MOLECULAR AND CELLULAR BIOLOGY, July 1996, p. 3420–3428 Vol. 16, No. 7
0270-7306/96/$04.00+0

Schizosaccharomyces pombe map1$^+$ Encodes a MADS-Box-Family Protein Required for Cell-Type-Specific Gene Expression

NAOYUKI YABANA AND MASAYUKI YAMAMOTO*

Department of Biophysics and Biochemistry, School of Science, University of Tokyo, Hongo, Tokyo 113, Japan

Received 7 December 1995/Returned for modification 16 January 1996/Accepted 11 April 1996

We cloned the *Schizosaccharomyces pombe map1* gene by virtue of its ability to stimulate transcription of the *sxa2* gene, which encodes a carboxypeptidase expressed specifically in h^- cells in response to mating-pheromone signaling. The cloned gene had a coding capacity of 398 amino acids split by two introns, and the deduced product was a protein of the MADS box family. This gene was most similar to *Saccharomyces cerevisiae MCM1*, which regulates cell-type-specific gene expression in budding yeast cells. Disruption of the *S. pombe* gene did not affect vegetative cell growth but conferred sterility. It blocked the mating ability of h^+ cells completely and that of h^- cells partially. Genetic and sequencing analysis indicated that the cloned gene is *map1*, which was originally defined by a mutation that caused h^+-specific sterility. Northern (RNA) blot analysis showed that the function of *map1* is absolutely essential for the expression of h^+-specific genes and is required for the full activation of h^--specific gene expression. Overexpression of *map1* resulted in enhanced transcription of cell-type-specific genes, but the range of genes affected by Map1 was restricted by the mating type of the cell. Results of yeast two-hybrid analysis suggested that Map1 may physically interact with Mat1-Pc, the product of the h^+-specific mating-type gene *mat1-Pc*. On the basis of these observations, we speculate that Map1 may be a transcriptional regulator of cell-type-specific genes similar to *S. cerevisiae* MCM1, whose activity is modulated by the α1 and α2 mating-type gene products.

Cells of the fission yeast *Schizosaccharomyces pombe* initiate sexual development when starved for nutrients, especially a nitrogen source. Haploid cells of the opposite mating types, termed h^+ (*P*) and h^- (*M*), communicate by secreting mating pheromones prior to mating: h^+ cells produce P-factor and the M-factor receptor, while h^- cells produce M-factor and the P-factor receptor, in response to nutritional starvation (17, 37). This cell-type-specific production of pheromones and receptors is observed only upon nutrient deprivation. Indeed, neither diploid- nor haploid-specific gene expression during mitotic growth in *S. pombe* has been reported.

The mating-pheromone response pathway of *S. pombe* provides a model system for the study of signal transduction in eukaryotic cells. This pathway involves a receptor-coupled G-protein system (33, 49, 64) and a mitogen-activated protein (MAP) kinase cascade (18, 44, 46, 65, 67). Mutants of *S. pombe* that exhibit sterility in a mating-type-specific manner have been useful in the identification of factors required for pheromonal communication. Four h^+-specific sterility genes, named *map1* through *map4*, and four h^--specific sterility genes, named *mam1* through *mam4*, have been identified (11, 26, 27a, 65). The *map2* gene encodes a precursor of P-factor, in which four repeats of the mature P-factor sequence are embedded (26). The gene products of *map3* and *mam2* are both putative seven-transmembrane proteins. Genetic evidence indicated that Map3 is the receptor for M-factor and that Mam2 is the receptor for P-factor (33, 64). The *map4* and *mam3* genes appear to encode putative aggulutinins (27a). A defect in these *map* or *mam* genes causes complete sterility in either h^+ or h^- cells. Although not identified by the isolation of sterile mutants, the structural genes for M-factor are also required for mating. M-factor is encoded by three genes, named *mfm1* through *mfm3* (6, 7, 34), and h^- cells become sterile only when all of these genes are knocked out (34). The expression and function of the *mfm* genes is h^- specific (34).

In addition to the *map* and *mam* genes, two other genes are known to affect mating efficiency in a mating-type-specific manner. These genes, namely, *sxa1* and *sxa2*, have been supposed to encode putative proteases that degrade M-factor and P-factor, respectively (27), and it has been demonstrated recently that an *sxa2*-dependent carboxypeptidase removes the C-terminal leucine residue of P-factor (36). The *sxa1* function is required for h^+ cells and the *sxa2* function is required for h^- cells, although disruption of neither *sxa1* nor *sxa2* abolishes the mating ability completely.

Molecular mechanisms that control differential gene expression in *Saccharomyces cerevisiae* **a** and α cells have been determined in detail (23). Transcription of cell-type-specific genes is regulated by the products of the mating-type locus, either *MAT***a** or *MAT*α in haploid *S. cerevisiae* cells. *MAT***a** encodes a single regulatory protein, **a**1, whereas *MAT*α encodes two regulatory proteins, α1 and α2. In α cells, α1 functions as a positive regulator of transcription of α-specific genes, whereas α2 functions as a negative regulator of transcription of **a**-specific genes. In **a** cells, **a**1 does not appear to play a role. Rather, **a**-specific genes are expressed because they are not repressed by α2 and α-specific genes are not expressed because there is no α1 to activate their transcription. **a**1 does have a role: in diploid cells, in conjunction with α2, it serves to repress transcription of haploid-specific genes (19). This regulatory circuitry is known as the α1-α2 model (62).

To carry out their roles as transcription regulators, α1 and α2 must work in conjunction with MCM1 (3, 29, 31), a protein present in all three cell types originally identified by isolation of mutants defective for the maintenance of minichromosomes (39, 51). In **a** cells, MCM1 protein binds to site P in the

* Corresponding author. Mailing address: Dept. of Biophysics and Biochemistry, School of Science, University of Tokyo, P.O. Hongo, Tokyo 113, Japan. Phone: 81-3-3814-9620. Fax: 81-3-5802-2042.

upstream region of **a**-specific genes and activates their transcription, but it exerts no effect on α-specific genes. In α cells, α1 binds to site Q adjacent to defective versions of the P site found in the upstream region of α-specific genes, thereby assisting MCM1 to bind to site P. This results in the activation of transcription of α-specific genes. Also in α cells, α2 binds cooperatively with MCM1 in the upstream region of **a**-specific genes and blocks transcriptional activation of these genes.

Cell-type specificity of *S. pombe* also stems from the mating-type locus on chromosome II, namely, *mat1-P* in h^+ cells and *mat1-M* in h^- cells. *mat1-P* consists of two transcription units, called *mat1-Pc* and *mat1-Pi*(*m*), and *mat1-M* consists of *mat1-Mc* and *mat1-Mi*(*m*) (32). In contrast to *S. cerevisiae*, *S. pombe* initiates sexual development only in the absence of nutrients. Expression of *mat1-Pc* and *mat1-Mc*, which are responsible for the production of mating pheromones and their receptors, is greatly enhanced by nitrogen starvation, an enhancement that requires the function of Ste11 transcription factor (63). Expression of *mat1-Pc* in h^+ cells and expression of *mat1-Mc* in h^- cells are crucial for mating, and the expression is also a prerequisite for meiosis in diploid cells because mating-pheromone signaling is essential for the initiation of meiosis (12, 33, 64, 71). In contrast, expression of *mat1-Pi* and *mat1-Mi* is induced in response to the mating-pheromone signal (47, 69). The expression of these two genes is critical for meiosis but dispensable for mating (71). How *mat1-Pc* and *mat1-Mc* regulate expression of their target genes in a cell-type-specific manner is an intriguing question which remains largely unsolved.

In the course of our attempt to identify new factors that regulate gene expression in response to the mating-pheromone signaling, we encountered a gene that apparently encodes a homolog of *S. cerevisiae MCM1*. Further analysis indicated that this gene is identical to *map1*, which has been known for decades as an h^+-specific sterility gene. Although loss of *map1* function causes complete sterility only in h^+ cells, our analysis indicated that Map1 is involved in the regulation of both h^+- and h^--specific gene expression. Furthermore, yeast two-hybrid analysis suggested a possible physical interaction between Map1 and Mat1-Pc. These observations provide preliminary evidence that analogous strategies may be used to regulate cell-type-specific gene expression in two distantly related yeast species, *S. cerevisiae* and *S. pombe.*

MATERIALS AND METHODS

Fission yeast strains, genetic procedures, and media. The *S. pombe* strains used in this study are listed in Table 1. General genetic procedures for *S. pombe* have been described previously (21). Sterile mutants were crossed by protoplast fusion (60). A lithium method was used for transformation of *S. pombe* (50). A qualitative assay of the mating efficiency was done by staining colonies with iodine vapor, which stains spores dark brown. Yeast media YPD and SD (58), SSA (13) and MM (43), and nitrogen-free MM-N (28) were used. SD, MM, and MM-N used in this study contained only 1% glucose.

Construction of an *sxa2-lacZ* fusion gene and strains carrying it. A 1.5-kb *Pvu*II-*Eco*RI fragment was excised from pST563-1, a plasmid carrying the *sxa2* gene (27), and inserted between the *Hin*cII and *Eco*RI sites of pBluescript KS(+) (Stratagene). A *Bam*HI fragment carrying the *lacZ* gene was isolated from pMC1871 (5) and inserted into the *Bam*HI site of the constructed plasmid. The resultant plasmid, named pFLB1, carried an *sxa2-lacZ* fusion gene, which consisted of a 1.1-kb-long segment carrying the promoter and the noncoding region of *sxa2*, part of the *sxa2* open reading frame (ORF) from the initiation codon to Asn-82, and the *lacZ* ORF connected in frame to the *sxa2* ORF. This *sxa2-lacZ* fusion gene was excised with *Kpn*I and *Xba*I, ligated to *Hin*dIII linkers after its termini were filled in with Klenow enzyme (Takara Shuzo), and then inserted into the *Hin*dIII site of pLU′, a plasmid that was designed to integrate the insert into the *leu1* locus of the *S. pombe* genome (42). A *Sal*I-*Eco*RI fragment carrying the *sxa2-lacZ* fusion gene and a *ura4*$^+$ cassette adjacent to it, the combination of which was flanked by segments of the *leu1* gene, was cut out from the resultant plasmid. *S. pombe* JZ47 (h^{90} *ade6-M216 ura4-D18*) was transformed with this fragment, and Ura$^+$ Leu$^-$ transformants were selected. Successful integration of the *sxa2-lacZ* fusion gene at the *leu1* locus was confirmed by Southern blotting for one of the transformants, which was named JZ925. JZ925 was crossed with a homothallic *spk1*Δ strain (JZ945) to construct an indicator strain for the colony color assay (JX494).

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

Strain	Genotype
JY333	h^- *ade6-M216 leu1*
JY334	h^+ *ade6-M216 leu1*
JY450	h^{90} *ade6-M216 leu1*
JY362	h^+/h^- *ade6-M216/ade6-M210 leu1/leu1*
JY745	h^- *ade6-M216 ura4-D18*
JY746	h^+ *ade6-M216 ura4-D18*
JY878	h^{90} *ade6-M216 leu1 ura4-D18*
JY919	h^{90}/h^{90} *ade6-M210/ade6-M216 leu1/leu1 ura4-D18/ura4-D18*
JZ47	h^{90} *ade6-M216 ura4-D18*
JZ272	h^{90} *ade6-M210 leu1 map1*
JZ925	h^{90} *ade6-M216 ura4-D18 leu1*::[*sxa2-lacZ* + *ura4*$^+$]
JZ945	h^{90} *ade6-M216 leu1 ura4-D18 spk1*::*ura4*$^+$
JX494	h^{90} *ade6-M216 leu1*::[*sxa2-lacZ* + *ura4*$^+$] *spk1*::*ura4*$^+$
JX496	h^- *ade6-M210 leu1 ura4-D18 map1*::*ura4*$^+$
JX498	h^+ *ade6-M210 leu1 ura4-D18 map1*::*ura4*$^+$
JX500	h^{90} *ade6-M216 leu1 ura4-D18 map1*::*ura4*$^+$
JX501	h^{90}/h^{90} *ade6-M216/ade6-M210 leu1/leu1 ura4-D18/ura4-D18 map1*::*ura4*$^+$/*map1*::*ura4*$^+$

Isolation of activators of *sxa2* transcription. A homothallic *spk1*Δ *sxa2-lacZ* strain (JX494) was transformed with an *S. pombe* genomic library that carried *Sau*3AI digests of the chromosomal DNA at the *Bam*HI site of the vector pDB248′ (2). Transformants were grown on SSA plates, and colonies were transferred to nitrocellulose filters. The level of β-galactosidase activity in each colony was assayed by detecting blue color generated by the cleavage of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) as previously described (4). Blue transformants were picked from the master plates, and plasmids were recovered from their crude lysates by transformation into an *Escherichia coli* strain. Plasmids that could activate *sxa2-lacZ* in JX494 upon retransformation were analyzed further. One of them, named pSX56, turned out to carry the *map1* gene.

DNA sequence analysis. A 2.7-kb *Kpn*I-*Eco*RV DNA fragment carried on pSX56 was subcloned into pUC118 in both orientations. Unidirectional deletion of DNA fragments was performed with exonuclease III and S1 nuclease (Takara Shuzo), according to the method of Henikoff (22). Single-stranded template DNA was prepared by using the helper M13KO7 bacteriophage. Nucleotide sequencing was performed by the chain-termination method of Sanger et al. (56). All parts of the sequence shown in Fig. 2 have been determined in both directions at least once. A cDNA clone corresponding to this genomic region was isolated from an *S. pombe* cDNA library constructed in the expression vector pREP3 (41) by complementation of the *map1* disruptant described below.

Gene disruption. For convenience, hereafter we call the gene carried on pSX56 *map1*, although this was not revealed until we performed the following experiments. Disruption of the chromosomal *map1* gene was carried out essentially according to a standard protocol for gene replacement (53). A 0.7-kb *Xho*I-*Bgl*II fragment (Fig. 1) was eliminated from the cloned *map1* ORF, and an *S. pombe ura4*$^+$ cassette (20) was inserted in its place. An *S. pombe* diploid strain, JY919 (h^{90}/h^{90}), was transformed with a *Hin*dIII fragment carrying the disrupted *map1* ORF (Fig. 1). Proper replacement of one of the two chromosomal *map1* alleles by the disruption construct in several transformants was confirmed by Southern blot analysis (data not shown). Ascospores produced by one of them were subjected to tetrad dissection. Most asci gave four viable spores. Two of them were Ura$^+$ and the other two were Ura$^-$, indicating that disruption of *map1* is not lethal. We then set out to construct a haploid strain carrying a larger deletion in *map1* from a haploid strain. A 1.5-kb *Hin*dIII-*Bcl*I fragment within the *map1* ORF was replaced by the *ura4*$^+$ cassette, and homothallic haploid strain JY878 was transformed with a *Kpn*I-*Hin*dIII fragment carrying this disruption construct (Fig. 1). Proper integration of the disrupted *map1* allele into the chromosome in some of the transformants was verified by Southern blot analysis (data not shown). They showed the same phenotypes as the disruptants derived from JY919. One of the disruptants derived from JY878, named JX500, was used in the subsequent experiments as a representative.

Cloning and sequence analysis of the original *map1* mutant allele. Genomic DNA was isolated from JY878 (h^{90} *map1*$^+$) and JZ272 (h^{90} *map1*), the latter of which carried the original *map1* mutation described by Egel (12), and was used as a template for the PCR to amplify the *map1* sequences. The reaction was carried out essentially as described previously (54), using primers MAP1-1 (5′-TTGGGTAAATGTGAAACGTT-3′) and MAP1R (5′-AACAAAAATGACT TTCTATAA-3′). The conditions employed for PCR were 94°C for 1 min, 45°C for 2 min, and 72°C for 2 min for a total of 30 cycles. The PCR products were

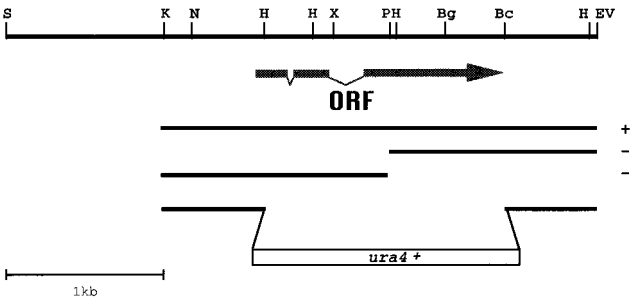

FIG. 1. Schematic illustration of the insert of pSX56, which carries the *map1* gene. Restriction sites on the insert are shown together with the extent and direction of the *map1* ORF (arrow). Restriction sites are abbreviated as follows: Bc, *Bcl*I; Bg, *Bgl*II; H, *Hin*dIII; K, *Kpn*I; N, *Nde*I; P, *Pst*I; and Xh, *Xho*I. Restriction fragments of the original insert were subcloned, and the ability of the clones to induce *sxa2-lacZ* expression in the tester strain was examined. The ability (+) or inability (−) of a subclone to give rise to blue colonies is indicated on the right. The structure of the linear fragment used to disrupt the *map1* gene in JY878 is shown.

separated by gel electrophoresis, and the major product was eluted from the gel. The amplified DNA was directly subjected to cycle sequencing with a Sequencing PRO kit (Toyobo) with [α-^{32}P]dCTP and the following primers: MAP1-1, MAP1-2 (5′-TGAGAAAGCGTATACTGGAA-3′), MAP1-3 (5′-AGAAAC AGGATTAGTGCATA-3′), MAP1-4 (5′-GCGCTTTTACTGAATGACGT-3′), MAP1-5 (5′-GAAAATGGGCCTAGTCACAT-3′), MAP1-6 (5′-TTATCAAT GCTGACTTCTCA-3′), and MAP1-7 (5′-AACAAAATCAGAGACTCCCT -3′). The mutation in the original *map1* allele was discovered by analysis using primer MAP1-4.

Preparation and Northern (RNA) blot analysis of *S. pombe* RNA. For Northern blot analysis, *S. pombe* cells were grown in MM to a cell density of 5×10^6/ml. One half of the culture was sampled, and the other half was transferred to MM-N and subjected to nitrogen starvation for 4 h. To activate mating-pheromone-responsive h^--specific genes, cells were resuspended in MM-N supplemented with 4 μg of synthetic P-factor per ml and incubated for 2 h. Total cellular RNA was prepared from each sample by disrupting the cells with glass beads, according to a standard extraction protocol (14). RNA was denatured with formaldehyde, separated by agarose gel electrophoresis, transferred to a membrane, and hybridized with a probe, as described elsewhere (55). The following DNA fragments were used as probes: *map1*, 0.5-kb *Pst*I-*Bgl*II (this study); *map2*, 0.6-kb *Nde*I-*Sca*I (26); *map3*, 0.5-kb *Xho*I-*Pst*I (64); *map4*, 1.8-kb *Hin*dIII-*Sac*I (27a); *sxa2*, 0.5-kb *Hin*dIII-*Hin*dIII (27); and *mam3*, 2.3-kb *Eco*RI-*Pst*I (27a).

Yeast two-hybrid analysis. An assay using the yeast two-hybrid system was performed essentially as described by Durfee et al. (10). The *mat1-Pc* sequence (32) was amplified by PCR (54) from a plasmid that carried the complete *P* cassette in the vector pDB248′. Oligonucleotides MATP5 (5′-AATAATCA TATGGATCCAAGAT-3′) and MATP3 (5′-ATTTTTCTGTAAGCTTATG CATATGC-3′) were used as primers. The PCR product was digested with *Hin*dIII and cloned into pUC119. The resultant plasmid was digested with *Nde*I, and the *mat1-Pc* ORF was inserted into the unique *Nde*I site of pAS1-CYH2 (10) and fused to the DNA-binding domain of *S. cerevisiae* GAL4 protein. The plasmid thus obtained was named pAS-Mat1-Pc. The *mat1-Mc* sequence was amplified similarly from a plasmid that carried the complete *M* cassette by using MATM5 (5′-CTCACAACTGCCATGGACTCA-3′) and MATM3 (5′-CAAAT GGGGATCCCAAAATATT-3′) as PCR primers. The product was digested with *Bam*HI and cloned into pUC119. The resultant plasmid was digested with *Nco*I and *Bam*HI, and the *mat1-Mc* ORF was inserted between the *Nco*I and *Bam*HI sites of pAS1-CYH2. The plasmid obtained was named pAS-Mat1-Mc. To construct a partner plasmid, a new *Eco*RI site was created in the *map1* cDNA sequence at the position corresponding to Arg-6–Lys-7 by localized mutagenesis (35) using oligonucleotide STY7 (5′-GATGAAAGAATTCTTTCAAAC-3′). The DNA fragment between this *Eco*RI site and the *Eco*RI site in the multicloning region, which covered most of the *map1* ORF, was excised and inserted into the *Eco*RI site of pACTII (10) so that most of Map1 was connected to the activation domain of GAL4. The resultant plasmid was named pACT-map1. An *S. cerevisiae* reporter strain, Y190, was cotransformed with pACT-map1 and either pAS-Mat1-Pc or pAS-Mat1-Mc, and histidine-independent growth of the transformant was examined.

Nucleotide sequence accession number. The nucleotide sequence of the *map1* gene (see Fig. 2) will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession no. D78483.

RESULTS

Isolation of genes involved in the regulation of *sxa2* expression. The *sxa2* gene, which apparently encodes a carboxypeptidase to inactivate P-factor, is expressed only in h^- cells exposed to P-factor (27). To identify factors involved in the induction of gene expression in response to mating-pheromone signaling, we screened an *S. pombe* genomic library for high-copy-number suppressors that could activate *sxa2* expression in the absence of pheromone signaling. We used a strain carrying the *sxa2-lacZ* fusion gene (JX494) as the indicator in this screening. JX494 could produce a fusion protein with β-galactosidase activity if transcription was started from the *sxa2* promoter. The enzyme activity was detected by a colony color assay, details of which are given in Materials and Methods. JX494 was defective in *spk1*, which encodes the MAP kinase that functions in the mating-pheromone response pathway (18, 65), because we sought to isolate factors that function downstream of the MAP kinase cascade. JX494 was transformed with an *S. pombe* genomic library based on high-copy-number vector pDB248′, and transformants were screened by the colony color assay. Plasmids were recovered from transformed cells that gave blue colonies and reexamined for the ability to promote expression of the *sxa2-lacZ* fusion gene in JX494. We obtained 12 positive plasmids from 80,000 transformants screened. Eleven of them were found to carry genes already sequenced and characterized, including *ste11* (63) (four clones), *mat1-M* (32) (six clones), and *spk1* (one clone). The remaining clone, called pSX56, appeared to carry a gene whose structure had not been reported before. We therefore characterized this gene further. As described below, this gene turned out to be identical to *map1*.

The gene carried on pSX56 has homology to *S. cerevisiae MCM1*. A restriction map of the insert of pSX56, which is 3.8 kb long, is shown in Fig. 1. The region responsible for the activation of *sxa2-lacZ* transcription was delimited to a 2.7-kb *Kpn*I-*Eco*RV fragment by subcloning (Fig. 1). Nucleotide sequence analysis of this fragment revealed a putative ORF which comprises three exons and potentially encodes 398 amino acid residues (Fig. 2). The assignment of exons shown in Fig. 2 has been confirmed by analysis of a cDNA clone isolated as described in Materials and Methods.

The deduced amino acid sequence of the ORF was compared with the Swiss Prot database. As shown in Fig. 3, it showed the highest degree of similarity to *S. cerevisiae* MCM1 (36.0% identity in the N-terminal 122 amino acids), which is a transcription factor regulating cell-type-specific gene expression (1, 51). It also showed 31.1% identity in the N-terminal region to the human serum response factor (SRF), which is a transcription factor that binds to the serum response element (SRE) of c-*fos* (48). The homologous region matched the MADS box, a conserved motif of 56 amino acids seen in the DNA-binding domains of a number of transcription factors and designated after the first letters of the names of the originally identified members (MCM1, AG, DEFA, and SRF) (57, 59). The deduced gene product also carried the loosely conserved motif C-terminal to the MADS box, called the SAM domain, which might be important for dimerization (59) (Fig. 3). Except for these motifs, the gene product showed no significant sequence similarity to any known protein.

Disruption of the gene. Disruption of the fission yeast *MCM1* homolog was carried out as described in Materials and Methods. The homothallic disruptants showed no apparent defect in cell growth but were unable to mate and sporulate within a colony. One of them, named JX500, was characterized further. Analysis of its mating ability revealed that JX500 could

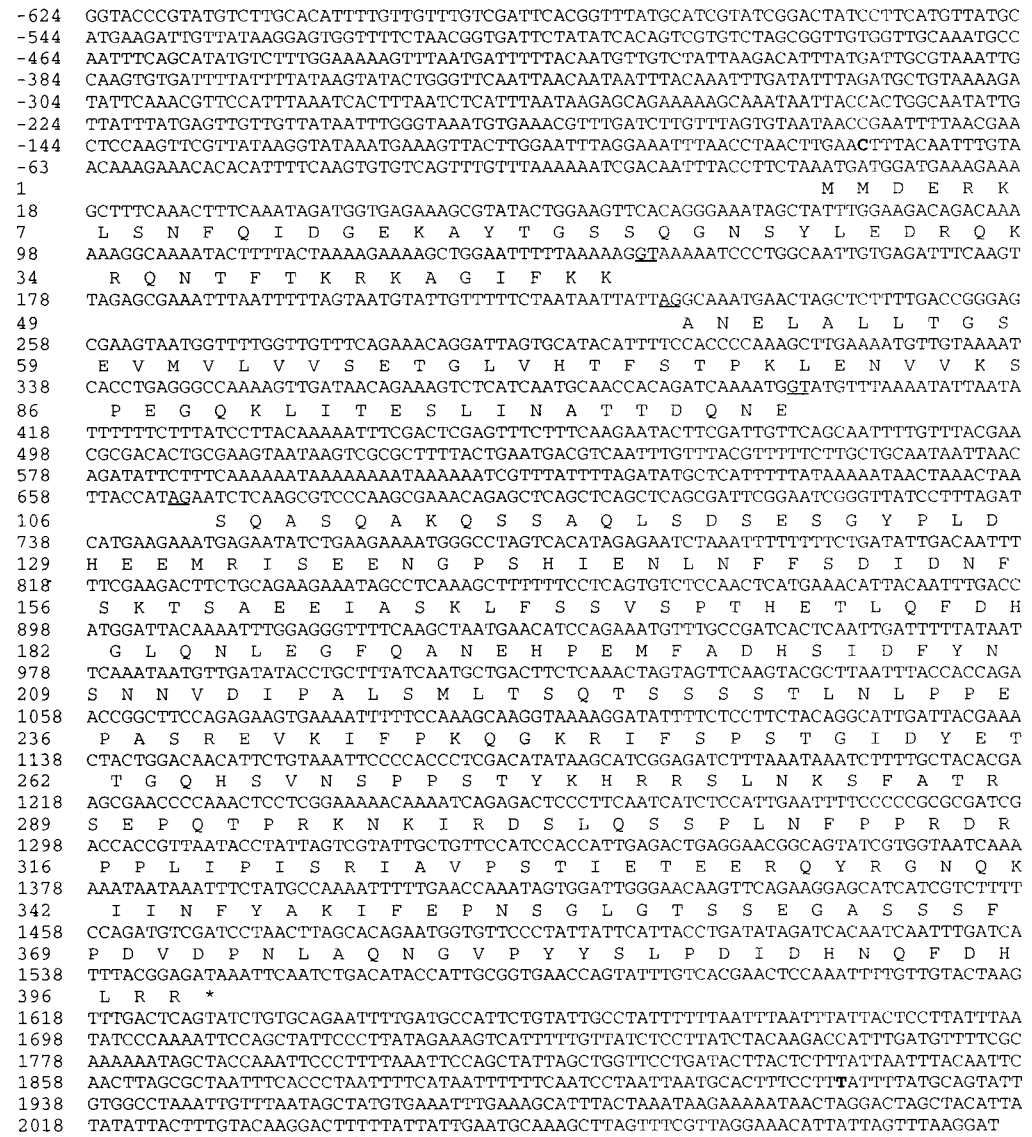

FIG. 2. The nucleotide sequence of the *map1* gene and its deduced amino acid sequence. The sequence of a 2,718-base *Kpn*I-*Eco*RV fragment that carries the entire *map1* ORF is shown. Numbering of the nucleotides starts with A in the first methionine codon of the ORF, and that of the amino acid residues starts with this methionine. The termini of introns (underlined GT and AG) and the 5′ end and the poly(A) addition site of the cDNA clone analyzed (boldface C and T, respectively) are indicated.

mate with h^+ cells but not at all with h^- cells, suggesting that disruption of the gene caused h^+-specific sterility.

Identification of the cloned gene as *map1*. The above results suggested that an h^+-specific sterility gene was disrupted in JX500. Among the four known h^+-specific sterility genes, *map2*, *map3*, and *map4* have previously been cloned and characterized (26, 27a, 64). The *MCM1* homolog identified in our analysis was obviously different from them. Thus, we examined whether the cloned gene might be allelic to the remaining *map* gene, *map1*. Cells of JX500 were fused with cells of a homothallic *map1* mutant (JZ272), which carried the original *map1* mutation described by Egel (12), and diploids were selected by intragenic complementation of the *ade6-M216* and *ade6-M210* alleles. The diploid strains could not sporulate at all, mimicking the phenotype of the *map1*/*map1* diploid strain (12). To see whether the cloned gene could complement the *map1* mutation, JZ272 was transformed with the original clone, pSX56. The transformants could mate with h^- cells efficiently, indicating that the gene indeed complemented the mutation. These results indicate that the cloned gene is *map1*.

To confirm that we had cloned the *map1* gene itself, chromosomal segments corresponding to the clone were amplified from *map1* mutant JZ272 and wild-type strain JY878 by PCR, as detailed in Materials and Methods, and sequenced directly. The PCR product derived from JY878 gave the same nucleotide sequence as the cloned gene. However, the product derived from JZ272 was different. It had four repeats of CTCAG instead of the three repeats located at nucleotides 695 to 709 of the cloned gene (Fig. 4; also see Fig. 2). The insertion of an extra CTCAG sequence caused a shift of the reading frame at amino acid residue 120 and generated a stop codon after residue 128. This suggested that the mutant gene product would be one-third smaller than the wild-type product. Thus, the cloned gene was obviously impaired in the *map1* mutant.

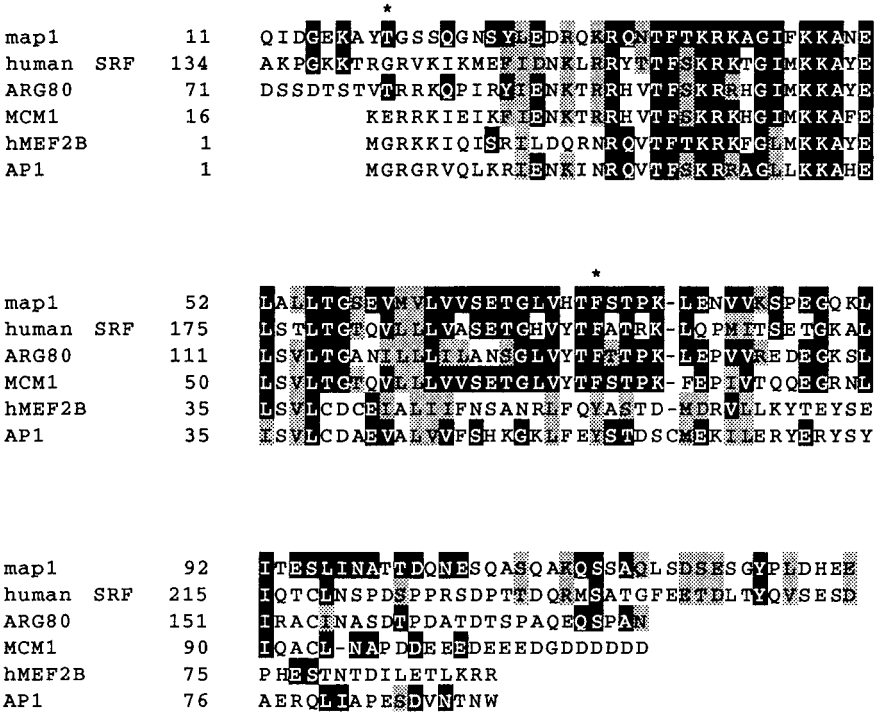

FIG. 3. Homology between the *map1* gene product and some MADS-box proteins. The proteins compared include human SRF (48), *S. cerevisiae* ARG80 (9) and MCM1 (1) proteins, human myocyte enhancer factor 2B (hMEF2B) (52), and *Arabidopsis thaliana* APETALA1 protein (AP1) (40). Amino acid residues identical to those of Map1 are shown in white against black; conservative changes are shaded. Conserved amino acid substitutions are grouped as follows: V, L, I, and M; F, Y, and W; K and R; E and D; Q and N; S and T; and A and G. The beginning and ending residues of the proposed MADS box (59) are indicated (asterisks).

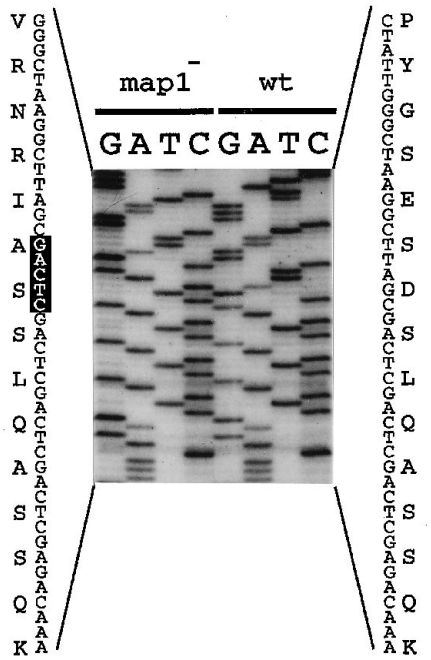

FIG. 4. Analysis of the mutant *map1* sequence. Nucleotides 687 to 719 of the *map1* gene isolated from JZ272, which carries the *map1* mutation described in reference 12 (map1$^-$), and the corresponding region isolated from the wild-type strain JY878 (wt) are shown.

From the above observations, we conclude that the gene we cloned is *map1* and call it *map1*. Such a conclusion, however, was rather surprising and even incredible at first, because we isolated the gene as an activator of *sxa2* transcription, which is strictly confined to h^- cells, whereas the *map1* gene had been believed to fulfill a function specifically required for h^+ cells. This apparent discrepancy was resolved by further analysis, as described below. (We have been informed by O. Nielsen that his group cloned the same gene by complementation of the *map1* mutation, details of which are to be published elsewhere.)

The function of *map1* is required to augment sexual development in h^- cells. To explore the possible involvement of *map1* in the regulation of sexual development in h^- cells, we examined the phenotypes of an h^- Δ*map1* strain (JX496) carefully. The mating frequency of this strain was lower than that of the wild-type h^- strain, especially on SSA plates (Fig. 5). We

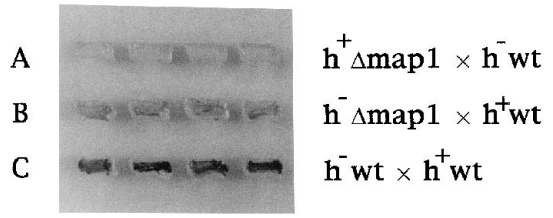

FIG. 5. Effects of *map1* disruption on the mating ability of h^+ and h^- cells. Cells were mixed as indicated, roughly in a 1:1 ratio, and spread in rectangular patches on an SSA plate. They were incubated for 2 days at 30°C and stained with iodine vapor. (A) JX498 (h^+ Δ*map1*) mixed with JY333 (h^- wild type); (B) JX496 (h^- Δ*map1*) mixed with JY334 (h^+ wild type); and (C) JY333 mixed with JY334. wt, wild type.

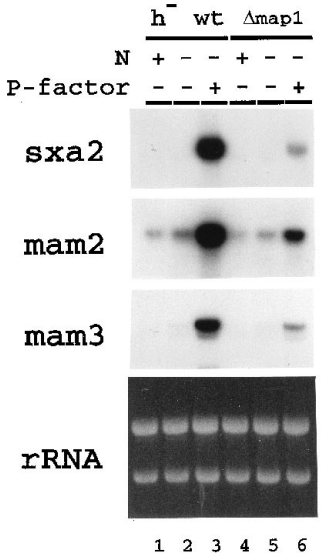

FIG. 6. Effects of *map1* disruption on the expression of mating-pheromone-responsive cell-type-specific genes. The control and the *map1*-disrupted h^- strains were grown to 5×10^6 cells per ml in MM. The culture was sampled, and the rest was shifted to nitrogen-free MM-N. At the medium change, P-factor was added to a portion of each culture to a final concentration of 4 μg/ml. Cells were incubated in MM-N for 2 h and sampled. RNA was prepared from each sample. The probes used to detect transcripts of *sxa2*, *mam2*, and *mam3* are described in Materials and Methods. The size of each transcript (in kilobases) is as follows: *sxa2*, 1.7 (27); *mam2*, 1.5 (33); and *mam3*, 3.9 (27a). Ethidium bromide staining of rRNA confirmed approximately equal loading of RNA in all lanes. wt, wild type.

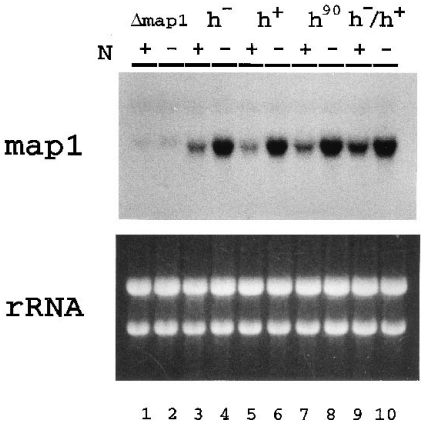

FIG. 7. Expression of the *map1* gene in cells of various mating types. Transcription of *map1* was analyzed by Northern blotting. A *Pst*I-*Bgl*II fragment within the *map1* ORF was used as the probe. Total RNA was prepared from either heterothallic h^- haploid cells (JY333), heterothallic h^+ haploid cells (JY334), homothallic h^{90} haploid cells (JY450), or heterozygous h^+/h^- diploid cells (JY362). Cells in which *map1* is deleted (JX500) were also examined. Samples were prepared from cells either during exponential growth in the presence of nitrogen or after being starved for nitrogen for 4 h. Northern blotting of the RNA preparations (top panel) and ethidium bromide staining of the preparations (bottom panel), which confirms approximately equal loading of RNA in all lanes, are shown.

then examined the level of *sxa2* transcription in JX496. To induce expression of *sxa2*, the h^- Δ*map1* cells and the control h^- cells were starved of nitrogen and exposed to P-factor (26). The expression of *mam3*, which is inducible in h^- cells under the same conditions (27a), was also tested. As shown in Fig. 6, transcriptional activation of both *sxa2* and *mam3* was blocked considerably in the *map1* disruptant.

Induction of transcription of *mam2*, which encodes the P-factor receptor, has been shown to occur in two steps. Transcription is induced by deprivation of nitrogen and is further enhanced by P-factor signaling (33, 70). Loss of *map1* function reduced both the induction of *mam2* expression by nitrogen starvation and its enhancement in response to P-factor (Fig. 6). These results suggest that the *map1* gene plays a significant role in the promotion of gene expression required for mating in h^- cells.

***map1* is expressed in cells of any mating type.** Expression of *map1* in various strains was examined by Northern blot analysis. The *map1* gene was transcribed into a single mRNA species of 1.7 kb in all strains tested. Unlike other *map* genes, which are transcribed in h^+ and h^+/h^- cells but not in h^- cells, *map1* was transcribed in all cell types at nearly the same level (Fig. 7), supporting the possibility that this gene might exert its function irrespective of the mating type of the cell. Expression of *map1* was enhanced two- to threefold by nitrogen starvation in all cell types (Fig. 7). This enhancement is consistent with the idea that *map1* gene function is required at the initial stage of sexual development.

Expression of cell-type-specific genes depends on *map1* function in a manner restricted by the mating type. The original *map1* mutant is apparently deficient in the production of P-factor and the M-factor receptor (12, 33, 38, 64). We examined transcription of *map2* and *map3*, which encode P-factor and the M-factor receptor, respectively (26, 64), in the homothallic *map1* disruptant (JX500) and a control strain (JY450). Total cellular RNA was prepared and subjected to Northern blot analysis. Expression of both *map2* and *map3* was completely blocked in JX500 (Fig. 8). Expression of the fourth *map* gene, *map4*, was also blocked (Fig. 8). These results suggest that Map1 is a critical positive factor for the transcription of the known h^+-specific genes.

To examine the consequences of *map1* overexpression in *S. pombe* cells of different mating types, we inserted the entire *map1* ORF downstream of the *nmt1* promoter carried on vector pREP1 (41). The resulting plasmid, pRX101, expressed *map1* strongly in the absence of thiamine. Strains transformed with this plasmid were grown in MM and then shifted to MM-N, neither of which contained thiamine. In h^+ cells, the enhanced *map1* expression led to activation of transcription of the three *map* genes. Their mRNA levels were higher than the levels seen under the physiological conditions (Fig. 8). Similar activation of transcription was observed with *sxa2* when *map1* was overexpressed in h^- cells (Fig. 8). These results confirm that *map1* controls expression of cell-type-specific genes in both h^+ and h^- cells. In addition, it was noted that the observed effects of *map1* overexpression were clearly cell type specific; in other words, it activated the *map* genes only in h^+ cells and the *sxa2* gene only in h^- cells. Overexpression of *map1* enhanced neither *mat1-Pc* nor *mat1-Mc* expression (data not shown), suggesting that the activation of cell-type-specific genes by *map1* overexpression was not mediated by overexpression of the mating-type genes.

Possible physical interaction of Map1 with Mat1-Pc. To explore the possibility that Map1 may physically interact with the products of the mating-type genes *mat1-Pc* and *mat1-Mc*, we carried out analysis using the yeast two-hybrid assay system, as described in Materials and Methods. Map1 protein fused to the activation domain of *S. cerevisiae* GAL4 and either Mat1-Pc or Mat1-Mc connected to the GAL4 DNA-binding domain were coexpressed in the reporter strain *S. cerevisiae* Y190. Expression of *HIS3* was under the control of GAL4 in

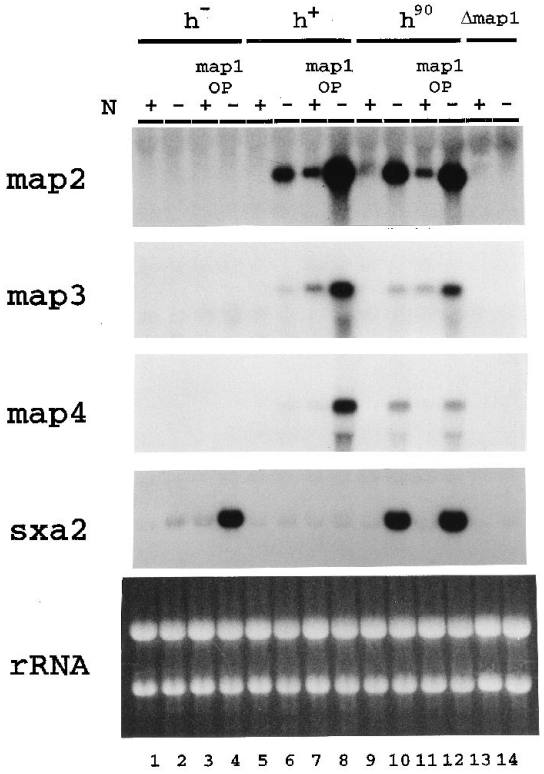

FIG. 8. Effects of *map1* overexpression on the expression of cell-type-specific genes. Total cellular RNA was prepared from cultures of h^-, h^+, and h^{90} strains transformed with either the vector pREP1 (lanes 1, 2, 5, 6, 9, and 10) or pRX101, which overexpresses *map1* (lanes 3, 4, 7, 8, 11, and 12). Transcription of the genes indicated was examined by Northern blot analysis. Lanes 1 through 4, heterothallic h^- haploid cells (JY333); lanes 5 through 8, heterothallic h^+ haploid cells (JY334); lanes 9 through 12, homothallic h^{90} haploid cells (JY450); and lanes 13 and 14, homothallic *map1* disruptant (JX500). RNA was prepared from each transformant either during exponential growth or after being starved for nitrogen, as in Fig. 7. The probes used here are described in Materials and Methods. The size of each transcript (in kilobases) is as follows: *map2*, 1.2 (26); *map3*, 2.4 (64); *map4*, 4.2 (27a); and *sxa2*, 1.7 (27). The ethidium bromide-stained patterns of rRNA verify approximately equal loading of RNA in all lanes (bottom panel). OP, overproducer.

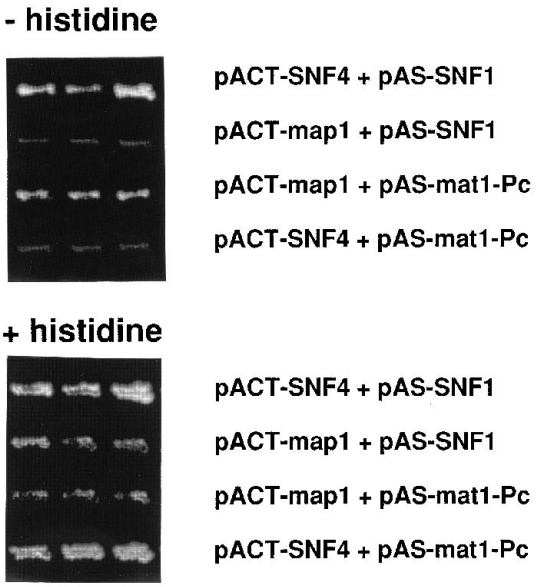

FIG. 9. Interaction of Map1 with Mat1-Pc in the yeast two-hybrid system. *S. cerevisiae* Y190, which carried a GAL4-dependent *HIS3* gene, was transformed with a plasmid that could express the GAL4-activating domain fused to either Map1 (pACT-map1) or SNF4 (pACT-SNF4). Y190 was transformed simultaneously with a plasmid that could express the GAL4 DNA-binding domain fused to either Mat1-Pc (pAS-mat1-Pc) or SNF1 (pAS-SNF1). Transformants that carried each combination of these plasmids, as indicated, were grown in the presence and absence of histidine at 30°C for 5 days. The plates were supplemented with 50 mM 3-aminotriazole to enhance the sensitivity of the assay. SNF4 and SNF1 are known to interact, and they serve as a positive control here. Cells carrying pACT-map1 grew more slowly, for an unclear reason. See Materials and Methods for more details.

this strain, and the host cells were expected to grow on the medium lacking histidine if the two fusion proteins could physically interact and form a complex (16). The results shown in Fig. 9 demonstrated that Map1 could interact with Mat1-Pc, although pACT-map1 encoding the GAL4-Map1 fusion protein apparently retarded cell growth, for an unclear reason. Essentially the same conclusion was reached when the interaction between Map1 and Mat1-Pc was evaluated by the level of expression of a reporter *lacZ* gene (data not shown). No positive interaction between Map1 and Mat1-Mc was detected in these analyses (data not shown). Although Mat1-Pc has not been well characterized biochemically, these results support the possibility that Map1 and Mat1-Pc may cooperate intimately to regulate expression of h^+-specific genes.

DISCUSSION

This study has shown that the *S. pombe map1* gene encodes a factor required for transcriptional activation of the cell-type-specific genes. The deduced *map1* gene product shows homology to transcription factors of the MADS-box family (57, 59). This family includes human SRF, which is involved in the coordination of c-*fos* transcription (48); *S. cerevisiae* MCM1, which regulates cell-type-specific gene expression in budding yeast cells (23); the MEF2 family of myocyte-specific enhancer factors (72); and the *Agamous* group of plant homeotic proteins (68). To our knowledge, Map1 is the first MADS-box protein identified in *S. pombe*. Several MADS-box proteins have been shown to interact with specific transcription factors and form multicomponent regulatory complexes. Protein-protein interactions are thought to be a common theme within the MADS-box family and to play a pivotal role in the regulation of target genes (59). Specificity of Map1 in the activation of its target genes also appears to depend on its interaction with factors representing the mating type of the cell, as shown in this study.

Disruption of *map1* dramatically affected the fertility of h^+ cells, and transcription of h^+-specific genes was not detectable in Δ*map1* cells. Unexpectedly, we found that disruption of *map1* also affects the expression of h^--specific genes and eventually reduces the efficiency of sexual development in h^- cells. This situation is reminiscent of that in *S. cerevisiae*, in which MCM1 is required for the transcriptional activation of both **a**- and α-specific genes (3, 29–31). Given that the two yeast species are only distantly related to each other and that the mating-type gene products of *S. cerevisiae* (α1, α2, and **a**1) and those of *S. pombe* (Mat1-Pc and Mat1-Mc) do not have sequence similarities, it is remarkable that they use similar MADS-box proteins to control cell-type-specific gene expression. However, it remains to be seen whether Map1 and MCM1 are totally homologous in function. It is unknown, for instance, whether Map1 participates in the maintenance of minichromosomes, for which MCM1 is required (39).

Map1 activates cell-type-specific gene expression in such a

way that its function is restricted by the mating type of the cell. This means that *mat1-Pc* is likely to restrict its function in h^+ cells, either directly or indirectly, and *mat1-Mc* is likely to do so in h^- cells. Perhaps Mat1-Pc or Mat1-Mc or both can physically interact with Map1 and modify its target specificity. The yeast two-hybrid analysis performed in this study suggested that Map1 and Mat1-Pc may function as a complex, although more direct evidence is required to draw a final conclusion. Unlike Mat1-Pc, Mat1-Mc failed to interact with Map1 in our two-hybrid assay system. Sequence analysis has shown that Mat1-Mc is a putative DNA-binding protein of the HMG-box family (8), and the possibility that it may also interact with Map1 under certain circumstances remains.

In *S. cerevisiae*, **a**- and α-specific genes are expressed during vegetative growth, and hence MCM1 is functional during the mitotic cell cycle. MCM1 functions also during sexual development. *S. cerevisiae* STE12 is a transcription factor regulated by FUS3/KSS1 MAP kinase, which mediates mating-pheromone signaling (61). STE12 forms a complex with MCM1, which binds to the upstream regulatory sequence of **a**-specific genes (15). This complex mediates basal transcription of these genes and also mediates pheromone-induced transcription when STE12 is phosphorylated by the MAP kinase, itself activated as a result of stimulation of the pheromone response pathway by binding of pheromone to cell surface receptor (61). Thus, MCM1 plays a role downstream of the MAP kinase cascade. STE12 is thought to function without the aid of MCM1 in other cases (15).

In *S. pombe*, expression of mating-type genes is regulated by nutrition. The *mat1-Pc* and *mat1-Mc* genes are only weakly expressed during vegetative growth, and *S. pombe* cells are virtually asexual when they are in the mitotic cell cycle. This study has shown that Map1 is required not only for expression of cell-type-specific genes induced by nutritional starvation, such as *map3*, but also for expression of cell-type-specific genes induced by mating-pheromone signaling, such as *sxa2*. This situation apparently parallels that of *S. cerevisiae*, in which MCM1 is involved in transcriptional activation both dependent on and independent of the mating-pheromone signaling. Although the precise function of Map1 in the mating-pheromone response pathway remains to be clarified, its involvement in that pathway does provide a rationale as to why *map1* was isolated in our screening.

Human SRF has been shown to bind to the promoter of the c-*fos* gene, forming a complex with a transcription factor Elk-1. If cells are stimulated with serum, Elk-1 is phosphorylated by MAP kinase and the complex activates the c-*fos* promoter (24, 66). Thus, the scheme that a MADS-box protein forms a complex with a transcription factor regulated by MAP kinase may be universal. Expression of *sxa2* occurs in response to stimulation of cells by P-factor, and the signaling is conveyed by the MAP kinase cascade involving Byr2, Byr1, and Spk1 (18, 25, 45, 46, 67). However, no transcription factor regulated by Spk1 has been identified yet in *S. pombe*. Identification of this factor, which may be a counterpart of *S. cerevisiae* STE12, is undoubtedly an important challenge. The fact that we isolated *mat1-M* and *ste11* as well as *map1* in our screening should prompt a critical examination of the possibility that Ste11, the predominant transcription factor for sexual development (63), may interact with Map1 to promote expression of the mating-pheromone-responsive genes.

ACKNOWLEDGMENTS

We thank Y. Imai, R. Tsuruta, Y. Iino, and A. Matsuyama for their kind assistance in the construction of *S. pombe* strains and plasmids used in this study. We also thank S. Elledge for supplying the yeast two-hybrid system. We are grateful to O. Nielsen for communicating results before publication.

This work was supported by grants-in-aid from the Ministry of Education, Science, Sports and Culture of Japan. N.Y. is the recipient of a JSPS Fellowship for Japanese Junior Scientists.

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