A Novel Gene, *msa1*, Inhibits Sexual Differentiation in *Schizosaccharomyces pombe*

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Manuscript received November 7, 2003
Accepted for publication January 22, 2004

ABSTRACT

Sexual differentiation in the fission yeast *Schizosaccharomyces pombe* is triggered by nutrient starvation or by the presence of mating pheromones. We identified a novel gene, *msa1*, which encodes a 533-aa putative RNA-binding protein that inhibits sexual differentiation. Disruption of the *msa1* gene caused cells to hypersporulate. Intracellular levels of *msa1* RNA and Msa1 protein diminished after several hours of nitrogen starvation. Genetic analysis suggested that the function of *msa1* is independent of the cAMP pathway and stress-responsive pathway. Deletion of the *ras1* gene in diploid cells inhibited sporulation and in haploid cells decreased expression of mating-pheromone-induced genes such as *mei2*, *mam2*, *ste11*, and *rep1*; simultaneous deletion of *msa1* reversed both phenotypes. Overexpression of *msa1* decreased activated $Ras1^{Val17}$-induced expression of *mam2*. Phenotypic hypersporulation was similar between cells with deletion of only *rad24* and both *msa1* and *rad24*, but simultaneous deletion of *msa1* and *msa2/nrd1* additively increased hypersporulation. Therefore, we suggest that the primary function of Msa1 is to negatively regulate sexual differentiation by controlling the expression of Ste11-regulated genes, possibly through the pheromone-signaling pathway.

THE haploid cells of *Schizosaccharomyces pombe* mate and initiate meiosis during nutritional starvation; these cells subsequently form ascospores after undergoing karyogamy, premeiotic DNA synthesis, meiosis I, and meiosis II (Yamamoto *et al.* 1997). Starvation induces expression of the *ste11* gene that encodes a key transcription factor, which, in turn, upregulates transcription of several genes involved in conjugation, meiosis, and sporulation (Sugimoto *et al.* 1991). One of these upregulated genes, *mei2*, encodes a well-characterized RNA-binding protein that is absolutely required for meiosis (Watanabe and Yamamoto 1994). Pat1/Ran1 kinase inhibits meiosis by negatively regulating both Ste11 and Mei2 through phosphorylation (Li and McLeod 1996; Watanabe *et al.* 1997). Phosphorylation of Mei2 inhibits its ability to bind meiRNA (Sato *et al.* 2002) and converts Mei2 into a substrate suitable for ubiquitin-dependent proteolysis (Kitamura *et al.* 2001). In nitrogen-starved diploid cells, transcription of *mei3* increases; its gene product, Mei3, inhibits Pat1/Ran1 through a pseudosubstrate mechanism, thereby allowing Mei2 to initiate meiosis (Li and McLeod 1996).

Sexual differentiation in *S. pombe* is regulated primarily by three signaling pathways: the cAMP pathway, the stress-responsive pathway, and the pheromone-signaling pathway (Yamamoto *et al.* 1997). Regulation of the cAMP pathway in *S. pombe* consists of many molecular interactions. For example, adenylyl cyclase, encoded by the *cyr1* gene, generates cAMP from ATP (Maeda *et al.* 1990; Kawamukai *et al.* 1991); trimeric G protein controls the activity of adenylyl cyclase through a nutrient-sensing mechanism (Isshiki *et al.* 1992); and cAMP phosphodiesterase, encoded by the *pde1* gene, downregulates the cAMP pathway by converting cAMP to AMP (Mochizuki and Yamamoto 1992). The intracellular level of cAMP decreases in nutrient-starved *S. pombe* cells as they exit the vegetative cycle to enter the stationary phase (Maeda *et al.* 1990; Kawamukai *et al.* 1991), but an experimentally high level of protein kinase A (PKA) activity inhibits initiation of sexual differentiation (Maeda *et al.* 1994; Yamamoto 1996). The cAMP-dependent PKA holoenzyme consists of a catalytic subunit encoded by *pka1* (Maeda *et al.* 1994) and a regulatory subunit encoded by *cgs1* (Devoti *et al.* 1991). PKA regulates expression of meiosis-specific genes such as *ste11* and, as a consequence, *mei2* (Devoti *et al.* 1991; Sugimoto *et al.* 1991; Wu and McLeod 1995). *In vitro*, PKA phosphorylates Rst2, a zinc-finger protein that binds the upstream region of *ste11* (Higuchi *et al.* 2002). Low levels of intracellular cAMP during starvation decrease activity of cAMP-dependent PKA, thereby decreasing downregulation of transcription factor Rst2 and triggering expression of *ste11* (Kunitomo *et al.* 2000).

The stress-responsive pathway in *S. pombe* is required for initiation of mating and progression through meiosis. The primary stress-responsive pathway components are the following: two MAPKK kinases, Wis4/Wik1/Wak1 and

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Win1; one MAPK kinase, Wis1, and one mitogen-activated protein (MAP) kinase, Phh1/Sty1/Spc1 (Warbrick and Fantes 1991; Kato *et al.* 1996; Samejima *et al.* 1997, 1998). Loss of function of *wis1*, *phh1/sty1/spc1*, or *atf1/gad7* greatly reduces *ste11* transcription (Takeda *et al.* 1995; Kanoh *et al.* 1996; Shiozaki and Russell 1996). MAP kinase Phh1/Sty1/Spc1 phosphorylates the heterodimeric transcription factor Atf1/Gad7-Pcr1 (Kon *et al.* 1998), which is required to activate expression of *ste11*.

The pheromone-signaling pathway includes Ras1, Byr2 (a MAPKK kinase), Byr1 (a MAPK kinase), and Spk1 (a MAP kinase; Fukui *et al.* 1986; Nadin-Davis and Nasim 1988; Toda *et al.* 1991; Wang *et al.* 1991; Neiman *et al.* 1993). Binding of the pheromone to its receptor transmits a signal to Gpa1 (Obara *et al.* 1991), the α-subunit of the trimeric G protein, which presumably activates Byr2 with the help of Ras1. Byr2 can also be activated by Ste4 (Okazaki *et al.* 1991; Barr *et al.* 1996), a leucine-zipper protein that is capable of self-activation through homodimerization (Tu *et al.* 1997); Byr2 can be downregulated by 14-3-3 proteins (Ozoe *et al.* 2002). Activated Byr2 in turn signals Byr1, which signals Spk1 to initiate conjugation and sporulation. Loss of function of any component of the pheromone-signaling pathway causes *S. pombe* cells to become sterile.

We previously developed nine distinguishable hypersporulating *S. pombe* mutants, ***s**porulation **a**bnormal **m**utant 1* (*sam1*)–*sam9*, by mutating wild-type strains using ethyl methanesulfonate; *sam1–9* sporulate on nutrient-rich medium (YEA) and have been partially characterized (Katayama *et al.* 1996).

In this study, we used a gene library to screen for a suppressor of hypersporulation in a *sam1* mutant and isolated a new gene, *msa1*, which encoded a putative RNA-binding protein and also a known gene, *msa2/nrd1* (Tsukahara *et al.* 1998). We report here that the *msa1* gene controlled sexual differentiation through inhibition of transcription of meiosis-inducing genes.

MATERIALS AND METHODS

Strains, media, and genetic manipulation: The strains of *S. pombe* used in this study are listed in Table 1. Standard yeast culture media and genetic manipulations were used, as described previously (Alfa *et al.* 1993; Kaiser *et al.* 1994). *S. pombe* strains were grown in complete YEA medium (0.5% yeast extract, 2% glucose, and 0.0075% adenine) or in the synthetic minimal medium, PM (0.3% potassium hydrogen phthalate, 0.22% sodium phosphate, 0.5% ammonium chloride, 2% glucose, vitamins, minerals, and salts), with added appropriate auxotrophic supplements (0.0075% adenine, leucine, uracil, and histidine) when required (Alfa *et al.* 1993). Electroporation was used to transform yeast cells (Prentice 1992). Sporulation was detected by iodine vapor staining (Gutz *et al.* 1974). *Escherichia coli* DH5α grown in Luria broth medium (1% polypepton, 0.5% yeast extract, 1% sodium chloride) hosted all plasmid manipulations, and standard methods were used for DNA manipulations (Sambrook *et al.* 1989).

Isolation of the *msa1* gene: HS412 cells were transformed using a *S. pombe* genomic library that had been constructed in the pWH5 vector (Kawamukai 1999). Cells were spread on PM medium agar plates and incubated at 30° for 5 days. Cells were exposed to iodine vapor and colonies that did not stain with iodine vapor (*i.e.*, no spores were present) were selected from the transformants. From ~10^5 transformants, nonsporulating clones were screened and plasmids from these strains were rescued in *E. coli*. Partial DNA sequencing and subcloning analysis of the *sam1* suppressor genes identified two types of clones, *msa1* (SPAC13G7.13c) and *msa2* (SPAC2F7.11). For genomic integration, a 5-kb *Bam*HI-*Pst*I fragment of the genomic region from pW1-12 was cloned into a pYC11 plasmid, which is the derivative of pBluescript KS(+) carrying the *Saccharomyces cerevisiae LEU2* gene (Takahashi *et al.* 1992), and the resulting plasmid was named pYC11-3601. The cDNA clone of *msa1* was isolated from a cDNA library constructed in λZAPII (Kawamukai *et al.* 1992) by plaque hybridization using the genomic fragment as a probe (Figure 1A).

Construction of *msa1* and *msa2* deletion mutants: A 4.6-kb *Hin*dIII-*Pst*I genomic fragment containing *msa1* from pW1-12 (Figure 1) was introduced into the plasmid vector pUC118. The 1-kb *Eco*RV-*Eco*RV region that contained 60% of the *msa1* open reading frame (ORF) was replaced with the 1.8-kb *Hinc*II DNA fragment of the *ura4* gene from pHSG398-ura4 (Tanaka *et al.* 1999) and the resulting plasmid was named pUC118-msa1::ura4$^+$. A 5.4-kb *Pst*I-*Hin*dIII fragment derived from pUC118-msa1::ura4$^+$ was used to transform the SP870 or SP335 strain (Kawamukai *et al.* 1992) to make *msa1* deletion mutants. Stable Ura$^+$ transformants were selected, and the *msa1* locus was analyzed by Southern hybridization with the probes of both *msa1* and *ura4*. Southern hybridization was done as previously described (Sambrook *et al.* 1989). Similarly, to make the *msa2* deletion mutants, the 1.4-kb *Sph*I-*Sph*I region containing 85% of the *msa2* ORF in pUC118-msa2 was replaced with the 1.8-kb *Sph*I DNA fragment of the *ura4* gene, yielding pUC118-msa2::ura4$^+$. pUC118-msa2 was derived by inserting the 4-kb *Hin*dIII fragment from originally screened clone pW136-19 into pUC118. The plasmid pUC118-msa2::ura4$^+$ was made linear to transform SP870 yielding HT211 (*msa2::ura4*$^+$). Proper disruption in HT211 was confirmed by Southern hybridization. HT81 (*msa1::ura4*$^+$ *msa2::ura4*$^+$) was isolated by crossing HT12 (*ade6-216 msa1::ura4*$^+$) with HT211 (*ade6-210 msa2::ura4*$^+$) and subsequent dissection of tetrad.

Construction of various double mutants: The double mutant of *mas1* with a variety of mutants, including *pde1, cgs1*, *phh1*, *rad24*, *gpa1*, *byr1*, *byr2*, *ras1*, *spk1*, and *ste4* in Table 1, are all derived by crossing their parental strains that retain different *ade* markers and subsequent dissection of tetrads. In tetrads, only a nonparental segregant (2Ura$^+$, 2Ura$^-$) was selected for isolating proper double mutants. Typically, HT11 (*ade6-210 msa1::ura4*$^+$) and JZ666 (*ade6-216 pde1::ura4*$^+$) were crossed, the diploids were allowed to sporulate, and spores were subjected to tetrad analysis. HT43 (*msa1::ura4*$^+$ *pde1::ura4*$^+$) was isolated as one of the nonparental tetrads. All other double mutants listed in Table 1 were derived in a similar way.

Plasmids: Plasmid manipulation and bacterial transformation were performed using standard techniques (Sambrook *et al.* 1989). pALmsa1 was constructed by inserting a 5-kb *Bam*HI-*Pst*I fragment of genomic region from pW1-12. The *msa1* gene was amplified by PCR using primer oligonucleotides (msa1FL) in Table 2. Amplified *msa1* was digested with *Sal*I and *Sma*I and then ligated with pREP1, pREP41, or pREP81 (Maundrell 1993), which had previously been digested with *Sal*I and *Sma*I to generate pREP1msa1, pREP41msa1, and pREP81msa1. pSLF273-msa1 was constructed by inserting the same fragment in the *Sal*I and *Sma*I sites of pSLF273 (Forsburg and Sherman 1997). The deletion derivatives from

TABLE 1

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

Strain	Genotype
SP66	*h^{90} ade6-210 leu1-32*
SP319	*h^{-} ade6-210 leu1-32 his3*
SP335	*h^{-} ade6-210 leu1-32 ura4 his3*
SP412	*h^{90} ade6-216 sam1*
HS412	*h^{90} ade6-210 leu1-32 ura4-D18 sam1*
SP870	*h^{90} ade6-210 leu1-32 ura4-D18*
SP870A	*h^{90} ade6-216 leu1-32 ura4-D18*
DY114	*h^{90} ade6-216 leu1-32 ura4-D18 cyr1::ura4^{+}*
JZ633	*h^{90} ade6-216 leu1-32 ura4-D18 pka1::ura4^{+}*
JZ666	*h^{90} ade6-216 leu1-32 ura4-D18 pde1::ura4^{+}*
JZ858	*h^{90} ade6-216 leu1-32 ura4-D18 cgs1::ura4^{+}*
TK105	*h^{90} leu1-32 ura4-D18 phh1::ura4^{+}*
JZ451	*h^{+} ade6-210 leu1-32 ura4-D18 gpa1::ura4^{+}*
SPRU	*h^{90} ade6-210 leu1-32 ura4-D18 ras1::ura4^{+}*
SPBU	*h^{90} ade6-210 leu1-32 ura4-D18 byr1::ura4^{+}*
SPSU	*h^{90} ade6-210 leu1-32 ura4-D18 byr2::ura4^{+}*
SPKU	*h^{90} ade6-210 leu1-32 ura4-D18 spk1::ura4^{+}*
SPFU	*h^{90} ade6-216 leu1-32 ura4-D18 ste4::ura4^{+}*
SP593	*h^{90} ade6-210 leu1-32 ras1^{val17}*
SP24U1	*h^{90} ade6-210 leu1-32 ura4-D18 rad24::ura4^{+}*
HT3	*h^{90} ade6-210 leu1-32 sam1*
HT5	*h^{90} ade6-216 leu1-32 msa1:3HA<<kanMX6*
HT11	*h^{90} ade6-210 leu1-32 ura4-D18 msa1::ura4^{+}*
HT12	*h^{90} ade6-216 leu1-32 ura4-D18 msa1::ura4^{+}*
HT14	*h^{90} ade6-216 ura4-D18 msa1::ura4^{+}*
HT21	*h^{-} ade6-210 leu1-32 ura4 his3 msa1::ura4^{+}*
HT43	*h^{90} ade6-216 leu1-32 ura4-D18 msa1::ura4^{+} pde1::ura4^{+}*
HT58	*h^{90} ade6-210 leu1-32 ura4-D18 msa1::ura4^{+} cgs1::ura4^{+}*
HT76	*h^{90} ade6-216 leu1-32 ura4-D18 msa1::ura4^{+} phh1::ura4^{+}*
HT77	*h^{90} ade6-210 leu1-32 ura4-D18 msa1::ura4^{+} rad24::ura4^{+}*
HT81	*h^{90} ade6-210 leu1-32 ura4-D18 msa1::ura4^{+} msa2::ura4^{+}*
HT89	*h^{90} ade6-210 leu1-32 ura4-D18 msa1::ura4^{+} gpa1::ura4^{+}*
HT90	*h^{90} ade6-216 leu1-32 ura4-D18 msa1::ura4^{+} gpa1::ura4^{+}*
HT91	*h^{90} ade6-210 leu1-32 ura4-D18 msa1::ura4^{+} byr1::ura4^{+}*
HT92	*h^{90} ade6-216 leu1-32 ura4-D18 msa1::ura4^{+} byr1::ura4^{+}*
HT93	*h^{90} ade6-210 leu1-32 ura4-D18 msa1::ura4^{+} byr2::ura4^{+}*
HT94	*h^{90} ade6-216 leu1-32 ura4-D18 msa1::ura4^{+} byr2::ura4^{+}*
HT95	*h^{90} ade6-210 leu1-32 ura4-D18 msa1::ura4^{+} ras1::ura4^{+}*
HT96	*h^{90} ade6-216 leu1-32 ura4-D18 msa1::ura4^{+} ras1::ura4^{+}*
HT97	*h^{90} ade6-210 leu1-32 ura4-D18 msa1::ura4^{+} spk1::ura4^{+}*
HT98	*h^{90} ade6-216 leu1-32 ura4-D18 msa1::ura4^{+} spk1::ura4^{+}*
HT99	*h^{90} ade6-210 leu1-32 ura4-D18 msa1::ura4^{+} ste4::ura4^{+}*
HT100	*h^{90} ade6-216 leu1-32 ura4-D18 msa1::ura4^{+} ste4::ura4^{+}*
HT101	*h^{90}/h^{90} ade6-210/ade6-216 leu1-32/leu1-32 ura4-D18/ura4-D18*
HT102	*h^{90}/h^{90} ade6-210/ade6-216 leu1-32/leu1-32 ura4-D18/ura4-D18 msa1::ura4^{+}/msa1::ura4^{+}*
HT103	*h^{90}/h^{90} ade6-210/ade6-216 leu1-32/leu1-32 ura4-D18/ura4-D18 msa1::ura4^{+}/msa1::ura4^{+} byr1::ura4^{+}/byr1::ura4^{+}*
HT104	*h^{90}/h^{90} ade6-210/ade6-216 leu1-32/leu1-32 ura4-D18/ura4-D18 msa1::ura4^{+}/msa1::ura4^{+} byr2::ura4^{+}/byr2::ura4^{+}*
HT105	*h^{90}/h^{90} ade6-210/ade6-216 leu1-32/leu1-32 ura4-D18/ura4-D18 msa1::ura4^{+}/msa1::ura4^{+} ras1::ura4^{+}/ras1::ura4^{+}*
HT106	*h^{90}/h^{90} ade6-210/ade6-216 leu1-32/leu1-32 ura4-D18/ura4-D18 msa1::ura4^{+}/msa1::ura4^{+} spk1::ura4^{+}/ spk1::ura4^{+}*
HT107	*h^{90}/h^{90} ade6-210/ade6-216 leu1-32/leu1-32 ura4-D18/ura4-D18 msa1::ura4^{+}/msa1::ura4^{+} ste4::ura4^{+}/ste4::ura4^{+}*
HT108	*h^{90}/h^{90} ade6-210/ade6-216 leu1-32/leu1-32 ura4-D18/ura4-D18 msa1::ura4^{+}/msa1::ura4^{+} gpa1::ura4^{+}/gpa1::ura4^{+}*
HT117	*h^{90} ade6-210 leu1-32 ura4-D18 gpa1::ura4^{+}*
HT118	*h^{90} ade6-216 leu1-32 ura4-D18 gpa1::ura4^{+}*
HT119	*h^{90} ade6-216 leu1-32 ura4-D18 ras1::ura4^{+}*
HT120	*h^{90} ade6-216 leu1-32 ura4-D18 byr2::ura4^{+}*
HT121	*h^{90} ade6-216 leu1-32 ura4-D18 byr1::ura4^{+}*
HT122	*h^{90} ade6-216 leu1-32 ura4-D18 spk1::ura4^{+}*
HT123	*h^{90} ade6-210 leu1-32 ura4-D18 ste4::ura4^{+}*
HT124	*h^{90}/h^{90} ade6-210/ade6-216 leu1-32/leu1-32 ura4-D18/ura4-D18 gpa1::ura4^{+}/ gpa1::ura4^{+}*
HT125	*h^{90}/h^{90} ade6-210/ade6-216 leu1-32/leu1-32 ura4-D18/ura4-D18 ras1::ura4^{+}/ ras1::ura4^{+}*
HT126	*h^{90}/h^{90} ade6-210/ade6-216 leu1-32/leu1-32 ura4-D18/ura4-D18 byr2::ura4^{+}/byr2::ura4^{+}*
HT127	*h^{90}/h^{90} ade6-210/ade6-216 leu1-32/leu1-32 ura4-D18/ura4-D18 byr1::ura4^{+}/byr1::ura4^{+}*
HT128	*h^{90}/h^{90} ade6-210/ade6-216 leu1-32/leu1-32 ura4-D18/ura4-D18 spk1::ura4^{+}/spk1::ura4^{+}*
HT129	*h^{90}/h^{90} ade6-210/ade6-216 leu1-32/leu1-32 ura4-D18/ura4-D18 ste4::ura4^{+}/ste4::ura4^{+}*
HT211	*h^{90} ade6-210 leu1-32 ura4-D18 msa2::ura4^{+}*

TABLE 2

Oligonucleotide primers used in this study

Primer	Sequence[a]
msa1 FL	5′-TTAGTCGAC(*Sal*I) AATGGTTGTTTCCTCT-3′
	5′-CAACCCGGG(*Sma*I) TCTAATTCCCATCCAT-3′
msa1 (Δ2-31)	5′-TTAGTCGAC(*Sal*I) AATGCCCCCAGGTTCTTTATC-3′
	5′-CAACCCGGG(*Sma*I) TCTAATTCCCATCCAT-3′
msa1 (Δ2-102)	5′-TTAGTCGAC(*Sal*I) AATGTTTCAACAGTGGGGTCC-3′
	5′-CAACCCGGG(*Sma*I) TCTAATTCCCATCCAT-3′
msa1 (Δ487-533)	5′-TTAGTCGAC(*Sal*I) AATGGTTGTTTCCTCT-3′
	5′-AGCCCCGGG(*Sma*I) CTAGTGAAATGTTGAAAACTG-3′
msa1 (w)	5′-CGTTGGCCAACTCGACCCAG-3′
msa1 (pFA6a-1,x)	5′-GGGGATCCGTCGACCTGCAGCGTACGAATTCCCATCCATAGGTGG-3′
msa1 (pFA6a-2,y)	5′-GTTTAAACGAGCTCGAATTCATCGATGTACATATTCTATAAAACAC-3′
msa1 (z)	5′-GGTTCTTTACCTCACGAACC-3′
rep1	5′-ATGGATTCTGATCGTTGTTTAACAGACGAA-3′
	5′-TTACCAATCACTGCAAAAACTCGAACCCAA-3′

[a] The top and bottom sequences indicate the sense and antisense primers, respectively. Restriction enzyme sites are underlined.

pSLF273-msa1 used in Figure 3 were constructed either by using a primer set described in Table 2 or by digestion with restriction enzymes. The *nmt1* promoters and its derivatives were repressed by addition of 50 μg/ml thiamine to the media (Maundrell 1990).

Genomic integration of 3HA epitopes: Sequences of three hemagglutinin (3HA) epitopes were integrated into the genomic locus of *msa1* at the C terminus by a PCR-based method using the pFA6a-3HA-kanMX6 modules (Krawchuk and Wahls 1999). DNA fragments ~600–700 bp and corresponding to the 5′ and 3′ region of the *msa1* gene were amplified by PCR using oligonucleotides msa1 (w) and msa1 (pFA6a-1,x) or msa1 (pFA6a-2,y) and msa1 (z) in Table 2. Both amplified fragments were used to attach with the ends of the *kanMX6* cassette by PCR. SP66 was transformed with the resulting *msa1-3HA-kanMX6* fragment. G418-resistant transformants were selected and protein expression was assessed by Western blot analysis.

Conjugation and sporulation efficiency assay: Cells were grown to midlog phase in PM medium, washed with nitrogen-free or glucose-free PM medium, inoculated in PM medium with various concentrations of nitrogen and glucose, and incubated at 30°. After incubation for selected times, 1 ml of cell suspension was removed and sonicated gently, and the number of zygotes were counted under the microscope. Conjugation frequencies were calculated by dividing the number of zygotes (one zygote counted as two cells) by the total number of cells present.

To determine the sporulation efficiency of diploid cells, the wild-type and each mutant strain were incubated at 30° for 5 days in PM plates that contained 0.5% nitrogen and 2% glucose. A minimum of three individual colonies from each strain was resuspended in water, 1000 cells/colony were microscopically examined for presence of ascospores, and sporulation efficiency was calculated.

Measurement of viability in stationary phase: Cells were grown to 10^7 cells/ml in PM at 30°. The cultures were maintained at this density and at daily intervals an aliquot was removed and plated onto YEA medium for incubation at 30°. The colonies formed were counted after 3 days.

Northern blot analysis: Total RNA was prepared and Northern blot analysis was performed as described previously (Ozoe *et al.* 2002). *S. pombe* cells were grown in PM medium at 30° to a density of 5×10^6 cells/ml. The cells were pelleted by centrifugation, washed with nitrogen-free or low-glucose PM medium, and resuspended in nitrogen-free or low-glucose PM medium at the same density. The cells were incubated for selected times and resuspended in 1 ml of isogen (RNA isolation reagent; Nippon Gene) and vigorously vortexed 6 min with glass beads. After centrifugation (10,000 × *g* for 15 min at 4°), the supernatant was precipitated with isopropanol. Approximately 10 μg of each sample of total RNA was applied to a 1% denaturing formaldehyde-agarose gel, electrophoresis was applied, and RNA was transferred to a hybridization membrane (Hybond-N+, Amersham Biosciences) in alkali transfer buffer (0.05 M sodium hydroxide) for 4 hr. The probes were labeled with [α-^{32}P]dCTP (Amersham Biosciences) by using BcaBEST labeling kit (Takara Biomedicals, Berkeley, CA). The transcription on the blot was analyzed by autoradiography with a BAS1500-Mac image analyzer (Fuji Film).

The hybridization probes used were the 1.3-kb *Pvu*II fragment for *ste11* from pSX1 (Sugimoto *et al.* 1991), the 3.2-kb *Cla*I fragment for *mei2* (Watanabe *et al.* 1988), the 3.5-kb *Hin*dIII fragment containing *mam2* (Kitamura and Shimoda 1991), and the 1.9-kb cDNA fragment for *rep1* amplified from the cDNA library using primers (rep1) described in Table 2 and the 1.7-kb cDNA fragment for *msa1*.

Western blot analysis: The *msa1-3HA* genomic integrated strain (HT5: *h^{90} ade6-216 leu1-32 msa1-3HA<<kanMX6*) was cultured to midlog phase in synthetic medium PM at 30°. Cells were then shifted to nitrogen-free medium, PM −N, and cell-free extracts were prepared at indicated times. About 1×10^8 cells of *S. pombe* were harvested. Pellets were washed with STOP buffer [150 mM NaCl, 50 mM NaF, 10 mM EDTA, 1 mM NaN_3 (pH 8.0)] and stored at −80°. The pellets were diluted in 100 μl of dH_2O and boiled at 95° for 5 min. Then 120 μl of 2× Laemmli buffer [4% SDS, 20% glycerol, 0.6 M β-mercaptoethanol, 0.12 M Tris-HCl (pH 6.8)] containing 8 M urea and 0.2% bromo phenol blue was added to the samples, which were vigorously vortexed with an equal volume of zirconia-silica beads for 3 min and then heated again at 95° for 5 min. The zirconia-silica beads and large debris were removed by centrifugation at 10,000 × *g* for 15 min. Approximately equal amounts of each sample were analyzed by SDS-polyacrylamide gel electrophoresis using a 10% polyacrylamide gel and then transferred to Immobilon transfer membranes (Millipore,

Bedford, MA) using a semidry-type transfer system. For detection of 3HA fusion proteins, membranes were incubated with an anti-HA polyclonal antibody (Molecular Probes, Eugene, OR) diluted 1:3000 in 5% dry milk in TBS-T (15 mM Tris, 137 mM NaCl, 0.1% Tween20), washed, and incubated with horseradish-peroxidase-conjugated anti-rabbit secondary antibody (Bio-Rad Laboratories, Richmond, CA) diluted 1:5000 in 5% dry milk in TBS-T. The secondary antibodies were detected with the enhanced chemiluminescence (ECL) system as described by the manufacturer (Amersham Biosciences). For detection of Cdc2p, membranes were incubated with an anti-PSTAIRE polyclonal antibody (Santa Cruz Biotechnology) diluted 1:3000 in 2% dry milk in TBS-T, washed, and incubated with horseradish-peroxidase-conjugated anti-rabbit secondary antibody diluted 1:2000 in 2% dry milk in TBS-T. The secondary antibodies were detected with the ECL system (Amersham Biosciences).

RESULTS

Isolation of the *msa1* gene: To isolate the gene that suppressed hypersporulation in *S. pombe sam1* cells (HS412; Katayama *et al.* 1996), HS412 cells were transformed using a *S. pombe* genomic library. In this screening, two types of clones, *msa1* (SPAC13G7.13c) and *msa2* (SPAC2F7.11), were identified as multicopy suppressors of *sam1*. We began to investigate both genes, but because we later showed that *msa2* was identical to the *nrd1* gene (Tsukahara *et al.* 1998), we concentrated on *msa1* in this study. The initial clone pW1-12 with the *msa1* gene contained a DNA fragment of ~5.3 kb. The region responsible for the suppressor activity in strain HT3 (*sam1*) was then isolated and sequenced (Figure 1). Although integration of plasmid pYC11-3601, which bears wild-type *msa1*, into the HT3 genome by homologous recombination was conducted, the genomic integration of pYC11-3601 did not suppress hypersporulation in the HT3 (*sam1*) strain, which indicated that the pW1-12 plasmid contains only a multicopy suppressor and not the *sam1* allele. We also sequenced the *msa1* locus of the HT3 (*sam1*) mutant after PCR cloning of the corresponding region but did not find alteration of the *msa1* sequence compared with the wild-type strain. It was for this reason that we tentatively named this suppressor gene ***m**ulticopy suppressor of **s**porulation **a**bnormal mutant* (*msa1*).

No intron was found in the sequence of the *msa1* gene after sequencing and comparison of cDNA and genomic DNA. Translation of the *msa1* gene revealed that the *msa1* gene encodes a 533-amino acid (aa) protein (Figure 1B). Homology searches using the DDBJ and GenBank databanks revealed no strong similarity with other proteins or genes, except for two putative RNA-recognition motifs (RRMs; Figure 1C). The most homologous protein (37% identity in 200 aa around the RNP motifs region) is *S. cerevisiae* Rim4, which is known to be a putative RNA-binding protein involved in meiosis (Soushoko and Mitchell 2000).

***msa1*Δ cells mate without nitrogen starvation:** To determine the function of the *msa1* gene, we made a chromosome deletion mutant of the *msa1* gene. The resulting *msa1* deletion mutants (*msa1*Δ) of homothallic and heterothallic strains were named HT11 (h^{90} *msa1::ura4*$^+$) and HT21 (h^- *msa1::ura4*$^+$), respectively. No distinguishable difference in cell growth or morphology was seen between heterothallic *msa1*Δ (HT21) and wild-type cells (SP319) cultured in PM medium (Figure 2A, Figure 8C). No aberrant spore formation as found in the *pat1*^{ts} mutant was observed in HT21.

We next examined the mating efficiencies of homothallic *msa1*Δ cells under various culture conditions. Neither wild-type nor *msa1*Δ (HT11) cells conjugated in growth medium containing 2% glucose and 0.5% ammonium chloride as the sole carbon and nitrogen sources. When the glucose concentration in the medium was decreased to 0.5%, *msa1*Δ cells conjugated with 30% efficiency and wild-type cells conjugated with 1.5% efficiency (Figure 2B). The *msa1*Δ cells conjugated very efficiently in nitrogen-free and glucose-rich medium (Figure 2C). Overexpression of *msa1* in the *msa1*Δ cells significantly inhibited the efficiency of conjugation under severe nutrient starvation (Figure 2D). Because the cells that lacked *msa1* conjugated with markedly increased efficiency under conditions of either glucose or nitrogen starvation, we concluded that the ability of *msa1* to inhibit sexual differentiation was not specifically associated with nutrient conditions of glucose and nitrogen.

RNA-binding domains are essential for Msa1 function: To investigate the region essential for the function of *msa1*, several deletion mutants of the *msa1* gene were constructed and examined for their ability to suppress the *msa1* deletion mutants. Deletion of 30 amino acids from the N terminus or deletion of 45 amino acids from the C terminus only slightly decreased the ability of Msa1 to suppress the high mating efficiency of *msa1* deletion mutants. However, when half of the N terminus RRM (pSLF273-DM2) or half of the C terminus RRM (pSLF273-DM3) was deleted, the function of Msa1 was completely inactivated (Figure 3). These results indicate that both RRMs are essential for the function of Msa1.

Expression of *msa1*: To examine the expression pattern of *msa1* mRNA, we performed Northern blot analysis, which showed that *msa1* mRNA was present in vegetatively growing cells, but the expression level was very low and was further reduced by nitrogen starvation (Figure 4A).

The level of the *msa1* product was examined by Western blotting. The *msa1* gene, tagged with 3HA at the C terminus, was integrated into the *msa1* genomic locus; expression of *mas1-3HA* was controlled by the authentic promoter. The h^{90} *msa1-3HA* strain (HT5) was incubated in PM medium and then shifted to nitrogen-free medium. Similar to mRNA level, the level of the Msa1-

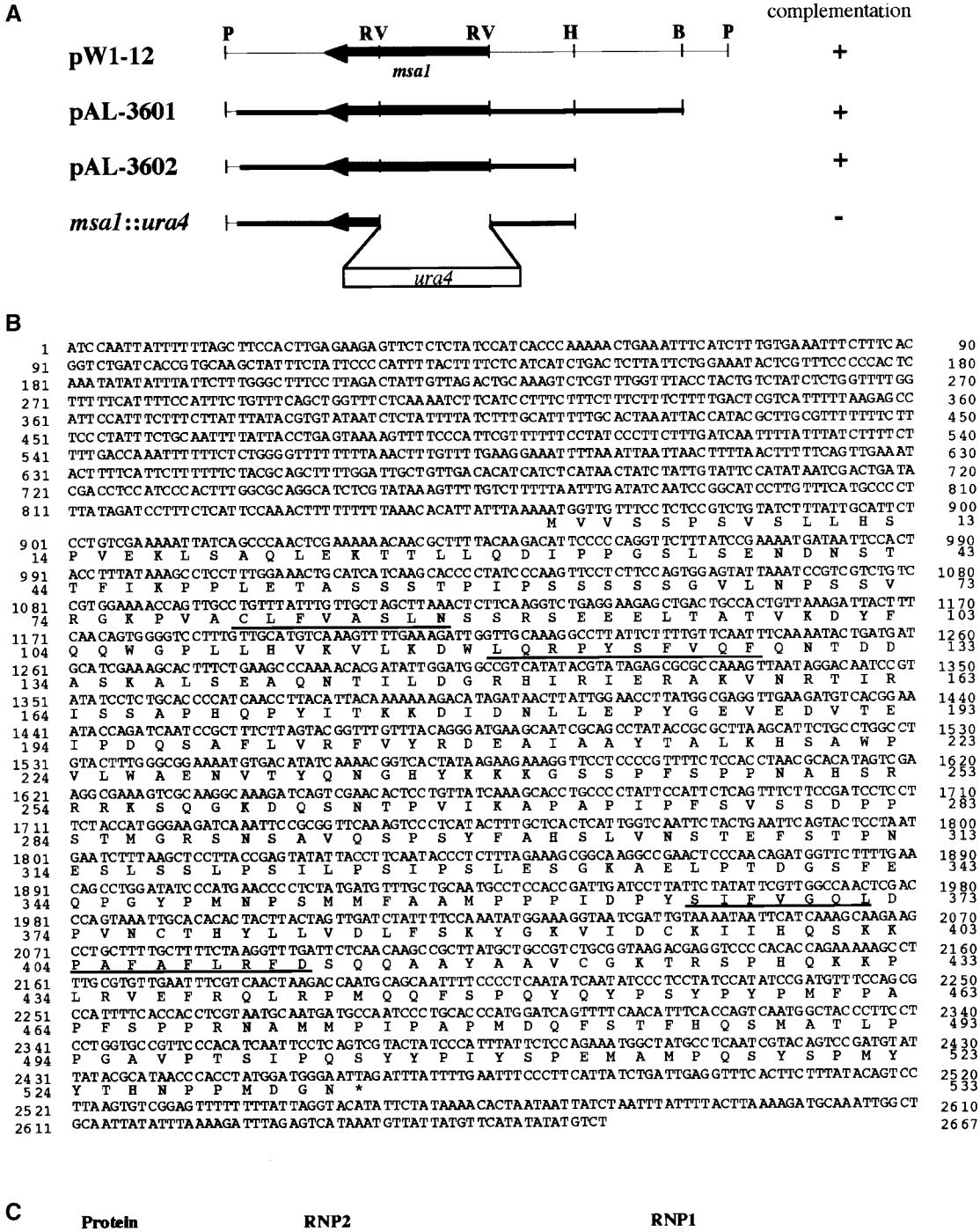

FIGURE 1.—Cloning, disruption, and the nucleotide sequence of the *msa1* gene. (A) Restriction map, subcloning, and disruption of the *msa1* gene. The arrow indicates the region and direction of the *msa1* ORF. Restriction sites for *Pst*I (P), *Eco*RV (RV), *Hin*dIII (H), and *Bam*HI (B) are shown. The complementation ability of each subclone was examined using HT3 (*h*^{90} *sam1 leu1-32*). Two *Pst*I sites are shown within the pWH5 vector. (B) Nucleotide sequence of *msa1* and its predicted amino acid sequence. The conserved amino acid sequences of RNP1 and RNP2 are underlined. (C) The RRM regions of *msa1* aligned with those of other RNA-binding proteins, which include *S. cerevisiae* Rim4, mouse RBM3, human Tra2, human SRp30c, and Arabidopsis SF2. Identical residues are shown as white letters on black background.

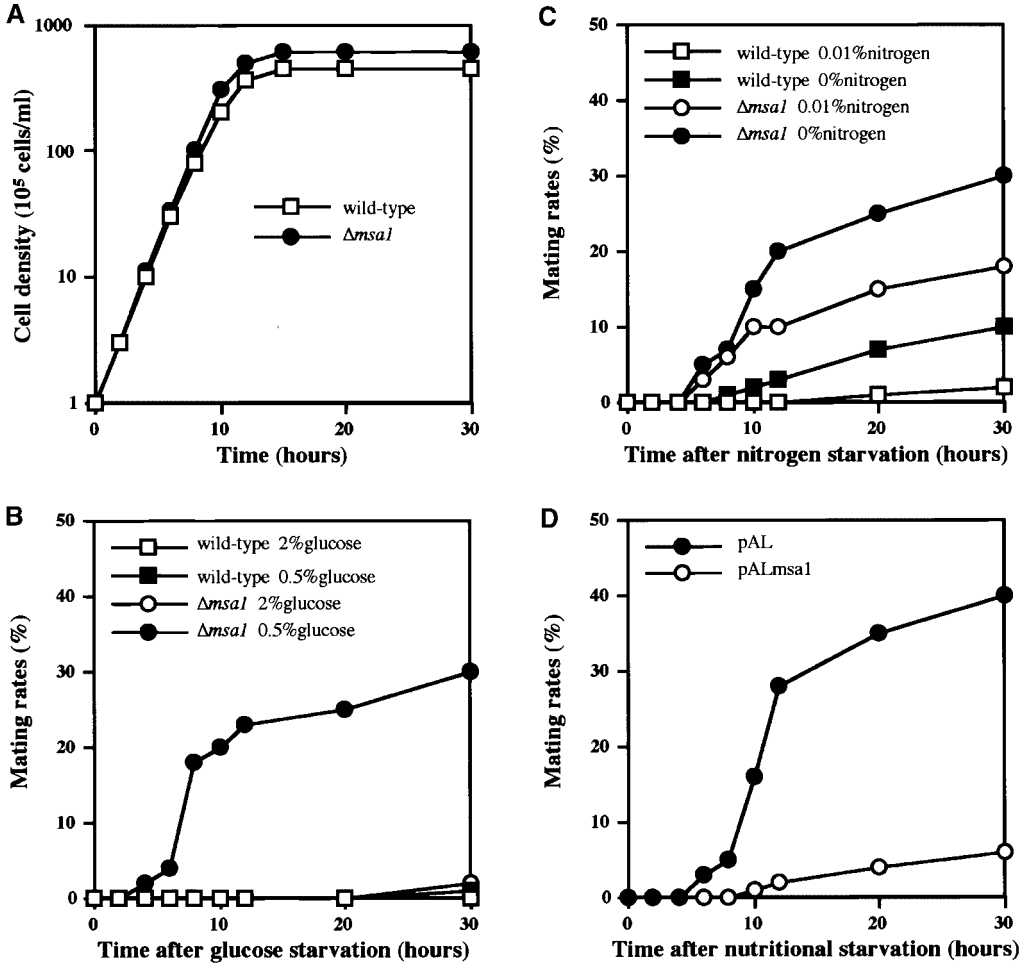

FIGURE 2.—Examining *msa1* function in cell growth and conjugation. (A) The h^- wild-type (SP319) cell and h^- *msa1*Δ (HT21) cell were grown in PM medium to midlog phase. Each strain was then inoculated into PM medium at 10^5 cells/ml and incubated at 30°. A portion of each culture was removed at the indicated times, and cells were counted using a hemocytometer. (B) Conjugation efficiency of *msa1*Δ cells at various glucose concentrations. The h^{90} wild-type (SP66) cell and h^{90} *msa1*Δ (HT11) cell were grown in PM medium to midlog phase. Each strain was then inoculated in PM medium containing 2% glucose or in PM medium containing 0.5% glucose and was incubated at 30° for the indicated time; the number of zygotes was counted. Mating rates shown here were calculated by dividing the number of zygotes (one zygote counted as two cells) by the number of total cells. (C) Conjugation efficiency of *msa1*Δ cells at various nitrogen concentrations. The h^{90} wild-type cell and h^{90} *msa1*Δ cell were grown in PM medium to midlog phase. Each strain was inoculated into PM medium containing 2% glucose and the indicated concentrations of nitrogen and incubated at 30°. At the indicated times, a portion of the cells was removed and the number of zygotes formed was counted. (D) Overexpression of *msa1* inhibits the efficiency of conjugation of the *msa1*Δ cell. The *msa1*Δ cells harboring pAL or pALmsa1 were incubated for the indicated times in nitrogen-free PM medium containing 0.5% glucose, and the number of zygotes were counted.

3HA fusion protein was also reduced during nitrogen starvation, but the timing of reduction is 3–4 hr later than that in mRNA expression, which we presumed to be the lag time of mRNA turnover (Figure 4B). These results indicate that the expression of *msa1* is inhibited by nitrogen starvation.

Meiosis-induced genes are derepressed in *msa1*Δ cells and repressed by *msa1* overexpression: Because signals

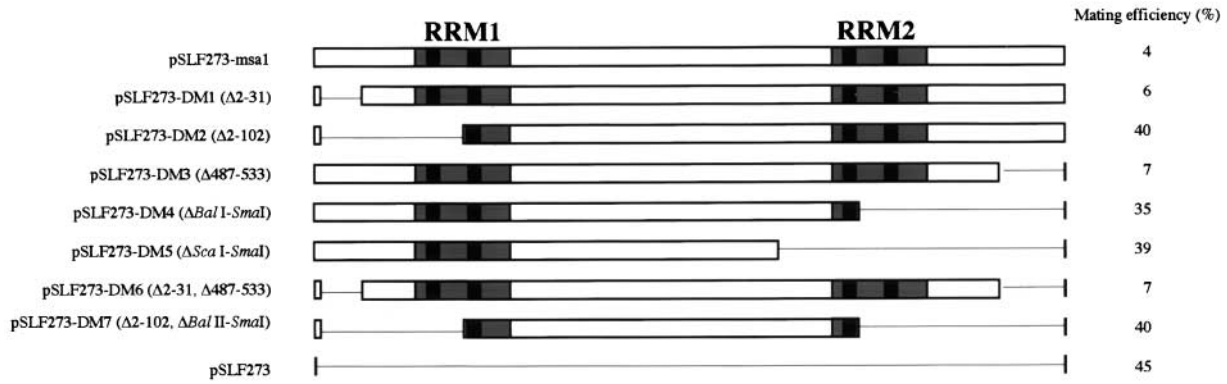

FIGURE 3.—Deletion analysis and identification of functionally essential regions of *msa1*. A series of *msa1* deletion mutants were cloned into the pSLF273 vector and HT11 (h^{90} *msa1::ura4*$^+$) was transformed with these plasmids. Mating efficiency was calculated by dividing the number of zygotes by the number of total cells.

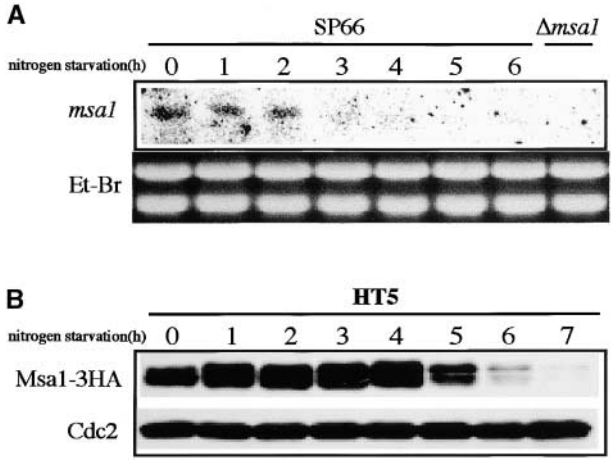

FIGURE 4.—Expression of *msa1*. (A) Northern blot analysis of *msa1* mRNA. Midlog phase cultures of SP66 (h^{90}) and HT11 (*msa1*Δ) were incubated in nitrogen-free medium for the periods indicated. Total RNA was extracted from each sample and analyzed with a hybridization probe for *msa1*. Ribosomal RNAs stained with ethidium bromide were used as equal loading controls. (B) Western blot analysis of Msa1-3HA fusion protein. A midlog phase culture of HT5 (h^{90} *msa1*-3HA<< *kanMX6*) was incubated in nitrogen-free medium. Cell-free extracts were subjected to SDS-PAGE and probed with anti-HA antibody. The Msa1-3HA fusion protein was detected as a band of ~70 kD. Immunodetection with anti-Cdc2 antibody was used as a loading control.

from nutrient starvation and pheromones are essential for both meiosis and conjugation in fission yeast (KITAMURA and SHIMODA 1991; OBARA *et al.* 1991; SUGIMOTO *et al.* 1991; TANAKA *et al.* 1993), we examined the mechanism used by the *msa1* gene product to interfere with signals from nutrient starvation and mating pheromones. We tested the regulation of expression of *mei2*, *rep1*, *ste11*, and *mam2* in wild-type and *msa1*Δ cells transferred to glucose-starved (0.5%) medium. These tested genes all positively regulate sexual differentiation; *mei2* encodes an RNA-binding protein essential for induction of meiosis (WATANABE and YAMAMOTO 1994); *rep1* encodes a zinc-finger protein required for onset of premeiotic DNA synthesis (SUGIYAMA *et al.* 1994); *mei2* and *rep1* are upregulated by *ste11*; and *mam2* encodes a mating-pheromone receptor and is upregulated by both *ste11* and mating pheromone (KITAMURA and SHIMODA 1991; SUGIMOTO *et al.* 1991; OZOE *et al.* 2002). Although *ste11* and *mei2* genes were expressed in both cell types after transfer to glucose-starved medium, expression was somewhat higher in *msa1*Δ cells than in wild-type cells (Figure 5A). Expression of both *mam2* and *rep1* was markedly higher and faster in *msa1*Δ cells than in wild-type cells.

Similarly, on the nitrogen-free medium, *ste11*, *mei2*, and *mam2* were induced significantly faster in the *msa1*Δ cells than in the wild-type cells (data not shown). These same genes were markedly repressed in the *msa1*Δ cells when *msa1* was overexpressed (Figure 5B). These data suggest that Msa1 represses both Ste11-regulated genes and the mating-pheromone signaling pathway.

The *msa1* function is independent of the cAMP pathway: We next examined whether the function of *msa1* was related to the cAMP pathway. Loss of function of *cyr1* (which encodes adenylate cyclase) usually results in hypersporulation (KAWAMUKAI *et al.* 1991); however, overexpression of *msa1* under the *nmt* promoter in *cyr1*-disruptant cells suppressed hypersporulation of the *cyr1* disruptants. The vector alone did not suppress hypersporulation (Figure 6A). A similar result was obtained with *pka1* disruptant cells (Figure 6B). Thus, overexpression of the *msa1* gene suppressed the effect of the loss of function of either *cyr1* or *pka1*.

Cellular cAMP levels regulate cell survival in stationary phase and *cgs1* codes for the regulatory subunit of PKA (DEVOTI *et al.* 1991). We therefore developed *cgs1* disruptant (*cgs1*Δ) and *msa1-cgs1* double-disruptant cells (*msa1*Δ *cgs1*Δ) and examined their phenotype. The *msa1*Δ *cgs1*Δ cells exhibited *cgs1*$^-$ phenotype and scarcely conjugated during nitrogen starvation (Figure 6D). In addition, *cgs1*Δ and *msa1*Δ *cgs1*Δ cells died after 3 days at G_0; conversely, *msa1*Δ and wild-type cells survived equally well at G_0 (Figure 6F). The same results, poor sporulation during nitrogen starvation and death after 3 days at G_0, were obtained with *pde1* disruptants (*pde1*Δ) and *msa1-pde1* double disruptants (*msa1*Δ *pde1*Δ; Figure 6, C and E). Thus, overexpression of *msa1* can reverse the hypermating phenotype of *cyr1* and *pka1* mutants, but deletion of *msa1* does not reverse the sterile phenotype of *cgs1* and *pde1* mutants. Sterility from *msa1* overexpression in *pka1*Δ cells is thought to be caused by inhibition downstream of Pka1. If Msa1 acted upstream of Pka1, Msa1 could not affect *pka1*Δ cells. Because *msa1*Δ *cgs1*Δ and *msa1*Δ *pde1*Δ double mutants have the same phenotype as *cgs1*Δ and *pde1*Δ (single) mutants, loss of functional Msa1 cannot overcome the hyperactive protein kinase A. These combined results suggest that Msa1 acts either downstream or independently of the cAMP pathway.

***msa1* is independent of the *spc1/sty1/phh1* pathway:** The Wis1-Spc1/Sty1/Phh1 pathway primarily mediates stress signals and also is partly required for *ste11* induction during the onset of sexual differentiation (YAMAMOTO *et al.* 1997). We investigated the possible relationship between *msa1* and this pathway. We constructed a homothallic *msa1* and *phh1* double mutant and compared it with each singly mutated cell for ability to perform conjugation. The *phh1* single mutant is not completely sterile but is nearly sterile (KATO *et al.* 1996), whereas the *msa1* deletion mutants conjugated efficiently even in nutrient-rich medium. The *msa1*Δ *phh1*Δ cells had an intermediate level of mating frequency and *ste11* expression, a result that indicated that *msa1* functions independently of the *phh1* pathway (Figure 7).

Loss of *msa1* can bypass the function of *ras1*: Msa1 significantly repressed the expression of *mam2* and *rep1* genes (Figure 5), genes that are induced by the phero-

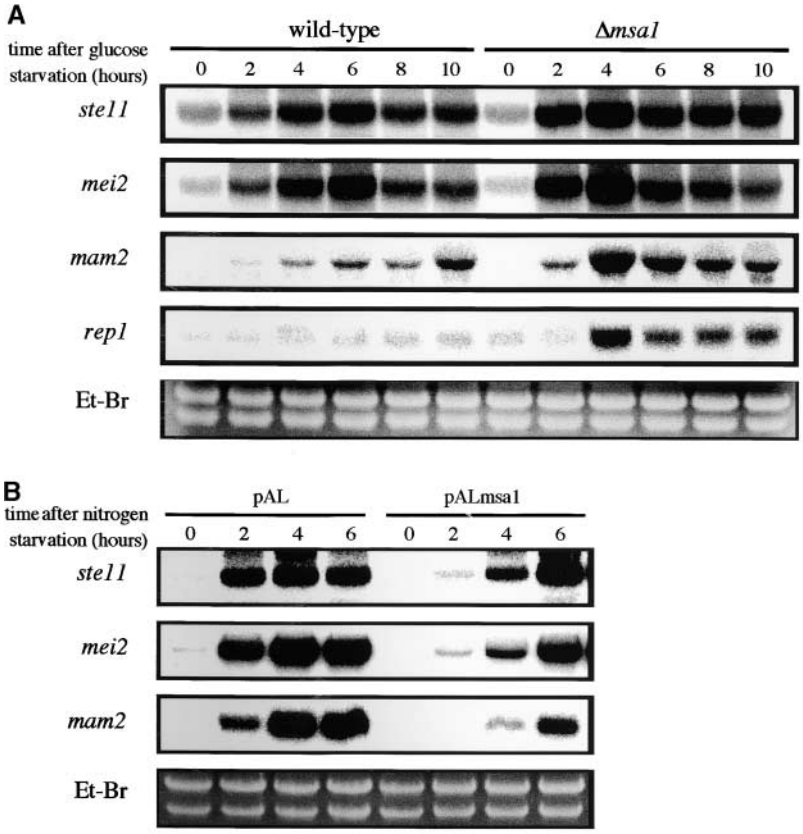

FIGURE 5.—Mating-pheromone-induced genes are derepressed in *msa1*Δ cells and repressed by *msa1* overexpression. (A) Northern blot analysis of cellular mRNA shows the time course for induction of *ste11*, *mei2*, *mam2*, and *rep1* transcripts in the wild-type cell (SP66) and the *msa1* deletion mutant (HT11) upon glucose starvation. Cells were grown in PM medium to midlog phase, washed, inoculated into low-glucose (0.5%) PM medium, and incubated for the indicated times. (B) Mating-pheromone-induced genes are repressed by *msa1* overexpression. The *msa1* deletion mutants harboring pAL or pALmsa1 were incubated for the indicated times in the nitrogen-free PM medium and cellular RNAs were prepared. The expression level of transcript was analyzed by Northern blotting. The equality of RNA loading was confirmed by staining with ethidium bromide (Et-Br).

mone-response pathway. To monitor negative regulation of the pheromone-response pathway, we examined the phenotype of *msa1* null cells, which also lacked genes for the pheromone-response pathway. Because *mam2* encodes the P-factor pheromone receptor in h^- cells and expression of *mam2* requires components of the pheromone-response pathway (XU *et al.* 1994), we constructed cells with mutations in both the *msa1* gene and different genes known to be important along the Ras-MAPK pathway and compared their expression of *mam2* by Northern blot analysis (Figure 8A). The levels of *mam2* expression in the disruptant for *ras1*, *byr2*, *byr1*, *spk1*, and *ste4* were very low, but the level of *mam2* expression in the *msa1*Δ *ras1*Δ and *msa1*Δ *ste4*Δ double mutants was higher than that of any of the single mutants. Interestingly, transcription of the *mam2* gene in the *msa1*Δ *ras1*Δ double mutant was similar to the transcription level in the wild-type cell (Figure 8A). In addition, the induction of *mam2* expression by the activated $ras1^{val17}$ gene was repressed by overexpression of *msa1* (Figure 8B). These results suggest that loss of *msa1* can bypass the function of *ras1* and that Msa1 negatively controls sexual differentiation (possibly) downstream of Ras1.

Because mating pheromone signaling is essential for meiosis in fission yeast (KITAMURA and SHIMODA 1991; TANAKA *et al.* 1993), we analyzed whether *msa1* can bypass the function of *ras1* in meiosis. All mutants were constructed in diploid form: *msa1*Δ *ras1*Δ cells sporulated, Δ*ras1* cells scarcely sporulated, and *msa1*Δ *byr2*Δ cells and *byr2*Δ cells appeared sterile (Figure 8C). The *gpa1*, *byr1*, *spk1*, and *ste4* mutants behaved in the same manner as the *byr2* mutant (data not shown). As shown in Table 3, the sporulation efficiency of *msa1*Δ *ras1*Δ diploid cells peaked ~90-fold higher than that of the *ras1*Δ diploid cell. However, deletion of *msa1* did not affect deletion-mutant *gpa1*, *byr2*, *byr1*, *spk1*, or *ste4* diploid cells. These results indicate that the loss of *msa1* can bypass the function of *ras1* in sporulation but not that of *gpa1*, *byr2*, *byr1*, *spk1*, or *ste4*, which suggests that Msa1 acts on the pheromone-response pathway downstream from Ras1.

Epistatic analysis of *msa1* and *rad24*: Rad24 acts as a negative regulator of the pheromone-response pathway by physically interacting with Byr2; this interaction affects the timing of Byr2 translocation in response to sexual differentiation signal (OZOE *et al.* 2002). We performed epistatic analysis to examine the relations of *msa1* and *rad24*. A homothallic *msa1-rad24* double mutant was constructed and was compared with each single mutant for mating efficiency (Figure 9A). The double mutant had a hypersporulation phenotype of *rad24*Δ in nitrogen-free medium. We compared expression of *mam2* between wild-type, *msa1*Δ, *rad24*Δ, and *msa1*Δ *rad24*Δ cells using Northern blot analysis (Figure 9B). The *mam2* mRNA began to appear in wild-type cells 6 hr after nitrogen starvation, 2 hr after nitrogen starvation in *msa1*Δ cells, and before nitrogen starvation in *rad24*Δ cells. The induction pattern of *mam2* mRNA in *msa1*Δ

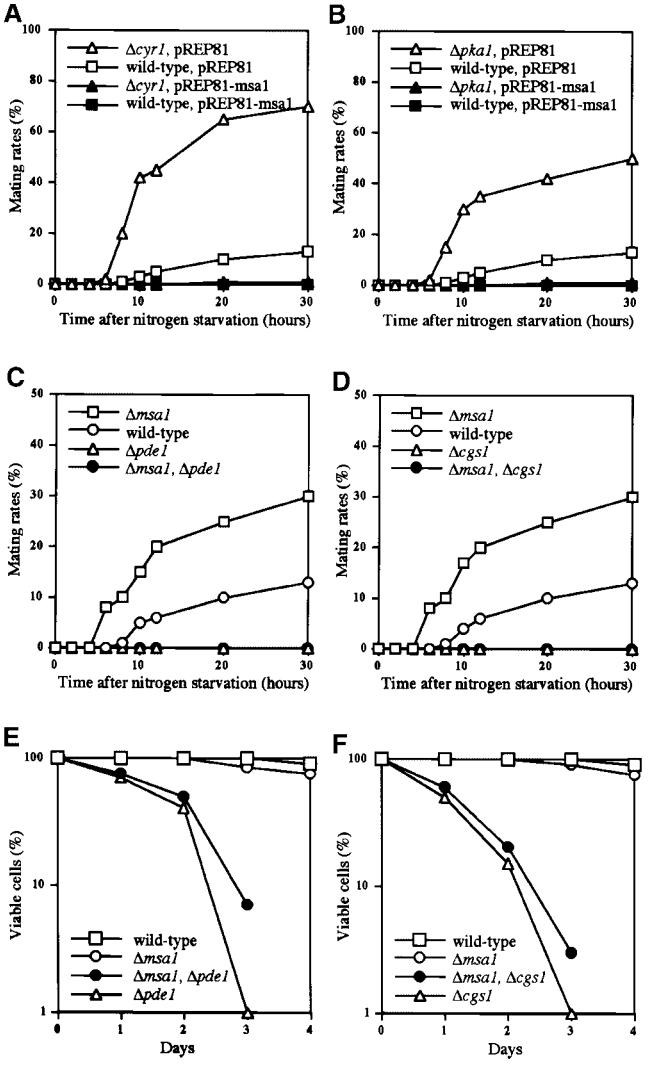

FIGURE 6.—*msa1* function is independent of the cAMP pathway. (A) Wild-type and *cyr1* deletion mutant cells were transformed with pREP81 or pREP81msa1. Transformed cells were grown in PM medium to midlog phase, washed, inoculated in nitrogen-free PM medium, incubated for the indicated times, and mating rates were calculated. (B) Wild-type and *pka1* disruptant cells were transformed with pREP81 or pREP 81msa1. Transformed cells were grown in PM medium to midlog phase, washed, inoculated in nitrogen-free PM medium, incubated for the indicated times, and mating rates were calculated. (C and D) Wild type and each disruptant were grown in PM medium to midlog phase, washed, inoculated in nitrogen-free PM medium, incubated for the indicated times, and mating rates were calculated. Cells used were the wild type (SP66), *msa1*Δ (HT11), *pde1*Δ (JZ666), *cgs1*Δ (JZ858), *msa1*Δ *pde1*Δ (HT43), and *msa1*Δ *cgs1*Δ (HT58). (E and F) Cells were grown to saturation (1×10^7 cells/ml; day 0) and incubated for an additional 4 days (days 1–4) in PM medium. A portion of the culture was removed each day and plated onto YEA plates for cultivation at 30°. The colonies formed were counted after 3 days.

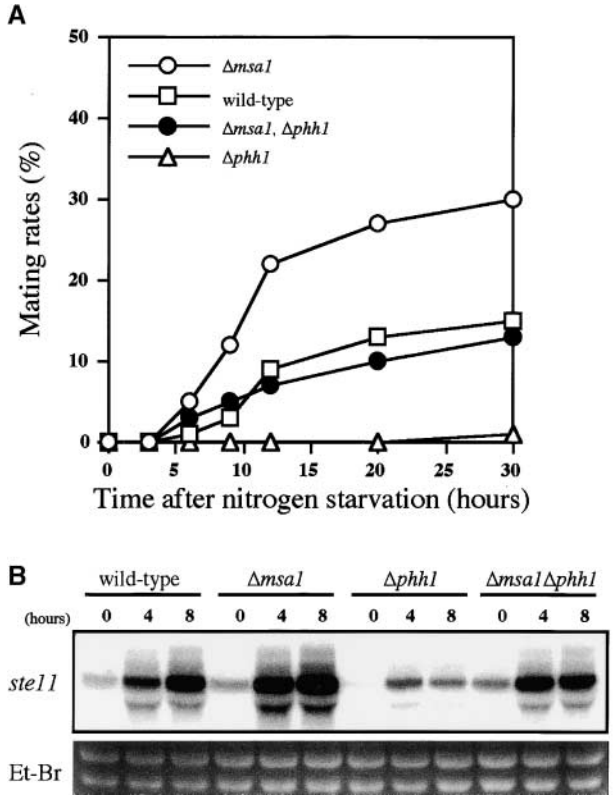

FIGURE 7.—*msa1* functions independently of the *phh1*-stress MAP kinase pathway. (A) The wild-type (SP66), *msa1*Δ (HT11), *phh1*Δ (TK105), and *msa1*Δ *phh1*Δ (HT76) cells were grown in PM medium to midlog phase. Each strain was then inoculated into nitrogen-free PM medium and incubated at 30° for the indicated times, and the number of zygotes was counted. (B) Expression of *ste11* during nitrogen starvation in the wild-type, *msa1*Δ, *phh1*Δ, or *msa1*Δ *phh1*Δ cells. Each strain was inoculated into nitrogen-free PM medium and incubated at 30° for the indicated times. Total RNA was prepared, and 10 μg was applied to each lane for Northern blot analysis. Et-Br, ethidium bromide.

*rad24*Δ cells was similar to that of *rad24*Δ cells, a result that suggests that *msa1* function is dependent on that of *rad24*.

Because the loss of function of *rad24* in cells showed a hypersporulation phenotype (OZOE *et al.* 2002), we next examined whether the function of *msa1* is related to the function of *rad24*. Wild type, *msa1*Δ, and *rad24*Δ were transformed with pREP81 or pREP81msa1 and mating frequencies were assayed (Figure 9C). Overexpression of the *msa1* gene under the *nmt1* promoter did not suppress well the hypersporulated phenotype of the *rad24* disruptant. Conversely, overexpression of *rad24* under the *nmt1* promoter suppressed the hypersporulated phenotype of a deletion mutant of *msa1* (Figure 9D).

***msa1* is independent of *msa2/nrd1*:** Of the two independent clones (*msa1*$^+$ and *msa2*$^+$) that were identified as multicopy suppressors of *sam1*, one gene (*msa2*) was identical to the *nrd1* gene. Msa2/Nrd1 is an RNA-binding protein that blocks commitment to conjugation until cells reach a critical level of nutrient starvation (TSUKAHARA *et al.* 1998). We independently constructed a *msa2/nrd1*-deleted strain and confirmed that it behaved as reported (TSUKAHARA *et al.* 1998). Cells that lack

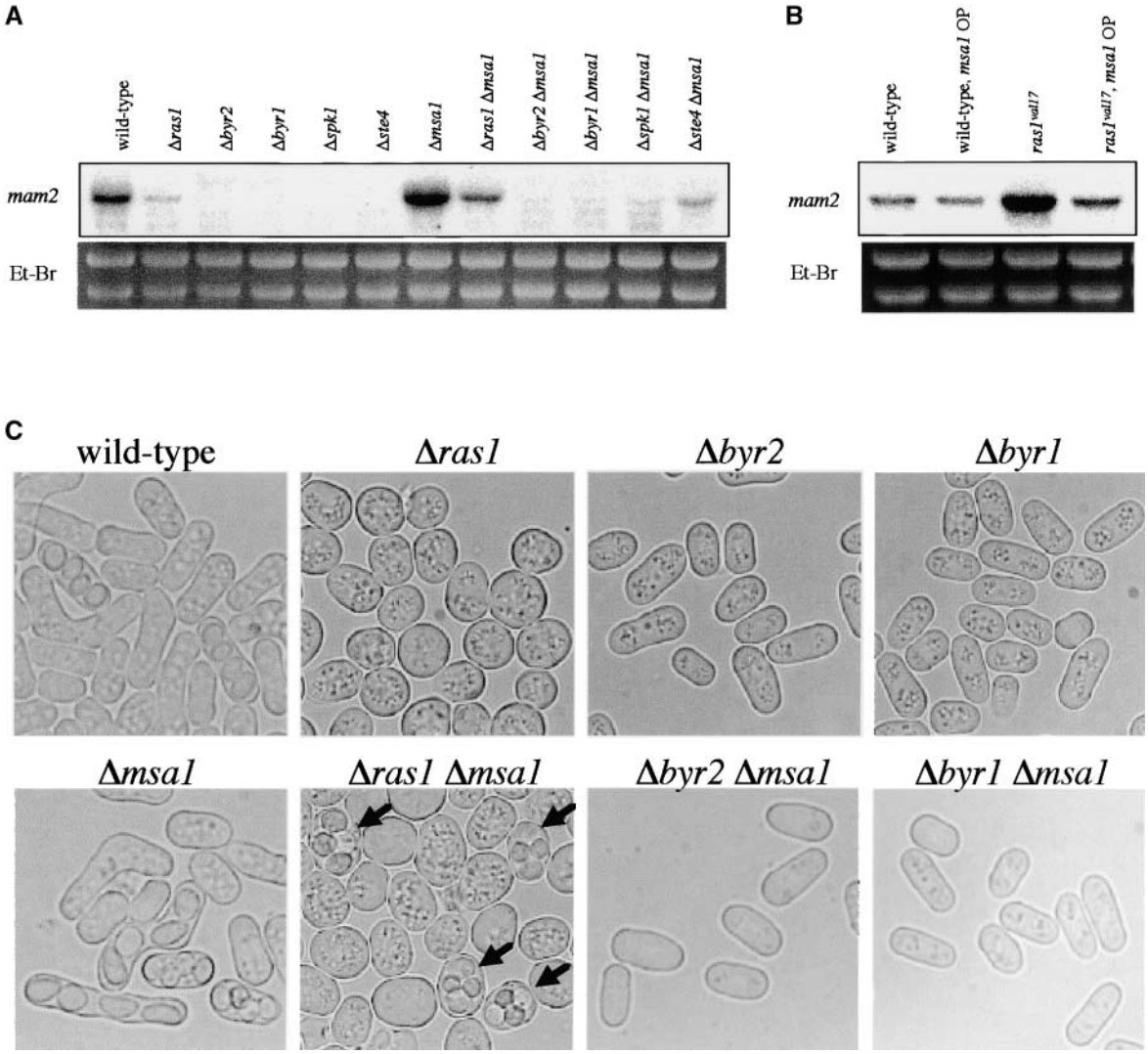

FIGURE 8.—Loss of *msa1* can bypass the function of *ras1*. (A) The level of *mam2* transcript was examined 6 hr after nitrogen starvation in SP66 (wild type), SPRU (*ras1*Δ), SPSU (*byr2*Δ), SPBU (*byr1*Δ), SPKU (*spk1*Δ), SPFU (*ste4*Δ), HT11 (*msa1*Δ), HT95 (*ras1*Δ *msa1*Δ), HT93 (*byr2*Δ *msa1*Δ), HT91 (*byr1*Δ *msa1*Δ), HT97 (*spk1*Δ *msa1*Δ), and HT99 (*ste4*Δ *msa1*Δ) strains. Total RNA was prepared from each strain and analyzed by Northern blot. (B) Wild-type cells and strain *ras1*^{val17} were transformed with either pREP41 or pREP41msa1. Transformed cells were cultured in nitrogen-free PM liquid medium for 6 hr and total RNA was analyzed by Northern blot. (C) Photomicrographs of homothallic diploid wild-type and *msa1*-null mutants. Arrows indicate sporulated diploid cells.

Msa2/Nrd1 resemble those that lack Msa1 in that they conjugate without starvation. The phenotypic similarity led us to perform epistatic analysis of the two genes. A homothallic *msa1-msa2* double mutant was constructed by crossing and was compared with each single deletion mutant for mating efficiency. The double-mutant cells had greater conjugation efficiency in nitrogen-free medium than either of the single-mutant strains (Figure 10), suggesting that *msa1* functions independently of *msa2/nrd1*.

DISCUSSION

We isolated two genes (*msa1* and *msa2/nrd1*) that negatively regulate sexual differentiation of *S. pombe*. Nrd1 has been characterized (TSUKAHARA *et al.* 1998) and Msa1 is analyzed in this study. Both proteins have the RNA-binding motifs that are essential for their functions (Figure 3; TSUKAHARA *et al.* 1998). In the regulation of sexual differentiation in fission yeast, Mei2, which is essential for meiosis and binds to meiRNA, is the best-characterized RNA-binding protein (WATANABE and YAMAMOTO 1994). Sla1, another RNA-binding protein, was recently characterized as the inducer of sexual differentiation when truncated (TANABE *et al.* 2003, 2004). Thus, at least four RNA-binding proteins are known to function as regulators of sexual differentiation in fission yeast. *S. cerevisiae* Rim4 (SOUSHOKO and MITCHELL 2000) is the most homologous protein of Msa1. It is interesting to note that Rim4 is a presumed

TABLE 3

Sporulation efficiency of *msa1* null mutants

Strain	Sporulation efficiency (%)[a]
Wild-type and single mutants	
HT101 (wild type)	38.5 ± 6.7
HT124 (*gpa1*Δ diploid)	0
HT125 (*ras1*Δ diploid)	0.1
HT126 (*byr2*Δ diploid)	0
HT127 (*byr1*Δ diploid)	0
HT128 (*spk1*Δ diploid)	0
HT129 (*ste4*Δ diploid)	0
msa1-null mutants	
HT102 (*msa1*Δ diploid)	51.4 ± 7.2
HT108 (*msa1*Δ *gpa1*Δ diploid)	0
HT105 (*msa1*Δ *ras1*Δ diploid)	9.5 ± 5.2
HT104 (*msa1*Δ *byr2*Δ diploid)	0
HT103 (*msa1*Δ *byr1*Δ diploid)	0
HT106 (*msa1*Δ *spk1*Δ diploid)	0
HT107 (*msa1*Δ *ste4*Δ diploid)	0

[a] Average of at least three separate measurements.

RNA-binding protein that positively regulates meiosis in opposition to the function of Msa1, which negatively regulates meiosis.

The *msa1* deletion mutant conjugated with great efficiency in either nitrogen-free or low-glucose medium compared with the wild-type cell (Figure 2). The ability of *S. pombe* cells to sense nitrogen and glucose levels and to thus regulate sexual differentiation is mediated partly by the cAMP pathway and partly by the stress-responsive MAP kinase pathway. Overexpression of *msa1* largely controlled hypersporulation in *cyr1*Δ and *pka1*Δ cells, and epistatic analysis showed that the function of *msa1* is either downstream or independent of the cAMP pathway (Figure 6). The inability of *msa1* deletion to suppress the phenotype of *cgs1* and *pde1* mutants suggested that *msa1* functions independently of the cAMP pathway. In the homothallic wild-type cell, the *msa1* gene is consistently and immediately expressed after cells are shifted from nitrogen-rich to nitrogen-free medium, but this expression was repressed after conjugation started (Figure 4, A and B, 6 hr after nitrogen starvation). Because we did not observe phenotypes other than involvement of sexual differentiation in the *msa1* deletion mutant and its combination with several mutants, the primary function of Msa1 is thought to be limited to sexual differentiation. A hypothetical function of Msa1 is as the threshold sensor, sensing the critical nutrient conditions independently of the cAMP pathway and transferring this signal to some factor or factors involved in sexual differentiation.

Msa1 controls expression of several genes that are necessary for the induction of sexual differentiation. Expression of genes usually induced by nutritional starvation, *ste11* and *mei2*, is mildly increased in *msa1*Δ cells compared with wild-type cells. Expression of genes usually induced by Ste11 and the mating-pheromone signals, *mam2* and *rep1*, is significantly increased in *msa1*Δ cells compared with wild-type cells (Figure 5). Msa1 influenced signaling of both nutritional starvation and mating pheromones. These results suggest to us that the primary function of Msa1 is to control the expression of Ste11-regulated genes through the pheromone-response pathway. DNA microarray experiments to compare gene expression level in wild-type and *msa1*Δ cells indicated that the pheromone-inducible genes like *mfm1*, *mfm3*, and *map2* were highly induced in the *msa1*Δ cells compared with wild type (our preliminary observation). Although wild-type diploid cells commonly formed azygotic spores, we observed that the zygotic spores increased in the diploid *msa1*Δ mutants compared with the wild-type diploid cells (data not shown). This also suggests that loss of functional Msa1 deregulates pheromone signaling.

The mating pheromone-response signal is transferred by the MAP kinase cascade, which consists of Byr2, Byr1, and Spk1 (Nadin-Davis and Nasim 1988; Toda *et al.* 1991; Wang *et al.* 1991; Neiman *et al.* 1993). Many proteins positively regulate this cascade, including Ras1, Gpa1, and Ste4 (Wang *et al.* 1991; Xu *et al.* 1994; Barr *et al.* 1996; Tu *et al.* 1997), and recently two negative regulators, Rad24 and Rad25, were reported (Ozoe *et al.* 2002). Because the deletion of *msa1* reversed the phenotype seen in *ras1*-deletion mutants (Figure 8) and increased the expression of mating-pheromone-induced genes, and because the expression of *msa1* reversed the hypersporulation seen with $Ras1^{Val17}$ (Figure 8), we further suggest that Msa1 acts as a negative regulator in the mating pheromone-response pathway.

Similar to cells with combined *rad24* and *ras1* deletion (Ozoe *et al.* 2002), deletion of *msa1* in the *ras1*Δ cells rescues *mam2* expression. But *msa1* is not epistatic to *rad24*. Because the 14-3-3 homologs, Rad24 and Rad25, have multiple targets that include Cdc25, Chk1, Plc1, Mei2, Ste11, Cap1, and Byr2 (Andoh *et al.* 1998; Chen *et al.* 1999; Lopez-Girona *et al.* 1999; Zhou *et al.* 2000; Kitamura *et al.* 2001; Ozoe *et al.* 2002; Sato *et al.* 2002), it is difficult to elucidate the relations between *msa1* and *rad24*. However, deletion of *msa1* did not increase hypersporulation in the *rad24* mutant, a result that suggests that the point of action of Msa1 is within the target of Rad24.

Several negative regulators that control sexual differentiation have been reported. Pat1 is the most essential regulator of sexual differentiation and works at several points during conjugation and meiosis (Nielsen and Egel 1990; Watanabe and Yamamoto 1994). Cig2/Cyc17, a B-type cyclin, promotes the cell cycle start and negatively regulates differentiation through cell cycle control (Obara-Ishihara and Okayama 1994; Mondesert *et al.* 1996). The 14-3-3 proteins are thought to play important roles in conjugation and meiosis through

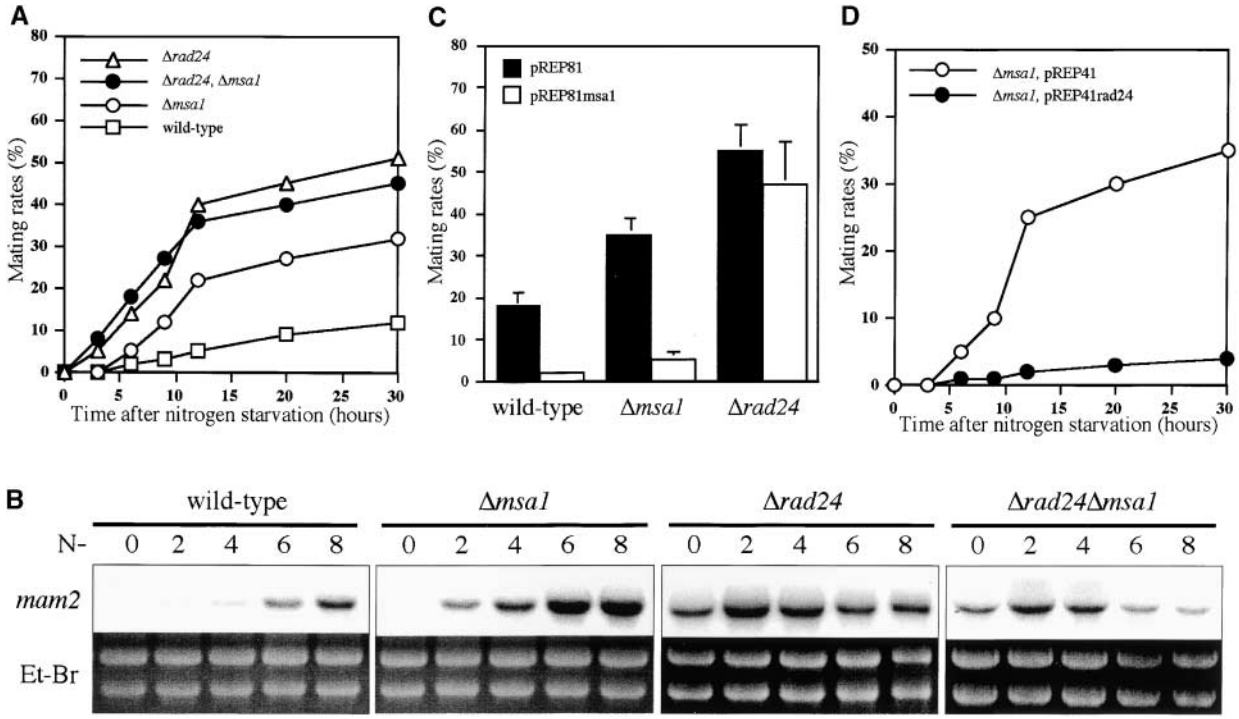

FIGURE 9.—Epistatic analysis of *msa1* and *rad24*. (A) Wild-type, *msa1*Δ, *rad24*Δ, and *mas1*Δ *rad24*Δ cells were grown to log phase in PM medium, transferred into nitrogen-free PM medium, and mating rates were calculated. (B) Expression of *mam2* during nitrogen starvation in the wild-type, *msa1*Δ, *rad24*Δ, or *mas1*Δ *rad24*Δ cells. Each strain was inoculated into nitrogen-free PM medium and incubated at 30° for the indicated times. Total RNA was prepared and Northern blot analysis was performed. Et-Br, ethidium bromide. (C) Wild-type, *msa1*Δ, and *rad24*Δ strains were transformed with pREP81 or pREP81msa1. Transformed cells were inoculated into nitrogen-free PM liquid medium and then cultured for 24 hr at 30°, and mating rates were calculated. Three independent samples were measured. (D) The *msa1*Δ cells were transformed with pREP41 or pREP41rad24. Transformed cells were inoculated into nitrogen-free PM liquid medium and then cultured for the times indicated, and mating rates were calculated.

different acting points (KITAMURA *et al.* 2001; OZOE *et al.* 2002). Pac1 and Pac2 also regulate sexual differentiation by repressing *ste11* expression using unknown mechanisms (IINO *et al.* 1991; KUNITOMO *et al.* 1995). An RNA-binding protein, Nrd1/Msa2, which we independently showed worked with Msa1, is a negative regulator of sexual differentiation, but its role is also not yet clear (TSUKAHARA *et al.* 1998). We described the new factor, Msa1, whose function is mainly as a negative regulator of sexual differentiation, possibly through the regulation of the pheromone-signaling-mediated pathway. The former three factors are relatively well characterized, but the functional points of the latter four are still obscure. Because sexual differentiation is undoubtedly a complicated process in cell events, it will be necessary to characterize each unknown factor one by one.

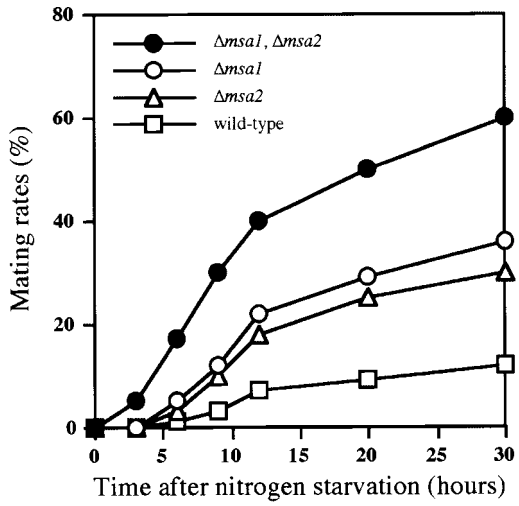

FIGURE 10.—*msa1* is independent of *msa2/nrd1*. Wild-type, *msa1*Δ, *msa2*Δ/*nrd1* deletion mutant, and *msa1*Δ *msa2*Δ cells were grown to log phase in PM medium, transferred into nitrogen-free PM medium for the times indicated, and mating rates were calculated.

We thank D. Beach, M. Yamamoto, Y. Watanabe, T. Kato, and K. Kitamura for strains and plasmids and E. Uchida and K. Nakasato for technical assistance. This work was supported by grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

LITERATURE CITED

ALFA, C., P. FANTES, J. HYAMS, M. MCLEOD and E. WARBRICK, 1993 *Experiments with Fission Yeast: A Laboratory Course Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

ANDOH, T., T. J. KATO, Y. MATSUI and A. TOH-E, 1998 Phosphoinositide-specific phospholipase C forms a complex with 14-3-3 pro-

teins and is involved in expression of UV resistance in fission yeast. Mol. Gen. Genet. **258:** 139–147.

Barr, M. M., H. Tu, L. Van Aelst and M. Wigler, 1996 Identification of Ste4 as a potential regulator of Byr2 in the sexual response pathway of *Schizosaccharomyces pombe*. Mol. Cell. Biol. **16:** 5597–5603.

Chen, L., T. H. Liu and N. C. Walworth, 1999 Association of Chk1 with 14-3-3 proteins is stimulated by DNA damage. Genes Dev. **13:** 675–685.

Devoti, J., G. Seydoux, D. Beach and M. McLeod, 1991 Interaction between *ran1*$^+$ protein kinase and cAMP-dependent protein kinase as negative regulators of fission yeast meiosis. EMBO J. **10:** 3759–3768.

Forsburg, S. L., and D. A. Sherman, 1997 General purpose tagging vectors for fission yeast. Gene **191:** 191–195.

Fukui, Y., T. Kozasa, Y. Kaziro, T. Takeda and M. Yamamoto, 1986 Role of a ras homolog in the life cycle of *Schizosaccharomyces pombe*. Cell **44:** 329–336.

Gutz, H., H. Heslot, U. Leupold and N. Loprieno, 1974 *Handbook of Genetics*. Plenum Press, New York.

Higuchi, T., Y. Watanabe and M. Yamamoto, 2002 Protein kinase A regulates sexual differentiation and gluconeogenesis through phosphorylation of the Zn finger transcriptional activator Rst2p in fission yeast. Mol. Cell. Biol. **22:** 1–11.

Iino, Y., A. Sugimoto and M. Yamamoto, 1991 *S. pombe pac1*$^+$, whose overexpression inhibits sexual development, encodes a ribonuclease III-like RNase. EMBO J. **10:** 221–226.

Isshiki, T., N. Mochizuki, T. Maeda and M. Yamamoto, 1992 Characterization of a fission yeast gene, *gpa2*, that encodes a G alpha subunit involved in the monitoring of nutrition. Genes Dev. **6:** 2455–2462.

Kaiser, C., S. Michaelis and A. Mitchell, 1994 *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Kanoh, J., Y. Watanabe, M. Ohsugi, Y. Iino and M. Yamamoto, 1996 *Schizosaccharomyces pombe gad7*$^+$ encodes a phosphoprotein with a bZIP domain, which is required for proper G1 arrest and gene expression under nitrogen starvation. Genes Cells **1:** 391–408.

Katayama, S., F. Ozoe, R. Kurokawa, K. Tanaka, T. Nakagawa *et al.*, 1996 Genetic analysis of the *sam* mutations, which induce sexual development with no requirement for nutritional starvation in fission yeast. Biosci. Biotechnol. Biochem. **60:** 994–999.

Kato, T., Jr., K. Okazaki, H. Murakami, S. Stettler, P. A. Fantes *et al.*, 1996 Stress signal, mediated by a Hog1-like MAP kinase, controls sexual development in fission yeast. FEBS Lett. **378:** 207–212.

Kawamukai, M., 1999 Isolation of a novel gene, *moc2*, encoding a putative RNA helicase as a suppressor of sterile strains in *Schizosaccharomyces pombe*. Biochim. Biophys. Acta **1446:** 93–101.

Kawamukai, M., K. Ferguson, M. Wigler and D. Young, 1991 Genetic and biochemical analysis of the adenylyl cyclase of *Schizosaccharomyces pombe*. Cell. Regul. **2:** 155–164.

Kawamukai, M., J. Gerst, J. Field, M. Riggs, L. Rodgers *et al.*, 1992 Genetic and biochemical analysis of the adenylyl cyclase-associated protein, cap, in *Schizosaccharomyces pombe*. Mol. Biol. Cell **3:** 167–180.

Kitamura, K., and C. Shimoda, 1991 The *Schizosaccharomyces pombe mam2* gene encodes a putative pheromone receptor which has a significant homology with the *Saccharomyces cerevisiae* Ste2 protein. EMBO J. **10:** 3743–3751.

Kitamura, K., S. Katayama, S. Dhut, M. Sato, Y. Watanabe *et al.*, 2001 Phosphorylation of Mei2 and Ste11 by Pat1 kinase inhibits sexual differentiation via ubiquitin proteolysis and 14-3-3 protein in fission yeast. Dev. Cell **1:** 389–399.

Kon, N., S. C. Schroeder, M. D. Krawchuk and W. P. Wahls, 1998 Regulation of the Mts1-Mts2-dependent ade6-M26 meiotic recombination hot spot and developmental decisions by the Spc1 mitogen-activated protein kinase of fission yeast. Mol. Cell. Biol. **18:** 7575–7583.

Krawchuk, M. D., and W. P. Wahls, 1999 High-efficiency gene targeting in *Schizosaccharomyces pombe* using a modular, PCR-based approach with long tracts of flanking homology. Yeast **15:** 1419–1427.

Kunitomo, H., A. Sugimoto, C. R. Wikinson and M. Yamamoto, 1995 *Schizosaccharomyces pombe pac2*$^+$ controls the onset of sexual development via a pathway independent of the cAMP cascade. Curr. Genet. **28:** 32–38.

Kunitomo, H., T. Higuchi, Y. Iino and M. Yamamoto, 2000 A zinc-finger protein, Rst2p, regulates transcription of the fission yeast *ste11*$^+$ gene, which encodes a pivotal transcription factor for sexual development. Mol. Biol. Cell **11:** 3205–3217.

Li, P., and M. McLeod, 1996 Molecular mimicry in development: identification of *ste11*$^+$ as a substrate and *mei3*$^+$ as a pseudosubstrate inhibitor of *ran1*$^+$ kinase. Cell **87:** 869–880.

Lopez-Girona, A., B. Furnari, O. Mondesert and P. Russell, 1999 Nuclear localization of Cdc25 is regulated by DNA damage and a 14-3-3 protein. Nature **397:** 172–175.

Maeda, T., N. Mochizuki and M. Yamamoto, 1990 Adenylyl cyclase is dispensable for vegetative cell growth in the fission yeast *Schizosaccharomyces pombe*. Proc. Natl. Acad. Sci. USA **87:** 7814–7818.

Maeda, T., Y. Watanabe, H. Kunitomo and M. Yamamoto, 1994 Cloning of the pka1 gene encoding the catalytic subunit of the cAMP-dependent protein kinase in *Schizosaccharomyces pombe*. J. Biol. Chem. **269:** 9632–9637.

Maundrell, K., 1990 *nmt1* of fission yeast: a highly transcribed gene completely repressed by thiamine. J. Biol. Chem. **265:** 10857–10864.

Maundrell, K., 1993 Thiamine-repressible expression vectors pREP and pRIP for fission yeast. Gene **123:** 127–130.

Mochizuki, N., and M. Yamamoto, 1992 Reduction in the intracellular cAMP level triggers initiation of sexual development in fission yeast. Mol. Gen. Genet. **233:** 17–24.

Mondesert, O., C. H. Mcgowan and P. Russell, 1996 Cig2, a B-type cyclin, promotes the onset of S in *Schizosaccharomyces pombe*. Mol. Cell. Biol. **16:** 1527–1533.

Nadin-Davis, S. A., and A. Nasim, 1988 A gene which encodes a predicted protein kinase can restore some functions of the *ras* gene in fission yeast. EMBO J. **7:** 985–993.

Neiman, A. M., B. J. Stevenson, H. P. Xu, G. F. Sprague, Jr., I. Herskowitz *et al.*, 1993 Functional homology of protein kinases required for sexual differentiation in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* suggests a conserved signal transduction module in eukaryotic organisms. Mol. Biol. Cell **4:** 107–120.

Nielsen, O., and R. Egel, 1990 The Pat1 protein kinase controls transcription of the mating-type genes in fission yeast. EMBO J. **9:** 1401–1406.

Obara, T., M. Nakafuku, M. Yamamoto and Y. Kaziro, 1991 Isolation and characterization of a gene encoding a G-protein alpha subunit from *Schizosaccharomyces pombe*: involvement in mating and sporulation pathways. Proc. Natl. Acad. Sci. USA **88:** 5877–5881.

Obara-Ishihara, T., and H. Okayama, 1994 A B-type cyclin negatively regulates conjugation via interacting with cell cycle "start" genes in fission yeast. EMBO J. **13:** 1863–1872.

Okazaki, N., K. Okazaki, K. Tanaka and H. Okayama, 1991 The *ste4*$^+$ gene, essential for sexual differentiation of *Schizosaccharomyces pombe*, encodes a protein with a leucine zipper motif. Nucleic Acids Res. **19:** 7043–7047.

Ozoe, F., R. Kurokawa, Y. Kobayashi, H. T. Jeong, K. Tanaka *et al.*, 2002 The 14-3-3 proteins Rad24 and Rad25 negatively regulate Byr2 by affecting its localization in *Schizosaccharomyces pombe*. Mol. Cell. Biol. **22:** 7105–7119.

Prentice, H. L., 1992 High efficiency transformation of *Schizosaccharomyces pombe* by electroporation. Nucleic Acids Res. **20:** 621.

Sambrook, J., E. F. Fritsch and T. Maniatis (Editors), 1989 *Molecular Cloning: A Laboratory Manual*, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Samejima, I., S. Mackie and P. A. Fantes, 1997 Multiple modes of activation of the stress-responsive MAP kinase pathway in fission yeast. EMBO J. **16:** 6162–6170.

Samejima, I., S. Mackie, E. Warbrick, R. Weisman and P. A. Fantes, 1998 The fission yeast mitotic regulator *win1*$^+$ encodes an MAP kinase kinase kinase that phosphorylates and activates Wis1 MAP kinase kinase in response to high osmolarity. Mol. Biol. Cell **9:** 2325–2335.

Sato, M., Y. Watanabe, Y. Akiyoshi and M. Yamamoto, 2002 14-3-3 protein interferes with the binding of RNA to the phosphorylated form of fission yeast meiotic regulator Mei2p. Curr. Biol. **12:** 141–145.

Shiozaki, K., and P. Russell, 1996 Conjugation, meiosis, and the osmotic stress response are regulated by Spc1 kinase through Atf1 transcription factor in fission yeast. Genes Dev. **10:** 2276–2288.

Soushoko, M., and A. P. Mitchell, 2000 An RNA-binding protein homologue that promotes sporulation-specific gene expression in *Saccharomyces cerevisiae*. Yeast **16:** 631–639.

Sugimoto, A., Y. Iino, T. Maeda, Y. Watanabe and M. Yamamoto, 1991 *Schizosaccharomyces pombe ste11*$^+$ encodes a transcription factor with an HMG motif that is a critical regulator of sexual development. Genes Dev. **5:** 1990–1999.

Sugiyama, A., K. Tanaka, K. Okazaki, H. Nojima and H. Okayama, 1994 A zinc finger protein controls the onset of premeiotic DNA synthesis of fission yeast in a Mei2-independent cascade. EMBO J. **13:** 1881–1887.

Takahashi, K., S. Murakami, Y. Chikashige, H. Funabiki, O. Niwa *et al.*, 1992 A low copy number central sequence with strict symmetry and unusual chromatin structure in fission yeast centromere. Mol. Biol. Cell **3:** 819–835.

Takeda, T., T. Toda, K. Kominami, A. Kohnosu, M. Yanagida *et al.*, 1995 *Schizosaccharomyces pombe atf1*$^+$ encodes a transcription factor required for sexual development and entry into stationary phase. EMBO J. **14:** 6193–6208.

Tanabe, K., N. Ito, T. Wakuri, F. Ozoe, M. Umeda *et al.*, 2003 Sla1, a *Schizosaccharomyces pombe* homolog of the human La protein, induces ectopic meiosis when its C-terminus is truncated. Eukaryot. Cell **2:** 1274–1287.

Tanabe, K., K. Tanaka, H. Matsuda and M. Kawamukai, 2004 Truncated Sla1 induces haploid meiosis through the Pat1-Mei2 system in fission yeast. Biosci. Biotechnol. Biochem. **68:** 226–270.

Tanaka, K., J. Davey, Y. Imai and M. Yamamoto, 1993 *Schizosaccharomyces pombe map3*$^+$ encodes the putative M-factor receptor. Mol. Cell. Biol. **13:** 80–88.

Tanaka, K., J. Nishide, K. Okazaki, H. Kato, O. Niwa *et al.*, 1999 Characterization of a fission yeast SUMO-1 homologue, pmt3p, required for multiple nuclear events, including the control of telomere length and chromosome segregation. Mol. Cell. Biol. **12:** 8660–8672.

Toda, T., M. Shimanuki and M. Yanagida, 1991 Fission yeast genes that confer resistance to staurosporine encode an AP-1-like transcription factor and a protein kinase related to the mammalian ERK1/MAP2 and budding yeast FUS3 and KSS1 kinases. Genes Dev. **5:** 60–73.

Tsukahara, K., H. Yamamoto and H. Okayama, 1998 An RNA binding protein negatively controlling differentiation in fission yeast. Mol. Cell. Biol. **18:** 4488–4498.

Tu, H., M. Barr, D. L. Dong and M. Wigler, 1997 Multiple regulatory domains on the Byr2 protein kinase. Mol. Cell. Biol. **17:** 5876–5887.

Wang, Y., H. P. Xu, M. Riggs, L. Rodgers and M. Wigler, 1991 *byr2*, a *Schizosaccharomyces pombe* gene encoding a protein kinase capable of partial suppression of the ras1 mutant phenotype. Mol. Cell. Biol. **11:** 3554–3563.

Warbrick, E., and P. A. Fantes, 1991 The *wis1* protein kinase is a dosage-dependent regulator of mitosis in *Schizosaccharomyces pombe*. EMBO J. **10:** 4291–4299.

Watanabe, Y., and M. Yamamoto, 1994 *S. pombe mei2*$^+$ encodes an RNA-binding protein essential for premeiotic DNA synthesis and meiosis I, which cooperates with a novel RNA species meiRNA. Cell **78:** 487–498.

Watanabe, Y., Y. Lino, K. Furuhata, C. Shimoda and M. Yamamoto, 1988 The *S. pombe mei2* gene encoding a crucial molecule for commitment to meiosis is under the regulation of cAMP. EMBO J. **7:** 761–767.

Watanabe, Y., S. Shimozaki-Yabana, Y. Chikashige, Y. Hiraoka and M. Yamamoto, 1997 Phosphorylation of RNA-binding protein controls cell cycle switch from mitotic to meiotic in fission yeast. Nature **386:** 187–190.

Wu, S. Y., and M. McLeod, 1995 The *sak1*$^+$ gene of *Schizosaccharomyces pombe* encodes an RFX family DNA-binding protein that positively regulates cyclic AMP-dependent protein kinase-mediated exit from the mitotic cell cycle. Mol. Cell. Biol. **15:** 1479–1488.

Xu, H., M. White, S. Marcus and M. Wigler, 1994 Concerted action of RAS and G proteins in the sexual response pathways of *Schizosaccharomyces pombe*. Mol. Cell. Biol. **14:** 50–58.

Yamamoto, M., 1996 The molecular control mechanisms of meiosis in fission yeast. Trends Biochem. Sci. **21:** 18–22.

Yamamoto, M., Y. Imai and Y. Watanabe, 1997 Mating and sporulation in *Schizosaccharomyces pombe*, pp. 1037–1106 in *The Molecular and Cellular Biology of the Yeast Saccharomyces*, edited by J. R. Pringle, J. R. Broach and E. W. Jones. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Zhou, G. L., T. Yamamoto, F. Ozoe, D. Yano, K. Tanaka *et al.*, 2000 Identification of a 14-3-3 homologue from *Lentinus edodes* as CAP (adenylyl cyclase-associated protein) interacting protein and its conservation in fission yeast. Biosci. Biotechnol. Biochem. **64:** 149–159.

Communicating editor: P. Russell