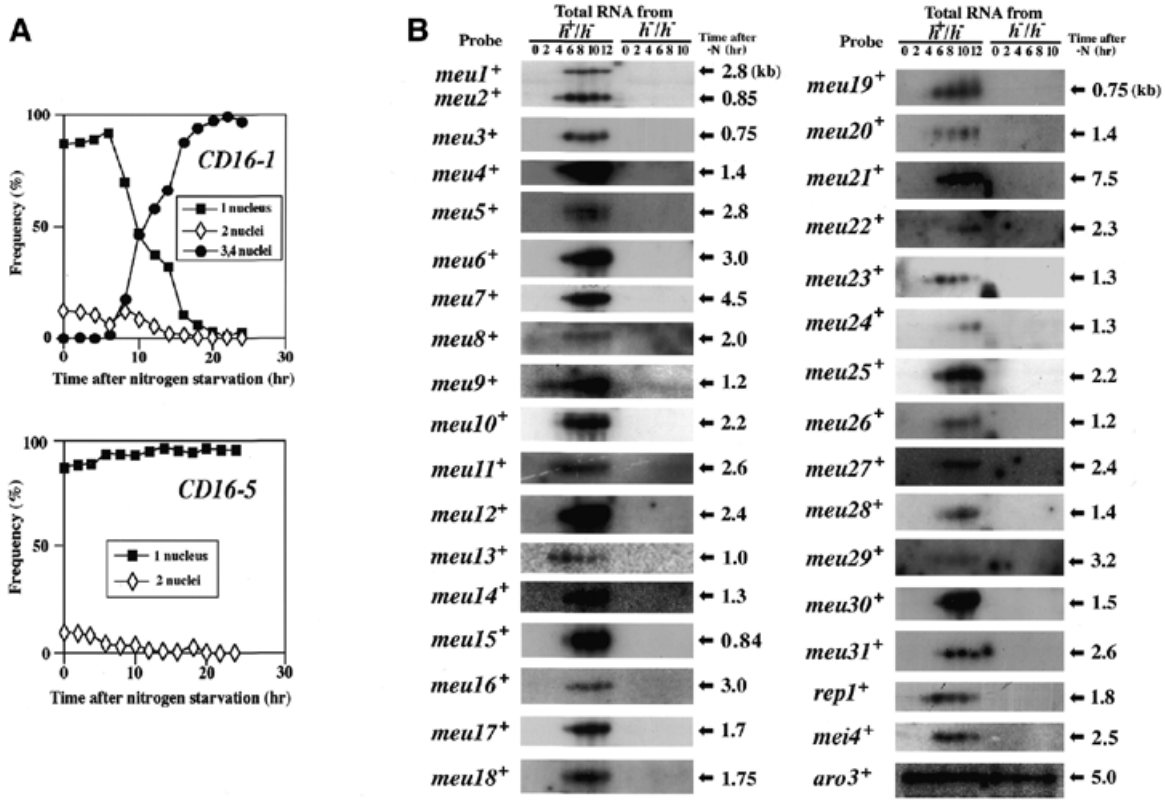


Figure 1. Isolation of the *S.pombe* genes from a subtracted cDNA library whose expression is induced during the meiosis and sporulation processes. (**A**) Frequency of cells with one, two, three or four nuclei as detected by staining with Hoechst33342. CD16-1 (h^+/h^-) and CD16-5 (h^-/h^-) cells were induced to enter meiosis by nitrogen starvation, and the population of cells with one, two, three or four nuclei were counted using a microscope after Hoechst33342 staining. (**B**) Northern blot analysis on isolated *meu* genes. Cells were collected for RNA preparation at 2 h intervals after nitrogen starvation. An aliquot of 10 µg of each RNA was electrophoresed in a 1% agarose gel and transferred to a nylon membrane. From each isolated plasmid, the cDNA insert was excised by *Eco*RI–*Not*I restriction enzymes, radiolabeled by [α-^{32}P]dCTP, and used as a probe. DNA fragments containing the *rep1*$^+$ and *mei4*$^+$ genes, which are known to behave as a *meu* gene, were also used as positive controls of meiosis-specific genes. The amount of loaded RNA was monitored by probing with the ^{32}P-labeled *aro3*$^+$ gene. Sizes of the transcripts are also shown in kb.

enrich the mRNA species induced during the meiotic or sporulation processes in a subtracted cDNA library. We took advantage of the fact that heterozygous diploid cells of distinct mating types (h^+/h^-) can be induced to initiate meiosis after nitrogen starvation, whereas homozygous diploid cells with the same mating type (h^-/h^-) never proceed to meiosis. Thus, we used two kinds of diploid strains, CD16-1 and CD16-5 (16) for subtraction. Upon nitrogen starvation, the heterozygous CD16-1 strain initiates meiosis, while the homozygous CD16-5 strain cannot proceed to meiosis. We confirmed this by counting the frequency of cells carrying one to four nuclei by staining the cells with Hoechst33342 (Fig. 1A).

We first prepared mRNA from CD16-1 cells that were collected at 1 h intervals (1–6 h) and pooled from the sporulation medium following nitrogen starvation. Using this mRNA sample, we prepared a cDNA library of 1.8×10^6 c.f.u. with an average insert size of 1.5 kb in a pAP3neo vector, which was designed to be converted to a single-stranded form by transfection with f1 helper phage (4). The number of independent clones in the original cDNA library was considered to be large enough to minimize the loss of the desired clones. In parallel with these experiments, we prepared mRNA from CD16-5 cells that were collected at 1 h intervals (0–8 h) after nitrogen starvation and pooled before mRNA preparation. This mRNA was labeled with biotin using the photobiotin system, and then mixed in excess in the hybridization buffer with the single-stranded form of the cDNA library from CD16-1 cells. After two rounds of subtractive hybridization, the single-stranded form of the subtracted cDNA library was converted to the double-stranded form by *Bca*BEST DNA polymerase (TaKaRa Shuzo, Japan) and transfected *E.coli* (MC1061A strain) by electroporation according to a protocol that maximizes the efficiency of transformation (11). Thus, we constructed a subtracted cDNA library of 1.2×10^4 c.f.u. (average insert size = 1.45 kb). The number of independent clones in the subtracted cDNA library was again judged to be large enough to cover almost all of the clones whose expression would be increased in CD16-1 cells after nitrogen starvation.

Isolation of meiosis- or sporulation-specific genes

The quality of this subtracted cDNA library was estimated as follows. First, we prepared plasmid DNA from randomly selected clones from the library, and investigated the size distribution of the cDNA inserts by digesting them with *Eco*RI and *Not*I restriction enzymes, whose digestion sites were situated in the multicloning site of the pAP3neo vector. Examination of their sizes by agarose gel electrophoresis revealed

that almost all of the plasmids contained cDNA inserts >100 bp, and that their size distribution was variable among the cDNA clones examined. The result suggested that the subtracted cDNA library contained sufficiently diverse clones to cover most of the cDNA species we desired to isolate. Therefore, we subjected this subtracted cDNA library to further analysis.

Next we cut cDNA inserts longer than 300 bp with *Eco*RI–*Not*I, purified the fragments by agarose gel electrophoresis, radiolabeled them with [α-^{32}P]dCTP and performed northern blot analyses (see Materials and Methods). In the northern blots, RNA samples taken at 2 h intervals from both CD16-1 and CD16-5 cells during the time course of incubation in the sporulation medium were loaded in each lane as shown in Figure 1B. By northern blot analysis we expected to detect the cDNA clones from the subtracted cDNA library whose expression would be induced in CD16-1 cells during the time course of meiosis, but not in CD16-5 cells. We also sequenced ~1 kb of DNA from the 5′ ends of the clones and found that most of the cDNA clones possessed distinct DNA sequences; this suggested that the redundancy of the cDNA component was very small.

We performed northern blot analyses of several hundred clones that were randomly isolated from the cDNA library. We found that the intensity of the bands for 80 clones displayed a band pattern whose intensity was abruptly increased at a certain stage of the meiotic process in CD16-1 cells; no band was observed before the induction of meiosis at 0 h. Since cells at 0 h are in the mitotic phase, the absence of a band implies that these genes are not expressed at the mitotic phase of the cell cycle, i.e., they are expressed in a meiosis-specific manner. In contrast, no band was detected throughout the time course of meiosis in CD16-5 cells, indicating that the bands appearing in CD16-1 cells are not due to nitrogen starvation but caused by progression into meiosis. We named these cDNA clones *meu* after meiotic expression upregulated. The DNA sequences of these cDNA clones revealed that they were independent clones except for the *meu1*$^+$*/meu2*$^+$, *meu3*$^+$ and *meu4*$^+$ cDNAs, which were isolated twice, three times and seven times, respectively. Thus, we isolated 31 types of *meu* cDNA clones.

Some *meu* genes encode identical or homologous proteins

Homology searches for these *meu* genes in the non-redundant database using the BLAST network service (http://www.genome.ad.jp/) revealed that most of them were uncharacterized novel genes. However, some matched previously identified genes, such as *meu4*$^+$, *meu12*$^+$, *meu21*$^+$, *meu28*$^+$ and *meu30*$^+$, which were identical to *isp3*$^+$, *ght6*$^+$, *bgs2*$^+$, *spn5*$^+$ and *mde5*$^+$, respectively (Table 1). The *isp3*$^+$ gene was previously isolated using a similar strategy, and the *isp3*-null mutant is partially defective for spore formation (3). *ght6*$^+$ codes for a putative hexose transporter (SPCC1235.13) with close similarity to the Ght5 protein. Bgs2, a putative 1,3-β-glucan synthase component, was identified from the database as a homolog of Drc1p/Cps1p, which is essential for the assembly of the septum during division (17). The spores resulting from meiosis of a *bgs2*-null mutant lyse upon release from the ascus and become inviable. *spn5*$^+$ encodes a septin homolog of the budding yeast. In *S.cerevisiae*, it has been reported that certain septin proteins are essential for sporulation (18,19). *mde5*$^+$ encodes the α-amylase precursor that is involved in the regulation of the late stage of meiosis/sporulation (20).

Other *meu* gene products were similar, but not identical, to known proteins. The gene product of *meu7*$^+$ is a protein similar to Meu30/Mde5 (data not shown). The gene products of *meu8*$^+$, *meu17*$^+$ and *meu22*$^+$ showed partial homology to enzymes involved in cellular metabolism: betaine aldehyde dehydrogenase precursor, glucoamylase precursor and amino acid permease, respectively (Table 1). *meu5*$^+$ is identical to SPAC1610.03c, which encodes a putative RNA binding protein (possibly the polyadenylase). *meu10*$^+$ encodes a protein similar to Sps2 in *S.cerevisiae*. Although a *sps2*-null mutant showed a normal phenotype in *S.cerevisiae*, the spores resulting from meiosis after overexpression of *SPS2* were reported to show an abnormal phenotype (21,22). Meu13, encoded by *meu13*$^+$, is a homolog of Hop2 of *S.cerevisiae*, which is required for pairing of homologous chromosomes in meiosis (23). *meu17*$^+$ encodes the glucoamylase precursor. One Meu17-like protein (SPAC4H3.03c) is found in *S.pombe*, and three [Sga1p, sporulation-specific glucoamylase (M16166) and intercellular glucoamylase (X13858)] are found in *S.cerevisiae*. *meu26*$^+$ encodes a protein identical to SPAC6B12.16, whose GFP fusion construct is localized in the nuclei of living cells (24).

Some *meu* genes encode uncharacterized proteins

The other 17 *meu* genes encode uncharacterized proteins with unknown functions. DNA sequencing of cDNAs and the corresponding genomic region revealed that the *meu1*$^+$ and *meu2*$^+$ genes are derived from the same genomic region. This region generates two kinds of transcripts that were detected as two bands in a northern blot, as shown schematically in Fig. 2A. Both genes contain an intron at the same location. Transcription of *meu2*$^+$ preceded *meu1*$^+$ by ~2 h, and *meu2*$^+$ displayed a decreased intensity after its peak. This suggests that their expression may play an important role in the modulation of the meiotic process, especially during the early phase (Fig. 1B). Northern blot analysis using four DNA fragments (a–d) in the vicinity of this genomic region indicated that the 2.9 kb band is detected with probes b and c, whereas a 0.8 kb band is detected with probe c alone. The result confirmed that these genes are actually derived from the same genomic region. The Meu1 protein is identical to SPAC1556.06, which encodes a coiled-coil protein of unknown function with a bipartite nuclear localization signal (NLS) and a leucine zipper motif (Fig. 2C). Meu2 protein lacks the coiled-coil and leucine zipper motifs, but retains an NLS at the C-terminus.

meu6$^+$ encodes a lysine-rich protein (SPBC428.07) with a bipartite NLS and a coiled-coil motif. *meu9*$^+$ codes for a protein (SPBC16A3.13) homologous to a hypothetical protein (SPCC1281.08) encoded by *meu24*$^+$, both of which belong to the Wtf family of proteins. Wtf proteins are the gene products of a putative transposable element found in *S.pombe* containing KVTAVFLAQCV repeats as shown in the data bank (The Sanger Centre). *meu14*$^+$ encodes an uncharacterized protein with a coiled-coil motif, whereas *meu15*$^+$ encodes an unknown protein with two NLS motifs. *meu18*$^+$ is a *S.pombe*-specific gene encoding a putative membrane protein carrying two NLSs and a coiled-coil motif. *meu23*$^+$ encodes a protein with a coiled-coil motif, and five other Meu23-like proteins (SPCC330.04cp, SPCC569.8, SPBC337.02c, SPBC106.08c

Table 1. Characterization of *meu* genes

Name	Cosmid	Gene Product	A.A.	Size (bp)	NLS	C-C	TM	H.R.[a]	A.N.	*S.p.*	*S.c.*	*A.t.*	*C.e.*	*D.m.*	*M.m.*	*H.s.*	Others
meu1+	SPAC1556.06	H.P.	776	2.9	+	+		11232 →11430	AB017034								
meu2+	SPAC1556.06	Truncated form of Meu1	60	0.8	+			11232 →11430	AB017033								
meu3+	SPCC1884	Putative non-coding RNA	x[b]	0.85				9595 → 8852	AB020594								
meu4+	SPAC1F8.05	=Isp3	182	1.6	+			15333 →16185	D14060								
meu5+	SPAC1610.03c	Putative RNA-binding polyadenylase	727	4.0	+			3199 →2545	AB054316	+	+	+	+	+	+	+	+
meu6+	SPBC428.07	H.P.	651	1.4	+	+		14731 →15554	AB054317								
meu7+	SPBC16A3.13	Similar to alpha-amylase precursor	774	2.3	+		+	14674 →14069	AB054318	+(6)							+
meu8+	SPCC550.10	Betain aldehyde dehydrogenase precursor	500	2.0				20747 →21447	AB054529	+	+	+	+	+	+	+	+
meu9+	SPCC548.02c	WTF11 psudogene	x[b]	2.0				2886 →2591	AB054530								
meu10+	SPCC1223.12c	Putative cell wall biogenesis protein	416	2.2			+	35408 →34704	AB017617	+	+(4)						
meu11+	SPBC18H10.04c	Putative non-coding RNA	x[b]	3.0				10576 →10163	AB054531								
meu12+	SPCC1235.13	Hexose transporter	535	4.4			+	30574 → 32074	AB054532	+	+	+	+	+	+	+	+
meu13+	SPAC222.15	*S.cervisiae* HOP2 homologue	216	1.0		+		36046 → 34961	AB017038		+	+			+	+	
meu14+	SPBC1347.03	H.P.	335	1.3		+		5360 →6156	AB016983	+(2)	+(2)						
meu15+	PJ732	H.P.	150	0.84	+		+	5597 →6663	AB054299								
meu16+	SPAC15A10	Putative non-coding RNA	x[b]	2.5				23807 → 23178	AB054300								
meu17+	SPBC14C8.5c	Glucoamylase precusor	450	1.2	+		+	9186 →8218	AB054301	+	+						+
meu18+	SPBC409.11	H.P.	553	4.0	+	+	+	24865 →25374	AB054302								
meu19+	SPCC569	Putative non-coding RNA	x[b]	0.85				6657 → 7405	AB054303								
meu20+	SPCC4F11	Putative non-coding RNA	x[b]	1.4				8882 → 8278	AB054304								
meu21+	SPAC24C9.07c	=Bgs2	1894	5.8	+	+	+	15429 →14833	AB054305	+(3)	+(3)	+(14)					
meu22+	SPBC19F8.06c	Amino acid permease	574	3.0			+	11569 → 10656	AB054306	+	+	+	+	+	+	+	+
meu23+	SPCC613.11c	H.P.	254	1.1		+		19696 →18775	AB054307	+(5)							
meu24+	SPCC1281.08	Hypothetical wtf11 protein	349	1.7			+	21991 → 22364	AB054308								
meu25+	SPBC27.03	Low similarity to YJL020C	622	4.1				3485 → 4289	AB054309								
meu26+	SPAC6B12.16	H.P.	344	2.0				36055 → 36199	AB054310								
meu27+	SPCC1259.14c	H.P.	736	4.0	+			30017 →29025	AB054311	+(5)							
meu28+	SPAC24C9.15c	=Spn5 =Mde9 ; septin homolog	464	1.2	+	+		36381 →35688	AB054312	+(6)	+(7)		+(2)	+(5)	+(8)	+(10)	+
meu29+	SPAC25H1.05	H.P.	217	2.0			+	6491 →6859	AB054313								
meu30+	SPAC4A8.01	= Mde5 ;Alpha-amylase precursor	514	1.2			+	787 → 1840	AB054314	+(6)							+
meu31+	SPAC1A6.06c	H.P.	185	1.2			+	15202 →14324	AB054315								

Meu proteins found in *S.pombe* itself and in other organisms identified by homology searches are also indicated. Numbers beside the plus signs indicate that such numbers of homologous proteins are found in each organism. For homology searches, we used BLAST network service (http://www.genome.ad.jp), the FASTA network service (http://fasta.genome.ad.jp/) and the PomPD database (http://www.proteome.com/databases/index.html). For motif searches, we used the PSORT II sever program (http://psort.ims.u-tokyo.ac.jp/). x[b] signifies that no apparent CDS is found in the gene product. A.A., amino acids; NLS, nuclear localization signal; C-C, coiled-coil; TM, transmembrane, H.R., homologous region; A.N., accession number; *S.p.*, *S.pombe*; *S.c.*, *S.cerevisiae*, *A.t.*, *A.thaliana*; *C.e.*, *C.elegans*; *D.m.*, *D.melanogaster*; *M.m.*, *M.musculus*; *H.s.*, *H.sapiens*.

[a]Numbers represent those registered in the data bank (The Sanger Centre).

and SPCC569.06) are found in the *S.pombe* genome. *meu27*+ encodes a protein identical to SPCC1259.14c with one NLS motif, and five kinds of Meu27-like proteins (SPAC11G7.06c, SPAC4G9.07, SPAC10F6.15, SPCC737.04 and SPBC1861.06c.) are found in the *S.pombe* genome. It is notable that Meu23- and Meu27-like proteins are not found in the genomes of other organisms. *meu29*+ encodes a putative membrane protein with a signal sequence of 21 amino acids at the N-terminus end. *meu31*+ encodes a putative membrane protein identical to SPAC1A6.06c with an ER-retention signal at the C-terminus.

Conservation of *meu* genes among species

A BLAST search for the genes conserved in other organisms revealed that many *meu* genes encode *S.pombe*-specific proteins (Table 1). They are *meu1*+, *meu2*+, *meu4*+, *meu6*+, *meu15*+, *meu18*+, *meu23*+, *meu24*+, *meu25*+, *meu26*+, *meu27*+, *meu29*+ and *meu31*+. Among these genes, *meu23*+ and *meu27*+ genes exist as duplicates in the *S.pombe* genome. These genes are not found in the genome of another yeast, *S.cerevisiae,* indicating that meiosis- or sporulation-related genes are highly species-specific, being distinct even between these two closely related yeast species. In contrast, *meu10*+ and *meu14*+ are found only in the genomes of *S.pombe* and *S.cerevisiae*, suggesting that they are yeast-specific. *meu17*+-like genes are also found in other fungi but not in other organisms. Meu13-homologous proteins are found in *S.cerevisiae* (Hop2), *Mus musculus* (TBP-1 interacting protein), *Homo sapiens* (TBP-1 interacting protein) and *Arabidopsis thaliana* (AC011810).

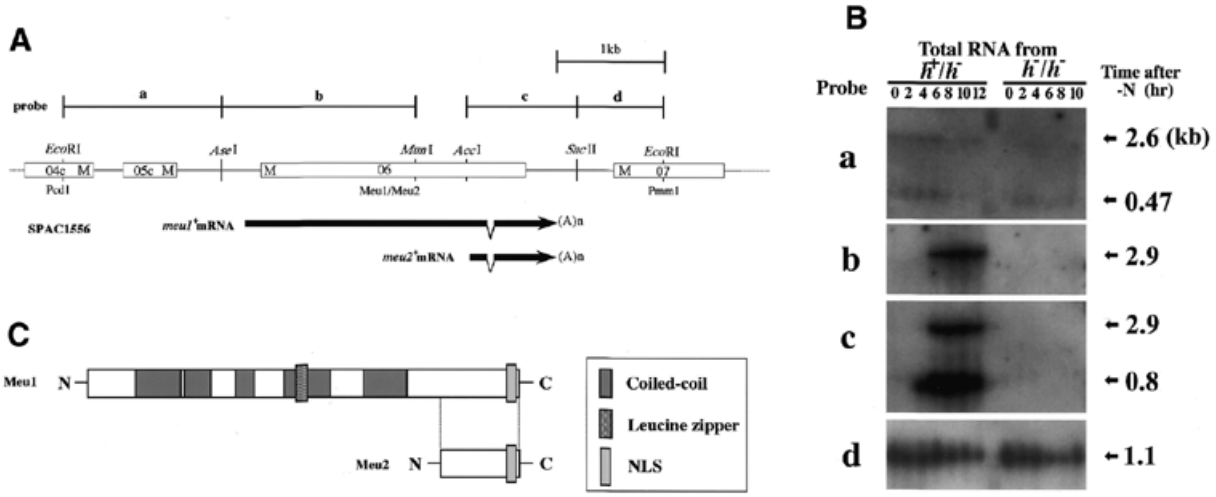


Figure 2. The *meu1*$^+$ and *meu2*$^+$ genes are transcribed from the same genomic locus. (**A**) Genomic structure of *meu1*$^+$ and *meu2*$^+$ genes and the surrounding regions in the *S.pombe* genome. Complementary DNAs (or mRNAs) for *meu1*$^+$ and *meu2*$^+$ are shown by horizontal arrows. The location of the poly(A) tail is denoted by (A)n. The sites for the restriction enzymes used to dissect the genomic DNA to create probes (a–d) for northern blots are shown below. The location and direction of other potential CDS in the vicinity of the *meu1*$^+$ and *meu2*$^+$ genes as determined from a sequence database (The Sanger Centre) are denoted by rectangles with the initiation methionine site (M) indicated. The cDNA clones obtained by colony hybridizations are shown by filled arrows, with a splicing site indicated. (**B**) Northern blot analysis for total RNA from CD16-1 (h^+/h^-) and CD16-5 (h^-/h^-) cells during meiosis after nitrogen starvation with the DNA fragments (a–d) as probes. The 0.8 kb band from the *meu2*$^+$ gene was detected only when probe c was used, indicating that *meu2*$^+$ cDNA is not an artifactual partial clone of *meu1*$^+$ cDNA, but is derived from a bona fide transcript of the *meu2*$^+$ gene. (**C**) Conserved structural motifs found in Meu1 and Meu2 proteins. Meu2 lacks the leucine zipper and coiled-coil motifs found in the N-terminus domain of Meu1, but retains an NLS at the C-terminus.

However, no Meu13-homolog is found in the whole genomic sequences of *Caenorhabditis elegans* and *Drosophila melanogaster*. These results indicate that many meiosis-specific genes have limited specificity among species, which may relate to the differences in fertility among species.

Five *meu* genes may generate non-coding RNAs

The transcripts from the *meu3*$^+$, *meu11*$^+$ and *meu19*$^+$ genes are small and possess no significant CDS (Fig. 3A). Although the size of the transcripts from *meu16*$^+$ and *meu20*$^+$ are large enough to encode proteins (Fig. 1B), they also harbor no apparent CDS (Fig. 3A). These results suggest that these genes do not code for proteins but rather generate non-coding RNA species. Using Zuker's computer program (25), with parameters in the algorithm presented in Jaeger *et al.* (26), we determined that the RNA transcribed from these genes forms stable hairpin structures, which also supports the idea that the gene products are RNA molecules (Fig. 3B). It is of note that 457 nt from the poly(A) site of the *meu16*$^+$ cDNA overlap the C-terminus region of the Mde6 CDS, indicating that these two genes are expressed in an overlapping manner. Such overlapped transcripts in the opposite strands of *spo6*$^+$ (27) and in *rec7*$^+$ (28) have been reported recently, but their physiological roles remain elusive.

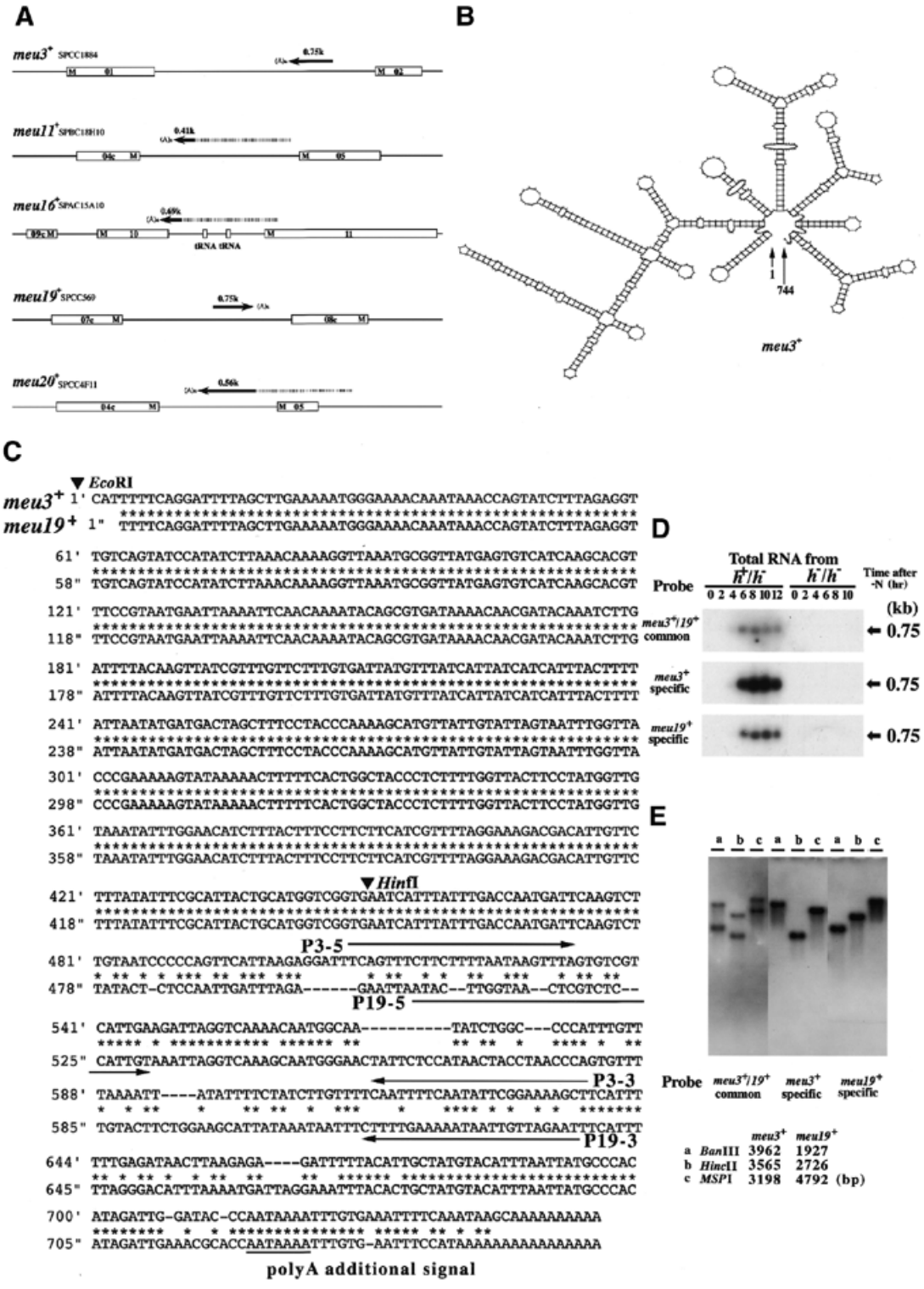
DNA sequencing showed that *meu3*$^+$ and *meu19*$^+$ are twin genes. Notably, the nucleotide sequences at the 5′ half of the molecules are identical, whereas those of the 3′ portion differ (Fig. 3C). We examined whether *meu3*$^+$ and *meu19*$^+$ cDNAs are derived from an alternative splicing or from distinct genomic regions, using genomic Southern blots digested with three kinds of restriction enzymes. A *meu3*$^+$/*meu19*$^+$ common probe prepared by digestion with restriction enzymes (*Eco*RI/*Hin*fI) detected two bands (Fig. 3D), whereas each set of bands was separately detected by the *meu3*$^+$- or *meu19*$^+$-specific probe

Figure 3. Some *meu* genes may generate non-coding RNAs. (**A**) The locations and the directions of the transcripts from *meu3*$^+$, *meu11*$^+$, *meu16*$^+$, *meu19*$^+$ and *meu20*$^+$ genes in the *S.pombe* genome. The location of the poly(A) tail is denoted by (A)n. The location and direction of other potential CDS as determined by sequence database (The Sanger Centre) are also shown by rectangles with the initiation methionine site (M) indicated. (**B**) Putative secondary structure of *meu3*$^+$ RNA *in silico*, in which the general algorithm for determination of the optimal and suboptimal secondary structures (25) and the folding parameters in the algorithm (26) are employed. The calculated free energy is –142.5 kcal/mol. (**C**) Alignment of *meu3*$^+$ and *meu19*$^+$ cDNA sequences to show their close homology. The sites for *Eco*RI and *Hin*fI restriction enzymes used to cut out the DNA fragment to be ^{32}P-labeled for the common probe are shown by vertical arrowheads. The location of a set of oligonucleotides used for PCR to generate the *meu3*$^+$- or *meu19*$^+$-specific probe is denoted by P3-5/P3-3 or P19-5/P19-3, respectively. (**D**) Genomic Southern blot analysis for the whole genome of *S.pombe* with either a *meu3*$^+$- or *meu19*$^+$-specific probe showed distinct band patterns, whereas the common probe detected both bands for all the restriction enzymes used. This indicates that *meu3*$^+$ and *meu19*$^+$ genes are in distinct locations of the *S.pombe* genome, as predicted from the genomic DNA sequencing. (**E**) Both the common probe, *meu3*$^+$- and *meu19*$^+$-specific probe detected a similar profile in the northern blots, indicating that these bands are not derived from cross-hybridization and that both the *meu3*$^+$ and *meu19*$^+$ genes are transcribed in a meiosis/sporulation-specific manner under similar transcriptional control.

that was generated by PCR. The result indicates that *meu3*$^+$ and *meu19*$^+$ are located in distinct genomic regions. It also indicates that there are no other *meu3*$^+$/*meu19*$^+$-like genes in *S.pombe*. Northern blot analysis with the *meu3*$^+$- or *meu19*$^+$-specific probe also showed a similar expression pattern as obtained by the common probe (Fig. 3E) indicating that both genes are separately expressed in a meiosis-specific manner under similar transcriptional regulation.

Expression of some *meu* genes depends on *mei4*$^+$

Mei4 is a transcription factor that regulates the expression of the genes functioning after commitment to meiosis in *S.pombe*



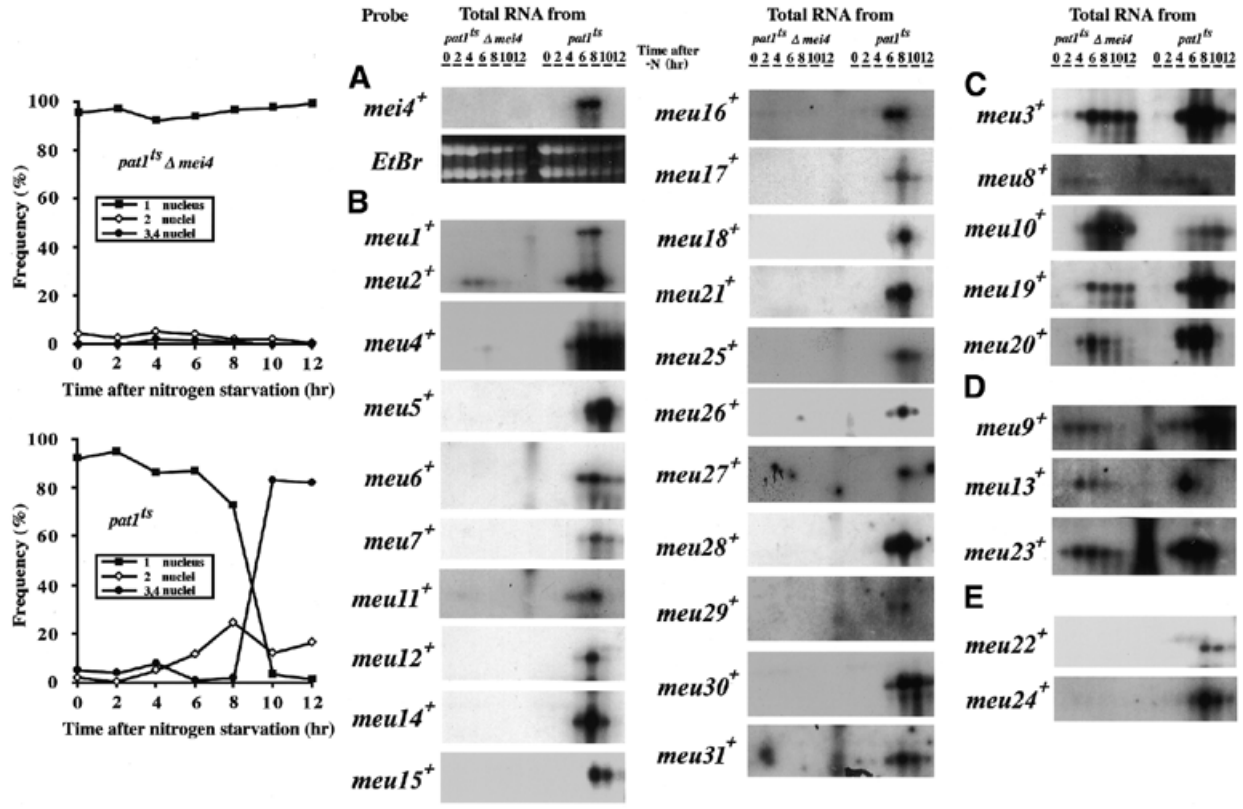


Figure 4. Identification of *mei4*$^+$-dependency for the expression of the *meu* genes by northern blot analysis. A temperature sensitive mutant, *pat-1-114*, was employed to attain a sharp synchronization for meiosis that was performed by shifting the incubation temperature. (**A**) Northern blots for *mei4*$^+$, whose expression is known to be under its own control. Ethidium bromide staining of the RNA was also conducted to show the loading amount of RNA in each lane. (**B**) Northern blots for the medial-onset *meu* genes whose expression was absent in the *mei4*-disrupted cells during meiosis, demonstrating that their expression is *mei4*$^+$-dependent. (**C**) Northern blots for the medial-onset *meu* genes whose expression was also observed in the *mei4*-disrupted cells. Nonetheless, the variation of the band intensity was observed by deletion of *mei4*$^+$ indicating that their expression is partly regulated by Mei4 transcription factor. (**D**) Northern blots for the early-onset *meu* genes, which were also expressed in the *mei4*-disrupted cells. (**E**) Northern blots for the late-onset *meu* genes, which are not expressed in the *mei4*-disrupted cells probably because they cannot proceed after prophase I. To collect RNA, the *pat-1-114 mei4* and *pat-1-114* cells were collected for RNA preparation at 2 h intervals after the temperature shift. Each RNA (10 µg) was electrophoresed in a 1% agarose gel and transferred to a nylon membrane. From each isolated plasmid, the cDNA insert was excised by *Eco*RI–*Not*I restriction enzymes, radiolabeled by [α-^{32}P]dCTP and used as a probe. DNA fragments containing *mei4*$^+$ were also used as a negative control for *mei4* disruption.

(13). Expression of *mei4*$^+$ can be detected at 6–10 h and is diminished at 12 h after nitrogen starvation in the CD16-1 strain (Fig. 1B). Thus, the transcriptional target genes of Mei4 or genes downstream of Mei4 regulation are expected to be transcribed after this time. To classify the isolated *meu* genes in view of Mei4 dependency, we examined the expression pattern of *meu* genes by northern blot analysis using RNA prepared from the *mei4*-disrupted strain (Fig. 4). In these experiments, we employed a temperature-sensitive mutant, *pat1* (8), instead of the wild-type strain in order to attain a better synchronization of meiosis. Expression of *mei4*$^+$ can be detected at 6–8 h but the refined synchronization enabled us to see it disappear at 10–12 h in the *pat*^{ts} strain (Fig. 4A).

Many of the medial-onset *meu* genes showed no expression in the *mei4pat1* strain alone indicating that their expression is Mei4-dependent (Fig. 4B). It is of note that the expression of *meu1*$^+$ is Mei4-dependent, but the expression of *meu2*$^+$ is not, suggesting that they have distinct roles in the regulation of meiosis despite the identity of their amino acid sequence at the C-terminus (Fig. 2A). Expression of other medial-onset *meu* genes is Mei4-independent, although the variation in the intensity of their bands indicates that they are also influenced by deletion of Mei4, which suggests that their expression is partially regulated by Mei4 (Fig. 4C). As expected, the early-onset *meu* genes that are expressed before the appearance of Mei4 showed similar expression patterns in both *mei4pat1* and *pat1* mutants (Fig. 4D). The expression of two late-onset *meu* genes that are usually induced after prophase I disappeared in the *mei4*-disrupted strain (Fig. 4E); this is probably because the *mei4*-disrupted strain cannot complete prophase I.

The Mei4 forkhead domain is known to bind to the FLEX motif, which is composed of 27 nt that contain the heptamer core, GTAAAYA (13). We could detect a FLEX-like sequence in the 5′ upstream region of *meu* genes whose expression was found to be dependent on Mei4, as listed in Table 2. These genes are candidate transcriptional target genes of Mei4. The other Mei4-dependent *meu* genes without the FLEX-like sequence may be located downstream of Mei4 regulation. On

Table 2. List of Mei-dependent *meu* genes and the FLEX-like sequences (denoted by italics) found at the 5′ upstream regions of the indicated *meu* genes

Gene name	FLEX-like element		
	Position	Direction	Sequence
meu 1$^+$	-	-	-
meu 4$^+$	-271 -245	→	AGCATCCACT ***GTAAACA***-AAAAAAAAAC
meu 5$^+$	-75 -59	→	TCATCGAAAT ***GTAAACA***AACAGCGATC
meu 6$^+$	-45 -71	←	TTTTTATGCT ***GTAAACA***AACAAATCGA
meu 7$^+$	-59 -85	←	ATTTTTAGGC ***GTAAACA***AAAGGACTGC
meu 8$^+$	-	-	-
meu12$^+$	-426 -452	←	AACAAATTAT ***GTAAACA***AACAATAAGG
meu14$^+$	-67 -41	←	AGATTTGCCT ***GTAAACA***-GAAGCACAAT
meu15$^+$	-299 -325	←	GTTTGGAAAA ***GTAAATA***ATAAGAGCGA
meu17$^+$	-144 -170	←	TTAAAGGTTT ***GTAAATA***TTACAACTCA
meu18$^+$	-	-	-
meu21$^+$	-949 -923	→	AGGATGGTTG ***GTAAACA***CTTACTAAGT
meu25$^+$	-58 -32	→	ATGATCCATA ***GTAAACA***AACATTCAAC
meu26$^+$	-155 -181	←	CGTAAAAACA ***GTAAACA***AACACACCCA
meu27$^+$	-256 -282	←	AAAGTGTTAA ***GTAAACA***TTTAATATTA
meu28$^+$	-253 -279	←	TGCGAGTCAT ***GTAAACA***AACAAACATT
meu29$^+$	-34 -8	→	GTTACCAAAA ***GTAAACA***ACTACCAGCC
meu30$^+$	-66 -92	←	TTACTTCATT ***GTAAACA***AACAAAAATA
meu31$^+$	-92 -118	←	TAATTAAAAT ***GTAAACA***AATTTCCATC

the other hand, Mei4 may bind to other, unknown, DNA sequences that exist in the 5′ upstream region of these *meu* genes.

DISCUSSION

Comprehensive isolation of meiosis- and sporulation-specific genes

The mechanism by which meiosis occurs is still relatively poorly understood. In order to learn more about its regulation, we isolated a large number of cDNA clones from *S.pombe* whose expression was upregulated during meiosis induced by nitrogen starvation. The isolation of such genes has been performed on the assumption that many such genes may be related to the regulation of meiosis. However, some genes induced by nitrogen starvation may be irrelevant to meiosis. To remove these unwanted genes, we employed a strategy of cDNA subtraction between mating types that respond differently to nitrogen starvation: namely, the heterozygous diploid (h^+/h^-) strain (CD16-1), which can initiate meiosis, and the homozygous diploid (h^-/h^-) strain (CD16-5), which cannot proceed to meiosis. To prevent loss of the desired clones from the cDNA library during the cDNA library preparation and subtractions, we kept the complexity of the cDNA inserts in the library above 10 times the expected number of cDNA species to be isolated. In *S.cerevisiae*, genetic and biochemical analysis has identified about 150 different genes that are specifically expressed during meiosis (29), and microarray experiments have identified about 500 genes that are either weakly or strongly induced during meiosis and sporulation (30). Considering these results, we expected that the number of genes in the *S.pombe* genome would be <10 000, and that there would be <1000 meiosis-specific genes. The average size of the cDNA inserts was also considered to be important for manipulation and subsequent analysis of the isolated clones. With these assumptions in mind, a complexity of 1.8×10^6 c.f.u. for the meiosis-oriented cDNA library (CD16-1) with an average insert size of 1.5 kb, and a complexity of 1.2×10^4 c.f.u. for the meiosis-specific subtracted cDNA library (CD16-1 minus CD16-5) with an average insert size of 1.45 kb were well above the required level. Northern blot analysis using randomly selected cDNA clones from the subtracted cDNA library as probes indicated that the transcription of nearly 10% of the genes in the library was induced following nitrogen starvation, and the intensity of the bands was stronger in the CD16-1 cells than those of the CD16-5 cells (data not shown). DNA sequencing of ~1 kb from the 5′ ends of each isolated cDNA clone indicated that they were derived from distinct genes, suggesting that the redundancy of the cDNA component in this subtracted cDNA library was very small. This equalized property signifies that isolation of such clones can be efficiently performed using this subtracted cDNA library. High scores of complexity and insert sizes imply that we have concentrated a large number of meiosis- or sporulation-specific cDNA clones in our subtracted cDNA library. Sequence homology searches with the isolated *meu* cDNA clones over the entire genome of *S.cerevisiae* revealed that less than half of them have a homolog. Homology searches also indicated that most of these *S.pombe*-specific genes had no homolog in the genomes of other species. These results indicate that the genes involved in meiosis are highly species-specific.

Some *meu* genes encode non-coding RNA

It is noteworthy that some of the *meu* cDNA clones had no apparent CDS. Stable hairpin formation found in the whole molecules of these gene products suggests that these genes may generate non-coding RNA molecules. Meiosis in *S.pombe* is known to be regulated by cooperation of a functional RNA and an RNA binding protein. For example, *mei2*$^+$, an essential gene for the initiation of premeiotic DNA synthesis and meiosis I, encodes an RNA binding protein (31). A polyadenylated RNA species called meiRNA, which suppresses a temperature-sensitive defect of *mei2*$^+$ by overexpression, specifically binds to Mei2 (32). The Mei2–meiRNA complex seems to function only at meiosis I. Another RNA species forming a complex with Mei2 to promote premeiotic DNA synthesis remains to be found. Expression of the functional *mes1*$^+$ product that is essential for meiosis II may also be regulated by meiosis-specific splicing with the aid of Mei2 (33). The putative RNA molecule required for this splicing reaction in association with Mei2 has not been found so far. It remains to be tested whether the RNA molecules generated by the *meu3*$^+$, *meu11*$^+$ and *meu19*$^+$ genes function as association partners of Mei2 in these meiosis-specific events.

meu16$^+$ was found to be transcribed on the strand opposite to, but overlapping with, *mde6*$^+$; it may have the same relationship to a gene encoding SPBC18H10. This result suggests that Meu16 RNA may function as an antisense RNA for these two genes. It has been reported that antisense RNAs may function as repressors of sense RNA. In *C.elegans*, a small non-coding RNA called *let-4* RNA is involved in the determination of developmental timing, and is expressed as an antisense RNA from the 3′ untranslated region of *lin-14* (34). The expression levels of the LIN-14 protein are decreased by *let-4* RNA expression at the end of the first larval stage. On the other hand, the expression of *lin-14* mRNA was unchanged, which implies that the repression of *lin-14* expression occurs after its transcription. In *S.pombe*, it was reported the coding region of

spo6$^+$ expressed a bi-directional transcript (27), and three kinds of transcripts in the opposite strands of *rec7*$^+$ have also been detected (28). Thus, antisense RNA may also play a pivotal role in meiosis in *S.pombe*.

Unusual expression of RNA in meiosis

We reported previously that a meiosis-specific clone of *S.pombe* named *eta1*$^+$ displayed two bands in a northern blot (35). DNA sequencing showed that *dmc1*$^+$ and *rad24*$^+$ genes are juxtaposed in this order in the genome and are co-transcribed to give a bicistronic *eta1*$^+$ mRNA of 2.8 kb. Dmc1 is a member of the RecA family of proteins, which plays a pivotal role in homologous recombination in *E.coli*. Dmc1 proteins from both *S.cerevisiae* (36) and *S.pombe* (35) have been shown to play essential roles in meiotic recombination. Rad24 is known to associate with target proteins via phosphoserine residues and is required for the function of a variety of cell events such as the DNA damage checkpoint (37). Rad24 also functions as an inhibitor in the regulation of the Mei2 protein, which plays an essential role in the progression of meiosis after nitrogen starvation (38). We found by northern blot analysis that another *eta1*$^+$-like cDNA clone in our subtracted cDNA library might also be derived from a putative bicistronic mRNA (data not shown). It remains to be determined whether this kind of unusual RNA expression has any significance in the progression of meiosis in fission yeast or in any other organisms.

Advances in DNA technology have resulted in the development of cDNA microarray technology and DNA chips for the quantitative analysis of gene expression at the mRNA level. DNA chips containing nearly every protein-coding yeast gene were used to assay changes in gene expression during sporulation, and revealed the existence of at least seven distinct temporal patterns of induction (30). Since the genome project of *S.pombe* is about to be completed, DNA chips will be available for the identification of meiosis-specific protein-coding genes in the near future. However, DNA chips cannot analyze the expression pattern of non-coding RNAs. Considering that we could identify many putative non-coding RNAs, overlapped transcription, and the generation of antisense RNA during meiosis of *S.pombe*, we would emphasize that the analysis of transcriptional modes of the genome by isolation of cDNA clones from a subtracted cDNA library is still powerful as an alternative choice in the post-genome era.

In summary, we have successfully constructed a subtracted cDNA library of *S.pombe*, in which meiosis- or sporulation-specific cDNA clones were highly enriched, and isolated a large number of such genes that were comprehensively named as *meu* genes. Our results indicated that some of the *meu* cDNA clones expressed putative non-coding RNAs. Analysis of *meu* genes will shed light on the events occurring during meiosis and sporulation.

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