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

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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* 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 stell 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 RNAbinding protein that is absolutely required for meiosis (WATANABE and YAMAMOTO 1994). Pat1/Ran1 kinase inhibits meiosis by negatively regulating both Stell 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 nitrogenstarved 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 stressresponsive 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 cyrl 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; Yамамото 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 stell 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 stell (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 stell (Кимитомо 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 (WAR-BRICK 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 selfactivation 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 pheromonesignaling pathway causes S. pombe cells to become sterile.

We previously developed nine distinguishable hypersporulating *S. pombe* mutants, *sporulation abnormal mutant* 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 DH5a 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 msal 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 $\sim 10^5$ 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 BamHI-PstI 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 \LAPII (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 HindIII-PstI genomic fragment containing msa1 from pW1-12 (Figure 1) was introduced into the plasmid vector pUC118. The 1-kb EcoRV-EcoRV region that contained 60% of the msa1 open reading frame (ORF) was replaced with the 1.8-kb HincII DNA fragment of the ura4 gene from pHSG398-ura4 (TANAKA et al. 1999) and the resulting plasmid was named pUC118msa1::ura4⁺. A 5.4-kb PstI-HindIII 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 SphI-SphI region containing 85% of the msa2 ORF in pUC118-msa2 was replaced with the 1.8-kb SphI DNA fragment of the ura4 gene, yielding pUC118-msa2::ura4⁺. pUC118-msa2 was derived by inserting the 4-kb HindIII 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). pALmsal 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 (FORS-BURG and SHERMAN 1997). The deletion derivatives from

TABLE 1

S. pombe strains used in this study

Strain	Genotype
SP66	
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}_{10} ade6-216 leu1-32 ura4-D18 cyr1::ura4 ⁺
JZ633	h ⁹⁰ ade6-216 leu1-32 ura4-D18 pka1::ura4 ⁺
JZ666	h^{ν_0} ade6-216 leu1-32 ura4-D18 pde1::ura4 ⁺
JZ858	$h^{\circ\circ}$ ade6-216 leu1-52 ura4-D18 cgs1::ura4
1K105 17451	h^{*} leu1-32 wra4-D18 phh1::wra4 http://doi.org/10.00000000000000000000000000000000000
SDD1	n^{-} <i>adeo-210 let1-52 urat-D16 gpd1::urat</i>
SPRU	$m = aaeo-210 \ ieu1-22 \ wat-D10 \ ius1wat-b00 \ adae 0.210 \ ieu1.22 \ wat-D18 \ bw1wat-$
SPSU	m^{-} and m^{-} 210 km ⁺ /2 km ⁺
SPKU	h^{00} also $h^{01} = 210$ [$\mu_1 = 32$] $\mu_1 = 400$ ($h^{11} = 100$ ($h^{11} = 100$)
SPFU	h^{90} ade6-216 lev1-32 $vra4$ -D18 ste4: $vra4^+$
SP593	h^{90} ade6-210 leu1-32 ras 1^{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< td=""></kanmx6<>
HT11	h_{2}^{00} ade6-210 leu1-32 ura4-D18 msa1::ura4 ⁺
HT12	h_{00}^{*0} ade6-216 leu1-32 ura4-D18 msa1::ura4 ⁺
HT14	h^{ν} ade6-216 ura4-D18 msa1::ura4 ⁺
H121	h^{-} adeb-210 leu1-32 ura4 his5 msa1::ura4'
H143	$n^{\prime\prime}$ adde-210 leu1-32 ura4-D18 msa1::ura4 p pde1::ura4
П100 НТ76	n addebig 10 left -22 und+D18 msa1: und+ $cgs1::und+$
HT77	m and 210 km r 22 km r r 210 km r r m r
HT81	h^{00} adde-210 [w1-32] wrd+D18 msa1: wrd+ msa2: wrd+
HT89	h^{90} add -210 leu 1-32 ura 4-D18 msa 1::ura 4+ spa 1::ura 4+
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_{00}^{90} ade6-210 leu1-32 ura4-D18 msa1::ura4 ⁺ ras1::ura4 ⁺
HT96	h^{*0} ade6-216 leu1-32 ura4-D16 msa1::ura4 ⁺ ras1::ura4 ⁺
H197	$h^{\circ \circ}$ ade6-210 leu1-52 ura4-D18 msa1::ura4 spk1::ura4
H198 UT00	n° adde-216 leu1-32 ura4-D18 msa1::ura4 spk1::ura4
П199 НТ100	h^{0} addeo 216 leu 1-32 urat-D18 msa1::urat stet::urat
HT101	m aue -210 teur -22 with -10 msur. with ster. with $msur = 0.0000000000000000000000000000000000$
HT102	m/m^{2} all $m = 210/m correction (m = 22/m = 1-52) m (m = 1-52) m $
HT103	h^{90}/h^{90} ade6-210/ade6-216 leu1-32/leu1-32 ura4-D18/ura4-D18 msa1::ura4 ⁺ /msa1::ura4 ⁺ hvr1::ura4 ⁺ /hvr1::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 [%] /h [%] ade6-210/ade6-216 leu1-32/leu1-32 ura4-D18/ura4-D18 msa1::ura4 ⁺ /msa1::ura4 ⁺ gpa1::ura4 ⁺ /gpa1::ura4 ⁺
HT117	h_{0}^{so} ade6-210 leu1-32 ura4-D18 gpa1::ura4 ⁺
HT118	h^{*0} ade6-216 leu1-32 ura4-D18 gpa1::ura4 ⁺
HT119	h^{29} ade6-216 leu1-52 ura4-D18 ras1::ura4
H1120	n° adde-216 leu1-32 ura4-D18 byr2::ura4
П1121 ЦТ199	m^{-} all $c^{-}210$ [w1-22 w1 d+D10 b)[1w1 d+ b^{00} and $c^{-}216$ [w1-32] wr d+D18 c b b) wr a d ⁺
HT193	h ⁹⁰ ade6-210 lev1-32 ura4-D18 ste4.ura4 ⁺
HT194	h^{90}/h^{90} ade 6-210/ade 6-216 lev 1-32/lev 1-32 ura 4-D18/ura 4-D18 oba 1···ura 4 ⁺ / oba 1···ura 4 ⁺
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 bvr2::ura4 ⁺ /bvr2::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%/h% 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 ^{<i>a</i>}
msa1 FL	5'-TTA <u>GTCGAC(Sal</u> I) AATGGTTGTTTCCTCT-3'
	5'-CAACCCGGG(SmaI) TCTAATTCCCATCCAT-3'
msa1 ($\Delta 2$ -31)	5'-TTAGTCGAC(SalI) AATGCCCCCAGGTTCTTTATC-3'
	5'-CAACCCGGG(SmaI) TCTAATTCCCATCCAT-3'
msa1 ($\Delta 2$ -102)	5'-TTAGTCGAC(SalI) AATGTTTCAACAGTGGGGTCC-3'
	5'-CAACCCGGG(Smal) TCTAATTCCCATCCAT-3'
msa1 ($\Delta 487-533$)	5'-TTAGTCGAC(SalI) AATGGTTGTTTCCTCT-3'
	5'-AGCCCCGGG(SmaI) 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'
1	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 μ g/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 $(10,000 \times g \text{ for } 15 \text{ min})$ 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 $[\alpha^{-32}P]dCTP$ (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 *PvuII* fragment for *stel1* from pSX1 (SUGIMOTO *et al.* 1991), the 3.2-kb *ClaI* fragment for *mei2* (WATANABE *et al.* 1988), the 3.5-kb *Hind*III 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: h⁹⁰ 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 cellfree extracts were prepared at indicated times. About 1×10^8 cells of S. pombewere harvested. Pellets were washed with STOP buffer [150 mм NaCl, 50 mм NaF, 10 mм EDTA, 1 mм NaN₃ (pH 8.0)] and stored at -80° . The pellets were diluted in 100 μ l of dH₂O and boiled at 95° for 5 min. Then 120 μ l of 2× Laemmli buffer [4% SDS, 20% glycerol, 0.6 M β-mercaptoethanol, 0.12 м Tris-HCl (pH 6.8)] containing 8 м 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 $10,000 \times g$ 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 *msal* 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 \sim 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 multicopy suppressor of sporulation abnormal 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 $msal\Delta$ (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%, $msa1\Delta$ 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-



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ATA CCAGA TCANT CCGCT TTCTT AGTAC GGTTT GTTTA CAGGA ATGGCAAT CGCA GC CTTA ACGCG CTTA ACGCG CTTA TCGCC CTGC CTTGT TCTCA CCGC CTTA CCGC CTTA L V R V R V R V R V R V A L K H S A W P141J D Q S A F L V R F V Y R D E A I A A Y T A L K H S A W P151GTA CTTTG GGCG AAAAT GTCAA TATTA CAAAG CGTCA CTATTA AGAAG AAAGG TTCCT CCCC GTTTC TTCCAC CTAAC GCCAATAGTC GA1621AG CGAAA GTGC AAAGA TCATT AAAA CGTCA CTCT TATA CAAAGACAC TCCCC GTTTC TTCCAC CTAAC GCCACATAGTC GA1621AG CGAAA GTGC AAAGA TCAGT CGAA ACTCC TCTTA TACAAAGCAC CTCCC CGATT CCAT CCA	1351	ATA TCCTC TGCAC CCCAT CAACC TTACA TACAA AAAA AGACA TAGAT AACTT ATTGG AACCT TATGG CGAGG TTGAA GATGT CACGG AA I S S A P H Q P Y I T K K D I D N L L E P Y G E V E D V T E	$1440 \\ 193$
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	1441	ATA CCAGA TCAAT CCGCT TTCTT AGTAC GGTTT GTTTA CAGGG ATGAA GCAAT CGCAG CCTAT ACCGC GCTTA AGCAT TCTGC CTGGC CT I P D Q S A F L V R F V Y R D E A I A A Y T A L K H S A W P	$1530 \\ 223$
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	1531 224	GTA CTTTG GGCGG AAAAT GTGAC ATATC AAAAC GGTCA CTATA AGAAG AAAGG TTCCT CCCCG TTTTC TCCAC CTAAC GCACA TAGTC GA V L W A E N V T Y Q N G H Y K K K G S S P F S P P N A H S R	$1620 \\ 253$
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	1621 254	AGG CGAAA GTCGC AAGGC AAAGA TCAGT CGAAC ACTCC TGTTA TCAAA GCACC TGCCC CTATT CCATT CTCAG TTTCT TCCGA TCCTC CT R R K S Q G K D Q S N T P V I K A P A P I P F S V S S D P P	$1710\\283$
1801GAA TCTTT AAGCT CCTTA CCGAG TATAT TACCT TCAAT ACCT TCTTA GAAAG CGCCA AGCC CGAACT CCCA CAGAT GGTT TTTTG AA18314ESLSSLPSILPSTFE31891CAG CCTGG TATAT CCTG ACCC CTTA TGATG TTGC TGCAA TGCCT CCAC CGATTG ATCCT TATT TCGTT GGCCA ACTCG AC19344QPGYPMNPSMMFAMPPIDPYSIFVGCLD31981CAG CTGGATAT CCACAC TACTT ACTAG TGGAT CTATT TTCCA AATAT GGAAAGGTAA TCGAT TGTAA AATAA TTCAT CAAAGCAGAAG2031981CCAGTAAA TTGCACACAC TACTT ACTAG TGGAT CTATT TTCCA AATAT GGAT GG	1711 284	TCT ACCAT GGGAA GATCA AATTC CGCGG TTCAA AGTCC CTCAT ACTTT GCTCA CTCAT TGGTC AATTC TACTG AATTC AGTAC TCCTA AT S T M G R S N S A V Q S P S Y F A H S L V N S T E F S T P N	$1800 \\ 313$
1891 CAG CCTGG ATATC CCATG AACCC CTCTA TGATG TTTGC TGCA TGCCT CACC GATTG ATCCT TATT TGTT GGCCA CTGG AC 19344 Q P G Y P M N P S M M F A A M P P P I D P Y S I F V G O L D 3149 CCA GTAAA TGCACAC TACTA TCATGAT GTAT CTTATT TCCA NATAT GGAAA GGTAA TCCAT TGTAA AATAA TTCAT CAAAG CAAGA AG 20374 P V N C T H Y L L V D L F S K Y G K V I D C K I I H Q S K K 2071 CCT GCTTT TCTTA AGGTT TGATT CTCAA CAAGC CGCTT ATGCAT GCGT CGCG GTAAG ACGAG GTCC CACAC CAGAAAAAGC CT 404 P A F A F L R F D S Q Q A A Y A A V C G K T R S P H Q K K P 4161 TTG CGTGT TGAAT TTCGT CAACT AAGAC CAATG CAGCA ATTTT CCCCT CAATA TCAAT ATCCC TCCAT ATCCG ATGTT TCCAG C 434 L R V E F R Q L R P M Q Q F S P Q Y Q Y P S Y P Y P M F P A 4251 CCA TTTTC ACCAC CTCGT AATGC AATGC AATGC TGCAC CTCAC CTCACT CAATG CTAAC CCTTC CT C 446 P F S P P R N A M M P I P A P M D Q F S T F H Q S M A T L P 2341 CCT GGTGC CGTTC CCACA TAGAT TCCCT AGGTG GTACT ATCCCA TTTT TCCTC CAGAA TGCCT TATCCA CAGT CCATG CCTTC CT T 449 P G A V P T S I P Q S Y Y I Y S P M M P Q S Y S P M Y 4494 P G A V P T S I P Q S Y Y I Y S P E M A M P Q S Y S P M Y 4511 TAT ACGCA TAACC CACTC ATGGA TGCGT AATTAC TATT CTCTC CAGAA TGCCT TACCTT CTTAT ACAGT CC 524 Y T H N P P M D G N * 2521 TAT AGGCA TAACC CACT ATGGAT ATGGAT ATTAT TTTT ATATT TATT	1801 314	GAA TCTTT AAGCT CCTTA CCGAG TATAT TACCT TCAAT ACCCT CTTTA GAAAG CGGCA AGGCC GAACT CCCAA CAGAT GGTTC TTTTG AA E S L S S L P S I L P S I P S L E S G K A E L P T D G S F E	$1890 \\ 343$
1981CCA GTAAA TTGCA CACAC TACTT ACTAG TTGAT CTATT TTOCA ATATT GGAAA GGTAA TCGAT TGTAA AATAA TTCATCAAAG CAAGA G20374PVNCTHYLLVDLFSXYDCKIIHQSKK42071CCT GCTTT TTGCT TTGCT TGATT CTCAA CAAGC CGCTT ATGCT GCCGT CTGCG GTAAG AGGAA GGCACC CAACC CAGAA AAAGC CT21404PAFLRFDSQQAAYAAVCGKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKKK </td <td>1891 344</td> <td>CAG CCTGG ATATC CCATG AACCC CTCTA TGATG TTTGC TGCAA TGCCT CCACC GATTG ATCCT TATTC TATAT TCGTT GGCCA ACTCG AC Q P G Y P M N P S M M F A A M P P P I D P Y <u>S I F V G O L</u> D</td> <td>$1980 \\ 373$</td>	1891 344	CAG CCTGG ATATC CCATG AACCC CTCTA TGATG TTTGC TGCAA TGCCT CCACC GATTG ATCCT TATTC TATAT TCGTT GGCCA ACTCG AC Q P G Y P M N P S M M F A A M P P P I D P Y <u>S I F V G O L</u> D	$1980 \\ 373$
2071 CCT GCTT TTGCT TTGCT AGGT TGATT CTCAA CAAGC CGCTT ATGCT GCCG GTAGA GAGAG GTCC CACAC CAGAAAAGC CT 404 $P \ A \ F \ A \ F \ L \ R \ F \ D \ S \ Q \ Q \ A \ A \ A \ C \ G \ X \ T \ R \ S \ P \ H \ Q \ K \ P \ 4 \ 4 \ C \ G \ X \ T \ R \ S \ P \ H \ Q \ K \ P \ 4 \ 4 \ C \ G \ X \ T \ R \ S \ P \ H \ Q \ K \ P \ 4 \ 4 \ C \ G \ X \ T \ R \ S \ P \ H \ Q \ K \ P \ 4 \ 4 \ C \ G \ X \ T \ R \ S \ P \ H \ Q \ K \ P \ 4 \ 4 \ C \ G \ X \ T \ R \ S \ P \ H \ Q \ X \ P \ 4 \ 4 \ C \ G \ X \ T \ R \ S \ P \ H \ Q \ K \ P \ 4 \ 4 \ C \ G \ X \ T \ R \ S \ P \ H \ Q \ K \ P \ 4 \ 4 \ C \ G \ X \ T \ R \ S \ P \ R \ Q \ L \ R \ P \ A \ R \ Q \ L \ R \ P \ A \ Q \ S \ P \ Q \ Y \ P \ S \ Y \ P \ Y \ P \ M \ F \ P \ A \ 4 \ 4 \ 4 \ 4 \ C \ G \ X \ T \ R \ S \ P \ R \ Q \ L \ R \ R \ R \ R \ R \ R \ R \ R \ R$	1981 374	CCA GTAAA TTGCA CACAC TACTTA CTAG TTGAT CTATT TTCCA AATAT GGAAA GGTAA TCGAT TGTAA AATAA TTCAT CAAAG CAAGA AG P V N C T H Y L L V D L F S K Y G K V I D C K I I H Q S K K	2070 403
2161TTG CGTGT TGAAT TTCGT CAACT AAGAC CAATG CAGCA ATTTT CCCCT CAATA TACCAT ATCCC ATGCT TCCAG CG22434LRVEFRQLRPMQQSYPYPMFPA2251CCA TTTTC ACCAC CTCGT AATGC ATGA TGACA ATCC TCCAC CCAC	2071 404	CCT GCTTT TGCTT TTCTA AGGTT TGATT CTCAA CAAGC CGCTT ATGCT GCCGT CTGCG GTAAG ACGAG GTCCC CACAC CAGAA AAAGC CT <u>P A F A F L R F D</u> S Q Q A A Y A A V C G K T R S P H Q K K P	2160 433
22 51CCA TTTTC ACCAC CTCGT ANTGC ANTGC ATGC ATGCC TGCAC CATE GATCA GTTTT CACAT TTTCA CCAGT CATG GCTAC CCTTC CT2323464PFSPPNAMMPPAPADQFSTFHQSNALP423 41CCT GOTGC CACAT CAAT CCTCA CATGCT GACGT ATTCCCA ATTTAT TCTCC AGAAA TGGCT ATGCC TCAAT CGAAC AGTCC GATGT AT2424SVPTSIPQSYYIYSPEMAMPQSYYYSPEMAMPQSYYYYSYPYSYSYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYYY <td< td=""><td>21 61 4 34</td><td>TTG CGTGT TGAAT TTCGT CAACT AAGAC CAATG CAGCA ATTTT CCCCT CAATA TCAAT ATCCC TCCTA TCCCA ATCCG ATGTT TCCAG CG L R V E F R Q L R P M Q Q F S P Q Y Q Y P S Y P Y P M F P A</td><td>2250 463</td></td<>	21 61 4 34	TTG CGTGT TGAAT TTCGT CAACT AAGAC CAATG CAGCA ATTTT CCCCT CAATA TCAAT ATCCC TCCTA TCCCA ATCCG ATGTT TCCAG CG L R V E F R Q L R P M Q Q F S P Q Y Q Y P S Y P Y P M F P A	2250 463
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	2251 464	CCATTTTC ACCAC CTCGT AATGC AATGA TGCCA ATCCC TGCAC CCATG GATCA GTTTT CAACA TTTCA CCAGT CAATG GCTAC CCTTC CT P F S P P R N A M M P I P A P M D Q F S T F H Q S M A T L P	2340 493
24 31 TAT ACGCA TAACC CACCT ATGGA TGGGA ATTAG ATTAT ATTTG AATTTCCCTT CATTA TCTGA GTTTC ACTTC TTTAT ACAGT CC 25 5 24 Y T H N P M D G N * 5 25 21 TTA ASTGT CGGAG TTTTT TTTAT TAGGT ACATA TTCTA TAAAA CACTA ATAAT TATCT AATTT ATTTT ACTTA AAAGA TGCAA ATTGG CT 26 26 26 CCA ATTAT ATTTA AAAGA TTTAG AGTCA TAAAAT GTTAT TATGT TCATA TATAT GTCT 26	23 41 4 94	CCT GGTGC CGTTC CCACA TCAAT TCCTC AGTCG TACTA TCCCA TTTAT TCTCC AGAAA TGGCT ATGCC TCAAT CGTAC AGTCC GATGT AT P G A V P T S I P Q S Y Y P I Y S P E M A M P Q S Y S P M Y	2430 523
25 21 TTA AGTGT CGGAG TTTTT TTAT TAGGT ACATA TTCTA TAAAA CACTA ATAAT TATCT AATTT ATTTT ACTTA AAAGA TGCAA ATTGG CT 26 26 11 GCA ATTAT ATTTA AAAGA TTTAG AGTCA TAAAT GTTAT TATGT TCATA TATAT GTCT 26	24 31 5 24	TAT ACCCA TAACC CACCT ATGGA TGGGA ATTAG ATTTA TTTTG AATTT CCCTT CATTA TCTGA TTGAG GTTTC ACTTC TTTAT ACAGT CC Y T H N P P M D G N *	2520 533
26 11 GCA ATTAT ATTTA AAAGA TTTAG AGTCA TAAAT GTTAT TATGT TCATA TATAT GTCT 26	25 21	TTA ASTGT CGGAG TTTTT TTTAT TAGGT ACATA TTCTA TAAAA CACTA ATAAT TATCT AATTT ACTTA AAAGA TGCAA ATTGG CT	2610
	2611	GCA ATTAT ATTTA AAAGA TTTAG AGTCA TAAAT GTTAT TATGT TCATA TATAT GTCT	2667



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 *Psd* (P), *Eco*RV (RV), *Hind*III (H), and *Bam*HI (B) are shown. The complementation ability of each subclone was examined using HT3 (h^{90} sam1 leu1-32). Two *Psd* 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.





 $msa1\Delta$ 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 $msa1\Delta$ cell. The $msa1\Delta$ 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 $msa1\Delta$ 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 (KITA-MURA 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*, *stel1*, 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 stell; and mam2 encodes a matingpheromone receptor and is upregulated by both *stell* and mating pheromone (KITAMURA and SHIMODA 1991; SUGIMOTO et al. 1991; OZOE et al. 2002). Although stell 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 wildtype cells.

Similarly, on the nitrogen-free medium, *stel1*, *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 ($cgs1\Delta$) and msa1-cgs1 double-disruptant cells $(msal\Delta cgsl\Delta)$ and examined their phenotype. The $msal\Delta$ $cgs1\Delta$ cells exhibited $cgs1^-$ phenotype and scarcely conjugated during nitrogen starvation (Figure 6D). In addition, $cgs1\Delta$ and $msa1\Delta$ $cgs1\Delta$ 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 $pka1\Delta$ cells. Because $msa1\Delta$ cgs 1Δ and $msal\Delta pdel\Delta$ double mutants have the same phenotype as $cgs1\Delta$ and $pde1\Delta$ (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 (YAMA-MOTO *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 stell, 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 nitrogenfree 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 ras $I\Delta$ 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 ras1val17 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 $byr2\Delta$ 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 $msa1\Delta$ $ras1\Delta$ diploid cells peaked ~90-fold higher than that of the $ras1\Delta$ 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 $rad24\Delta$ in nitrogen-free medium. We compared expression of mam2 between wild-type, $msa1\Delta$, $rad24\Delta$, and $msa1\Delta$ $rad24\Delta$ 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 cyrl 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), $msa1\Delta$ (HT11), $pde1\Delta$ (JZ666), $cgs1\Delta$ (JZ858), $msa1\Delta \ pde1\Delta$ (HT43), and $msa1\Delta \ cgs1\Delta$ (HT58). (E and F) Cells were grown to saturation $(1 \times 10^7 \text{ cells/ml; day 0})$ 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.

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

Because the loss of function of rad24 in cells showed



FIGURE 7.—*msa1* functions independently of the *ph1*-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.

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-bind-ing protein that blocks commitment to conjugation until cells reach a critical level of nutrient starvation (Tsu-KAHARA *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 (WATA-NABE 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
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Strain	Sporulation efficiency (%) ^a
Wild-type and single mutants	
HT101 (wild type)	38.5 ± 6.7
HT124 ($gpa1\Delta$ diploid)	0
HT125 ($ras1\Delta$ diploid)	0.1
HT126 (byr2 Δ diploid)	0
HT127 (byr1 Δ diploid)	0
HT128 ($spk1\Delta$ diploid)	0
HT129 (<i>ste4</i> Δ diploid)	0
<i>msa1</i> -null mutants	
HT102 ($msa1\Delta$ diploid)	51.4 ± 7.2
HT108 ($msa1\Delta$ $gpa1\Delta$ diploid)	0
HT105 ($msa1\Delta$ $ras1\Delta$ diploid)	9.5 ± 5.2
HT104 ($msa1\Delta$ byr2 Δ diploid)	0
HT103 ($msa1\Delta$ by $r1\Delta$ diploid)	0
HT106 ($msa1\Delta spk1\Delta$ diploid)	0
HT107 (msa1Δ ste4Δ 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 stressresponsive 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.

Msal controls expression of several genes that are necessary for the induction of sexual differentiation. Expression of genes usually induced by nutritional starvation, *stel1* and *mei2*, is mildly increased in *msal* 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 Ste11regulated genes through the pheromone-response pathway. DNA microarray experiments to compare gene expression level in wild-type and $msa1\Delta$ 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 $ras1\Delta$ 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. Patl 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; MONDE-SERT *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 nitrogenfree 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 *stel1* expression using unknown mecha-



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

nisms (IINO *et al.* 1991; KUNITOMO *et al.* 1995). An RNAbinding 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.

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