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Analysis of the Structural Genes Encoding M-Factor in the Fission Yeast *Schizosaccharomyces pombe*: Identification of a Third Gene, *mfm3*

SØREN KJÆRULFF,[1] JOHN DAVEY,[2] AND OLAF NIELSEN[1]*

Department of Genetics, Institute of Molecular Biology, University of Copenhagen, DK-1353 Copenhagen K, Denmark,[1] and School of Biochemistry, The University of Birmingham, Birmingham B15 2TT, United Kingdom[2]

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We previously identified two genes, *mfm1* and *mfm2*, with the potential to encode the M-factor mating pheromone of the fission yeast *Schizosaccharomyces pombe* (J. Davey, EMBO J. 11:951–960, 1992), but further analysis revealed that a mutant strain lacking both genes still produced active M-factor. Here we describe the isolation and characterization of a third M-factor gene, *mfm3*. A mutant lacking all three genes fails to produce M-factor, indicating that all functional M-factor genes now have been identified. The triple mutant exhibits an absolute mating defect in M cells, a defect that is not rescued by addition of exogenous M-factor. A mutational analysis reveals that all three *mfm* genes contribute to the production of M-factor. Their transcription is limited to M cells and requires the *mat1-Mc* and *ste11* gene products. Each gene is induced when the cells are starved of nitrogen and further induced by a pheromone signal. Additionally, the signal transduction machinery associated with the pheromone response is required for transcription of the *mfm* genes in both stimulated and unstimulated cells.

The fission yeast *Schizosaccharomyces pombe* exists in one of two mating types, minus (M) or plus (P), and under the appropriate conditions, cells of opposite mating type can conjugate to form a diploid zygote (reviewed in reference 45). Prior to conjugation, the cells communicate with each other via diffusible mating pheromones that prime the recipient cell for mating; M cells release M-factor, which prepares P cells for mating, and P cells secrete P-factor, which stimulates M cells. Pheromone-induced changes include a G_1 arrest of the cell cycle (9, 26), an altered pattern of gene transcription (46), and elongation of the cell (7, 19, 36).

The response begins when the pheromone binds to a G-protein-coupled receptor on the surface of the target cell; receptors for P-factor are encoded by the *mam2* gene (29), while those for M-factor are encoded by *map3* (58). Stimulation of the receptor is thought to cause dissociation of the G protein such that the Gα subunit (encoded by *gpa1* [49]) is able to trigger an intracellular signalling pathway which includes a cascade of protein kinases encoded by the *byr1*, *byr2*, and *spk1* genes (42, 44, 56, 59, 61). A number of these enzymes are functionally homologous to the mitogen-activated protein kinases believed to be involved in controlling the proliferation and differentiation of mammalian cells (reviewed in reference 16). Transmission of the pheromone signal also requires the *ras1* gene product, the *S. pombe* homolog of the mammalian *ras* oncogene (20, 46).

The M-factor pheromone is a nonapeptide in which the C-terminal cysteine residue is S farnesylated and carboxyl methylated (7, 8, 60); P-factor is a peptide made of 23 residues and is probably unmodified (26). Although the biogenesis of M-factor is not fully characterized, it is likely to be similar to that of the **a**-factor pheromone from the budding yeast *Saccharomyces cerevisiae*. The two pheromones are similar in structure (3, 8), and each appears to be synthesized as a precursor which undergoes proteolytic processing and post-translational modifications to produce the mature, active pheromone (reviewed in reference 39). A previous study identified two genes, *mfm1* and *mfm2*, with the potential to encode M-factor (8), but as part of our present investigation, we found that a mutant lacking both genes could still produce active M-factor. This finding suggested that there was at least one more M-factor gene, and here we describe the isolation and characterization of a third *mfm* gene, *mfm3*. Additionally, we have examined the transcriptional regulation of the three M-factor genes.

MATERIALS AND METHODS

Yeast strains, media, and genetic methods. The yeast strains used in this study are shown in Table 1. Standard genetic procedures were carried out as described by Moreno et al. (41). Strains were grown in YEA or YEL (22) at 30°C, and mating and meiosis were induced in MSA or nitrogen-deficient MSL (MSL lacking arginine) (14, 15). DNA manipulations were performed as described by Sambrook et al. (52), and amplification by PCR was performed in 50-μl volumes as described by Kocher et al. (31). M-factor was prepared as described previously (8). P-factor (26) was synthesized by solid-phase methodology using FMOC protection chemistry on a Biotech Instruments BT7300 peptide synthesizer with materials and conditions recommended by the manufacturer. This work was performed by Alta Bioscience, University of Birmingham.

Cloning and sequencing of the *mfm3* gene. To confirm the presence of a third *mfm* gene, we initiated a PCR-based screen using degenerate primers based on the conserved sequences at the ends of the *mfm1* and *mfm2* genes: A (N-terminal end), 5′-TA(A/G)CCATGGACTC(A/C)AT(G/T)GC-3′; and B (C-

* Corresponding author. Mailing address: Department of Genetics, Institute of Molecular Biology, University of Copenhagen, Øster Farimagsgade 2A, DK-1353 Copenhagen K, Denmark. Phone: 45 3532 2102. Fax: 45 3532 2113. Electronic mail address: ONIGEN@Biobase.AAU.DK.

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

Strain	Genotype	Reference
EG282	h^{90}	36
EG410	h^{90} *mat1-Pc*$^-$	48
EG494	h^{90} *ste11 leu1*	57
EG495	h^{90} *leu1 Δbyr1::LEU2*	This study[a]
EG533	h^{90} *leu1 ras1::LEU2*	20
EG544	h^- *Δmat2,3::LEU2 leu1*$^+$	This study[b]
EG545	h^+ *Δmat2,3::LEU2 leu1*$^+$	46
EG559	h^{90} *Δste8::ura4*$^+$ *ura4-D18*	56
EG570	*mat1-Mc*$^-$*::ura4*$^+$ *Δmat2,3::LEU2*$^+$ *ura4-D18 leu1*	63
EG637	h^{90} *mam2*	12
EG640	h^{90} *leu1 ura4-D18*	This study
EG662	h^{90} *Δmfm1::LEU2*$^+$ *leu1 ura4-D18*	This study
EG663	h^{90} *Δmfm2::ura4*$^+$ *leu1 ura4-D18*	This study
EG664	h^{90} *Δmfm1::LEU2*$^+$ *Δmfm2::ura4*$^+$ *leu1 ura4-D18*	This study
EG667	h^- *Δmfm1::LEU2 Δmfm2::ura4*$^+$ *leu1 ura4-D18*	This study
EG670	*M Mc::ura4*$^+$*/P Δmat2,3/Δmat2,3 ura4-D18/ura4-D18 ade6/+*	63
EG677	h^{90} *Δmfm1::LEU2 Δmfm2-D4 leu1 ura4-D18*	This study
EG699	h^- *sxa2-563*	This study[c]
EG709	*Δmat-1::ura4*$^+$ *leu1 ura4-D18*	63
EG710	h^{90} *gpa1::ura4*$^+$ *ade6 leu1 ura4-D18*	49
EG728	h^- *Δmfm1::LEU2 leu1 ura4-D18*	This study
EG773	h^{90} *Δmfm1::LEU2 Δmfm2-D4 Δmfm3::ura4*$^+$ *leu1 ura4-D18*	This study
EG774	h^{90} *Δmfm1::LEU2 Δmfm2::ura4*$^+$ *leu1 ura4-D18*	This study
EG775	h^{90} *Δmfm2-D4 leu1 ura4-D18*	This study
EG776	h^{90} *Δmfm2-D4 Δmfm3::ura4*$^+$ *leu1 ura4-D18*	This study
EG777	h^- *Δmfm1::LEU2 Δmfm3::ura4*$^+$ *leu1 ura4-D18*	This study
EG781	h^- *Δmfm2-D4 leu1 ura4-D18*	This study
EG782	h^- *Δmfm2-D4 Δmfm3::ura4*$^+$ *leu1 ura4-D18*	This study
EG783	h^- *Δmfm1::LEU2 Δmfm2-D4 Δmfm3::ura4*$^+$ *leu1 ura4-D18*	This study
EG784	h^{90} *Δmfm3::ura4*$^+$ *leu1 ura4-D18*	This study
EG788	h^{90} *Δspk1::ura4*$^+$ *ura4-D18*	This study[d]
EG800	h^- *Δmfm3::ura4*$^+$ *leu1 ura4-D18*	This study

[a] The *Δbyr1::LEU2* construction is described by Nadin-Davis and Nasim (42).
[b] The *Δmat2,3::LEU2* construction is described by Klar and Miglio (30).
[c] The *sxa2-563* mutation is described by Imai and Yamamoto (25).
[d] The *Δspk1::ura4*$^+$ construction is described by Toda et al. (59).

terminal end), 5′-CCAAGCTTA(A/T)GCAAT(T/G)ACAC TA-3′. Use of DNA from EG664 (h^{90} *Δmfm1::leu2 Δmfm2::ura4*$^+$) as the template produced a 204-bp fragment which was sequenced by using the Hot Tub system (Amersham) and confirmed the presence of a third *mfm* gene. Because of the high degree of conservation between the three M-factor genes, it was not possible to obtain a probe that would be specific for *mfm3*, and so the *S. pombe* genomic DNA library was rescreened with the probe originally used to isolate *mfm1* and *mfm2* (8). Positive clones containing either *mfm1* or *mfm2* were discarded after being identified by PCR using gene-specific primers, and the *mfm3* clones were further analyzed as described previously (8).

Gene disruptions. The one-step disruption method was used to generate null mutants of each *mfm* gene (50). Replacement of the wild-type alleles by the disrupted genes was confirmed by Southern analysis and/or PCR of several transformants. To obtain an *mfm1* null allele, a 1,900-bp *Bgl*II-*Hin*dIII fragment containing *mfm1* was cloned into the *Bam*HI-*Hin*dIII sites of Bluescript pSK (Stratagene). A 467-bp *Eco*RI-*Nhe*I fragment including the open reading frame (ORF) of *mfm1* was replaced by a 2,400-bp *Hin*dIII-*Xba*I fragment containing the *LEU2* gene from *S. cerevisiae* (the *Eco*RI and *Hin*dIII sites were end filled with the Klenow fragment) (Fig. 1A). A *Hin*dIII-*Xba*I fragment carrying the *Δmfm1::LEU2* allele was used to transform an h^{90} *leu1 ura4-D18* strain (EG640).

The *mfm2* gene was deleted by the sequence overlap extension technique, a PCR-based method which uses overlapping primers (24). Four primers were used: A, 5′-GCCGGATCC TCCATTTTATGAATG-3′; B, 5′-**TGCCTATCGATGACTG**C CAATTCCCCAGAAA-3′; C, 5′-**CAGTCATCGATAGGCA**C GTGCCTTAACTTTC-3′; and D, 5′-GGGGCATGCAGAAT TCTTTGCCAA-3′. The sequences underlined in primers A and D indicate *Bam*HI and *Sph*I sites, respectively, while those in primers B and C represent *Cla*I sites. The boldface region of primer B is complementary to the boldface region of primer C. Primers A and B were used to amplify a 585-bp region just upstream of the ORF of the *mfm2* gene, while primers C and D were used to amplify a 700-bp region just downstream of the ORF (Fig. 1B). The two PCR products were purified on a low-melting-point agarose gel (SeaPlaque GTG) and mixed to allow the overlapping ends to anneal and enable the 3′ overlap of each strand to serve as a primer for the extension of the complementary strand (PCR for 10 cycles without primers). The fused products were amplified further by 30 cycles of PCR with primers A and D, and the final product was cloned between the *Bam*HI and *Sph*I sites of pGEM3 (Promega). The construct was linearized at the *Cla*I site, and a 1,800-bp *Cla*I fragment of the *ura4*$^+$ gene from pON163 was inserted (21, 62). A 3,100-bp *Bam*HI-*Sph*I fragment containing the *Δmfm2::ura4*$^+$ allele was used to transform an h^{90} *leu1 ura4-D18* strain (EG640).

To delete the *mfm3* gene in the *Δmfm1 Δmfm2* double disruptant, we chose to remove the *ura4*$^+$ marker from the *Δmfm2::ura4*$^+$ genomic construction. The original *mfm2* deletion construct lacking the *ura4*$^+$ gene was therefore used to

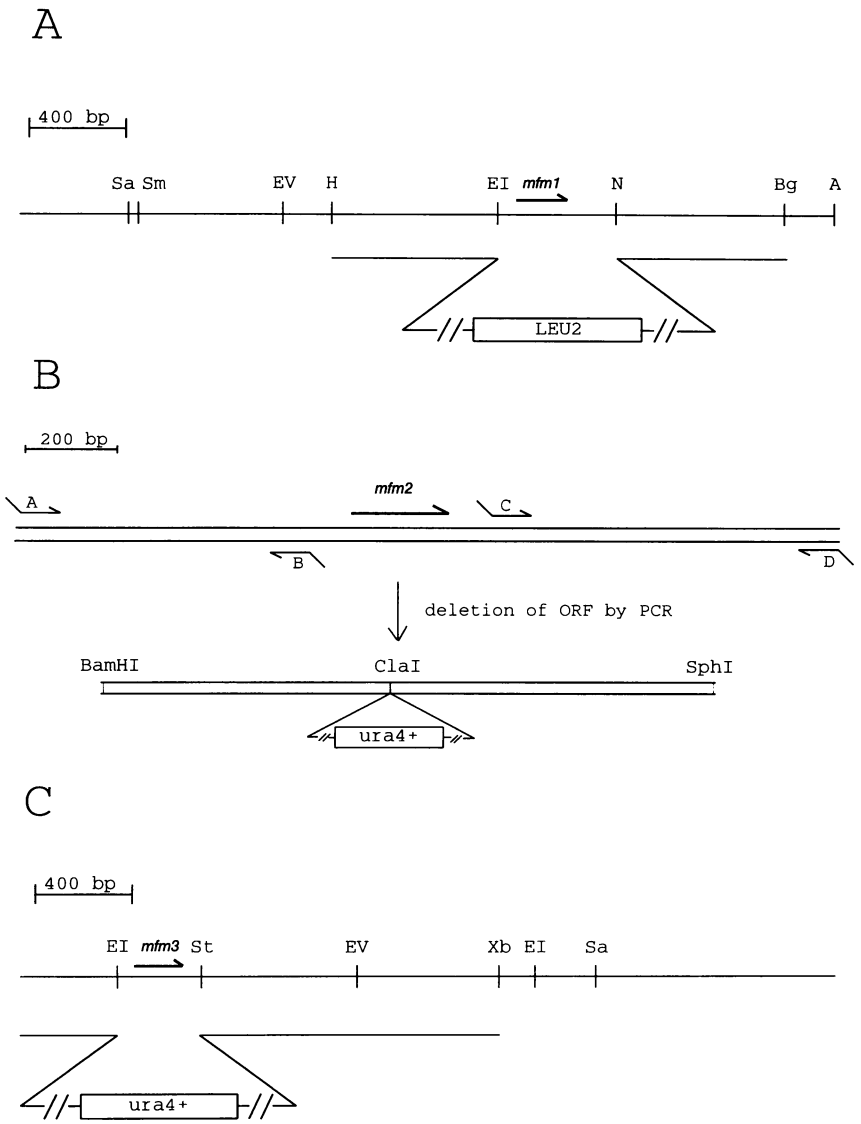


FIG. 1. (A and C) Restriction maps of the *mfm1* and *mfm3* loci. Arrows show the locations and directions of the ORFs. The fragment used in disruption of each *mfm* gene is shown at the bottom of each panel. Abbreviations for restriction endonucleases: A, *Apa*I; Bg, *Bgl*II; EI, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; N, *Nhe*I; Sa, *Sac*I; Sm, *Sma*I; St, *Stu*I; Xb, *Xba*I. (B) Schematic diagram showing how the deletion of the *mfm2* gene was generated by the overlap extension method. For this purpose, primers A, B, C, and D were used. Primer A includes a *Bam*HI site and primer D includes an *Sph*I site, which made it possible to clone the PCR fragment into the pGEM3 vector. 5′ ends of primers B and C are complementary to each other and include a *Cla*I site in which the *ura4*$^+$ gene was inserted.

transform the h^{90} Δ*mfm1*::*LEU2* Δ*mfm2*::*ura4*$^+$ (EG664) strain, and Ura$^-$ cells were selected with 5-fluoro-orotic acid (5) (Fig. 1B). The *mfm3* gene was contained in a 2,000-bp *Sau*3AI-*Xba*I fragment cloned between the *Xho*I and *Xba*I sites of Bluescript pSK, and a 343-bp *Eco*RI-*Stu*I fragment including the ORF of *mfm3* was replaced by a 1,800-bp *Eco*RI-*Hin*dIII fragment containing the *ura4*$^+$ gene (the *Hin*dIII site was end filled with the Klenow fragment) (Fig. 1C). A 3,800-bp *Apa*I-*Not*I fragment containing the disrupted *mfm3* allele was used for transformation of an h^{90} Δ*mfm1*::*LEU2* Δ*mfm2 ura4-D18* strain (EG677).

Southern analysis. *S. pombe* chromosomal DNA was isolated from 10-ml YEL cultures as described by Nielsen and Egel (47) except that the citrate-phosphate buffer containing sorbitol was replaced with 0.65 M KCl and the proteinase K treatment was omitted. The DNA was digested, separated on a 0.7% agarose gel, and blotted onto a nylon membrane (Hybond-N) as instructed by the manufacturer. The membranes were hybridized to single-stranded RNA probes as described by Nielsen and Egel (47) before being washed and exposed to Agfa Curix X-ray film as described by Sambrook et al. (52). Single-stranded RNA probes were transcribed from the pGEM3 vector containing a probe-specific insert, either a 1,900-bp *Hin*dIII-*Bgl*II fragment of the *mfm1* gene, a 1,728-bp *Bam*HI-*Sph*I fragment of the *mfm2* gene, or a 1,400-bp *Sau*3AI-*Eco*RV fragment of the *mfm3* gene.

Northern (RNA) analysis. The effect of nitrogen starvation and/or addition of pheromone on *mfm* RNA synthesis was examined as described previously (46). Total RNA was extracted from *S. pombe* cells as described by Nielsen and Egel

(48). Ten-microgram aliquots of total RNA were run on 1.5% formaldehyde gels in 1× MOBS buffer (52) and transferred to Hybond-N membranes (Amersham) as instructed by the manufacturer. The membranes were hybridized to single-stranded RNA probes as described by Nielsen et al. (46) before being washed and exposed to Agfa Curix and Agfa Structurix X-ray films as described by Sambrook et al. (52). RNA levels were quantitated with either a scanning densitometer (Hoefer Scientific Instruments) or a Packard InstantImager. Single-stranded RNA probes were transcribed from the pGEM3 vector containing a probe-specific insert, either a 467-bp *Eco*RI-*Nhe*I fragment of the *mfm1* gene, a 1,728-bp *Bam*HI-*Sph*I fragment of the *mfm2* gene (*Bam*HI and *Sph*I sites were introduced by PCR), or a 343-bp *Eco*RI-*Stu*I fragment of the *mfm3* gene. Probes specific for *mat1-Mc* and *cdc2* have been described previously (46, 48).

Mating assays. To monitor mating efficiency in solid medium, homothallic strains were grown in MSL (with appropriate supplements) to a density of 5×10^6 cells per ml. Five-microliter aliquots of these exponentially growing cultures were spotted on MSA agar plates and incubated for 48 h before the number of asci and zygotes was recorded. At least 500 cells were counted for each sample, and the efficiency of mating was calculated as the following ratio: 2 × number of asci and zygotes formed/(total number of cells + 2 × number of asci and zygotes).

M-factor assays. M-factor production was monitored in a halo assay based on the induction of meiosis in the diploid *mat1-Mc*$^-$/*mat1-P* strain (EG670) (15, 63). A mixture containing the strain being assayed and a 100-fold excess of the responsive diploid strain is spotted on MSA (with appropriate supplements) and incubated for 48 h. Iodine staining then detects the halo of azygotic asci surrounding the M-factor-producing cells, and the width of this halo provides a semiquantitative measure of M-factor production.

RESULTS

Identification, cloning, and sequencing of the *mfm3* gene. We previously identified two genes that appeared to encode the M-factor mating pheromone, *mfm1* and *mfm2* (8). To further investigate these genes, disrupted versions were constructed in vitro and introduced into the chromosome by the one-step gene replacement technique (50). The *mfm1* and *mfm2* functions were not expected to be essential for cell viability, and so the disruptions were performed in the haploid, homothallic strain EG640 (*h*^{90} *leu1 ura4-D18*), producing *h*^{90} Δ*mfm1*::*LEU2* (EG662) and *h*^{90} Δ*mfm2*::*ura4*$^+$ (EG663). Replacement of the wild-type alleles was confirmed by Southern analysis, and disruption of neither *mfm1* nor *mfm2* had an obvious effect on mating ability (Table 2). We next created the double disruptant by crossing the two singly disrupted strains and selecting for Leu$^+$ and Ura$^+$ progeny. To our surprise, the double mutant was still mating proficient (Fig. 2B; Table 2), suggesting that *S. pombe* contains at least one more M-factor gene in addition to *mfm1* and *mfm2*.

We used a two-step approach to identify and clone the additional M-factor gene(s). Using degenerate oligonucleotide primers based on the conserved regions at either end of the *mfm1* and *mfm2* genes (see Materials and Methods), we performed PCR-based screening of genomic DNA from wild-type cells and from a strain (EG664) lacking both *mfm1* and *mfm2*. Wild-type cells produced three PCR fragments: one from *mfm1*, one from *mfm2*, and one of 204 bp (data not shown). Repeating the reaction with DNA from the double disruptant (EG664) gave rise to only the 204-bp fragment, and sequencing showed this to be from a third M-factor gene, which we call *mfm3*. Because of the high degree of conservation between the three M-factor genes (see below), it was not possible to obtain a probe that would be specific for *mfm3*, and so the *S. pombe* genomic DNA library was rescreened with the probe originally used to isolate *mfm1* and *mfm2* (8). Of 78 positive clones, 38 carried *mfm1*, 36 carried *mfm2*, and only 4 carried *mfm3*, which appears to be underrepresented in the library. Sequence analysis of *mfm3* confirmed its similarity to *mfm1* and *mfm2*. Each *mfm* gene contains an intron and encodes M-factor as part of a precursor (Fig. 3). Excision of the intron from *mfm3* was confirmed by DNA sequencing of the product generated by PCR amplification of transcripts extracted from cells deleted for the *mfm1* and *mfm2* genes (data not shown).

TABLE 2. Mating efficiency of mutants defective in *mfm1*, *mfm2*, and *mfm3*

Homothallic strain[a]	% Mating[b]
Wild type	53.9 ± 2.1
Δ*mfm1*	49.7 ± 2.5
Δ*mfm2*	47.2 ± 4.1
Δ*mfm3*	51.0 ± 1.2
Δ*mfm1* Δ*mfm2*	37.0 ± 2.4[c]
Δ*mfm1* Δ*mfm3*	38.5 ± 0.9[c]
Δ*mfm2* Δ*mfm3*	40.5 ± 1.0[c]
Δ*mfm1* Δ*mfm2* Δ*mfm3*	<0.2[c]
Δ*mfm1* Δ*mfm2* Δ*mfm3* + M-factor[d]	<0.2[c]

[a] The strains tested were EG282, EG662, EG663, EG784, EG664, EG774, EG776, and EG773.

[b] On sporulation medium. The mating procedure and calculation of mating efficiency are described in Materials and Methods. Values represent means of three separate trials ± standard deviations.

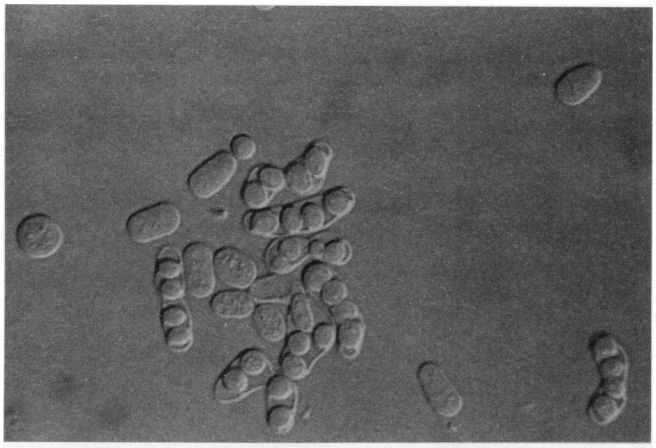
[c] Mating is significantly reduced compared with that of wild-type cells ($P < 5.0\%$; tested by Student's *t* test).

[d] Medium supplemented with 5 to 20 U of M-factor per ml.

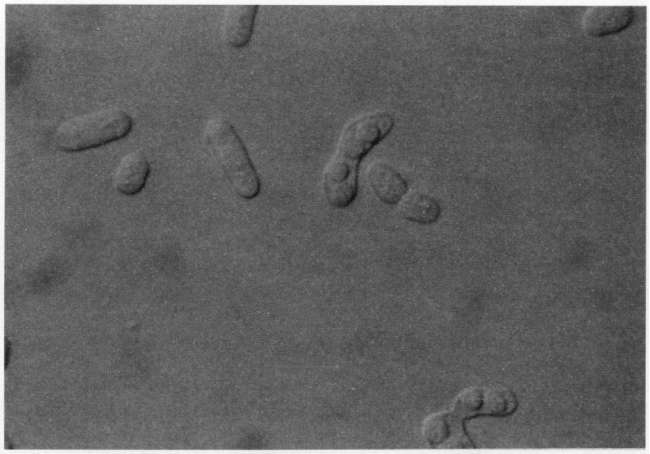
Disruption of all three M-factor genes causes sterility. Disruption of *mfm3* was achieved by replacing the complete coding region with the *ura4*$^+$ gene (Fig. 1C), and replacement of the wild-type allele was confirmed by Southern analysis (data not shown). The homothallic strain lacking all three M-factor genes (EG773) is sterile (Fig. 2C; no zygotes or asci have been observed in liquid or solid medium [Table 2]) but has no other obvious mutant phenotype. Sterility is M cell specific, since the triple disruptant is able to mate with *h*$^-$ but not *h*$^+$ tester cells (data not shown). These results demonstrate that M-factor is essential for mating and that *mfm1*, *mfm2*, and *mfm3* are the only functional M-factor genes.

All three *mfm* genes contribute to M-factor production. Strains containing zero, one, two, or three M-factor genes in all possible combinations were constructed (Table 1). Northern analysis of RNA extracted from nitrogen-starved cultures showed that all three genes are transcribed in wild-type cells and that each is transcribed when present as the only M-factor gene in a homothallic strain lacking the other two (Fig. 4). Furthermore, the Northern analysis suggests that the level of the *mfm3* transcript is raised when the *mfm1* and *mfm2* genes are deleted. The basis for this is currently unknown. Transcripts of *mfm1* and *mfm2* are approximately 450 nucleotides long, while the *mfm3* transcript is about 400 nucleotides long.

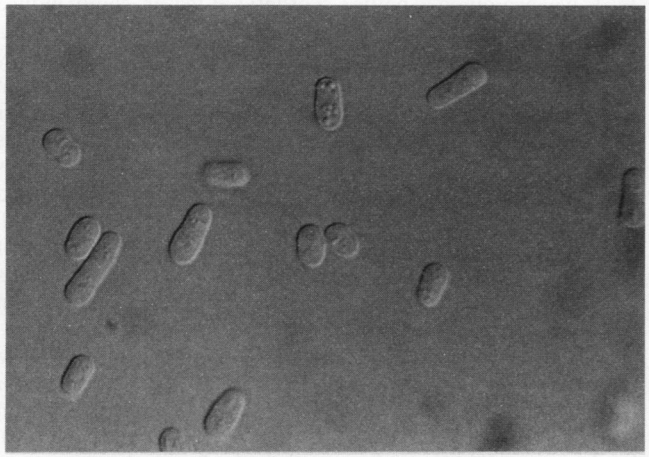
Production of M-factor by strains possessing one, two, or three M-factor genes was determined in a semiquantitative M-factor-dependent sporulation assay (see Materials and Methods). The level of activity observed was related to the number of genes present in the strain being examined (Fig. 5; Table 3): the triply disrupted mutant produced no M-factor,



A h^{90}



B h^{90} *Δmfm1::LEU2 Δmfm2::ura4*$^+$



C h^{90} *Δmfm1::LEU2 Δmfm2 Δmfm3::ura4*$^+$

FIG. 2. Mating behavior of *mfm* disrupted strains on sporulation medium. Cells were grown on MSA plates to induce mating and micrographed with Nomarski optics after 2 days of incubation at 30°C. The presence of asci in panels A and B indicates that mating has occurred. (A) EG280; (B) EG664; (C) EG773.

and strains possessing one gene produced a low level of activity which increased in strains possessing a second gene and increased further in the wild-type cells possessing all three genes. These results not only demonstrate that all three genes are able to produce active M-factor but also suggest that all three genes are normally used by wild-type cells.

Efficient mating requires expression of only one of the *mfm* genes. To investigate the requirement for M-factor during mating, we measured the mating efficiency of the various *mfm* null mutants on solid medium (Table 2). Mating was almost unaffected by the loss of one of the three genes. The mating efficiencies of the double mutants were lowered by 25 to 30%. This result demonstrates that each of the double mutants is capable of producing enough M-factor to sustain efficient mating.

To further analyze the involvement of M-factor in mating, we examined whether the sterility of the *Δmfm1 Δmfm2 Δmfm3* triple disruptant could be rescued by addition of exogenous pheromone. Mating was monitored in both liquid and solid media, and both were supplemented with purified M-factor to between 5 and 20 U/ml: a nitrogen-depleted culture of wild-type h^- cells produces 8 to 10 U of M-factor per ml (7). Addition of M-factor did not alleviate the sterility of the triple disruptant (Table 2), suggesting that the cells must produce M-factor themselves in order to mate.

Transcription of the M-factor genes is limited to M cells and induced by nitrogen starvation. M-factor is produced by M cells but not by P cells (19, 36), and only M cells would therefore be expected to transcribe the *mfm* genes. This was confirmed by Northern analysis, which revealed transcripts of *mfm1*, *mfm2*, and *mfm3* in h^- cells but not in h^+ cells (Fig. 6). The mating type of an *S. pombe* cell is determined by the allele present at the *mat1* locus, *mat1-Mc* specifying M cells and *mat1-Pc* specifying P cells (4, 28). This finding, coupled with the observation that M-factor is produced only in M cells, could suggest that the *mat1-Mc* product controls transcription of the *mfm* genes, and a positive role for Mat1-Mc was confirmed when Northern analysis of a heterothallic strain carrying a *mat1-Mc* allele failed to detect transcription of *mfm1*, *mfm2*, or *mfm3* (Fig. 6A; data shown only for *mfm1* and *mfm3*).

Many of the genes required for sexual differentiation in *S. pombe*, including the mating-type genes (28), are induced by nitrogen depletion, and we next examined whether starvation is required for expression of the *mfm* genes. All three genes are transcribed during vegetative growth, but all three are further induced by nitrogen starvation (Fig. 6). Quantitation of the transcript levels revealed that expression of *mfm2* is only 4- to 5-fold induced, while expression of *mfm1* and *mfm3* is 20- to 30-fold induced by nitrogen starvation (data not shown). Transcription of *mfm3* appears low at all times, at least compared with transcription of *mfm1*.

The product of the *ste11* gene is believed to be a transcription factor required for the expression of several genes involved in sexual differentiation, including all four *mat1* genes (57). It was therefore expected to be required for expression of the *mfm* genes, and this was confirmed by Northern analysis of an h^{90} *ste11* strain: no M-factor transcripts could be detected, even under conditions of nitrogen depletion (Fig. 6A; data shown only for *mfm1* and *mfm3*).

Transcription of the M-factor genes is enhanced by a pheromone signal. In *S. cerevisiae*, many genes involved in the mating pathway are induced when the cells are exposed to mating pheromones (33), an induction that is believed to be mediated by the STE12 transcription factor (10). Even the constitutively expressed pheromone genes themselves are few-

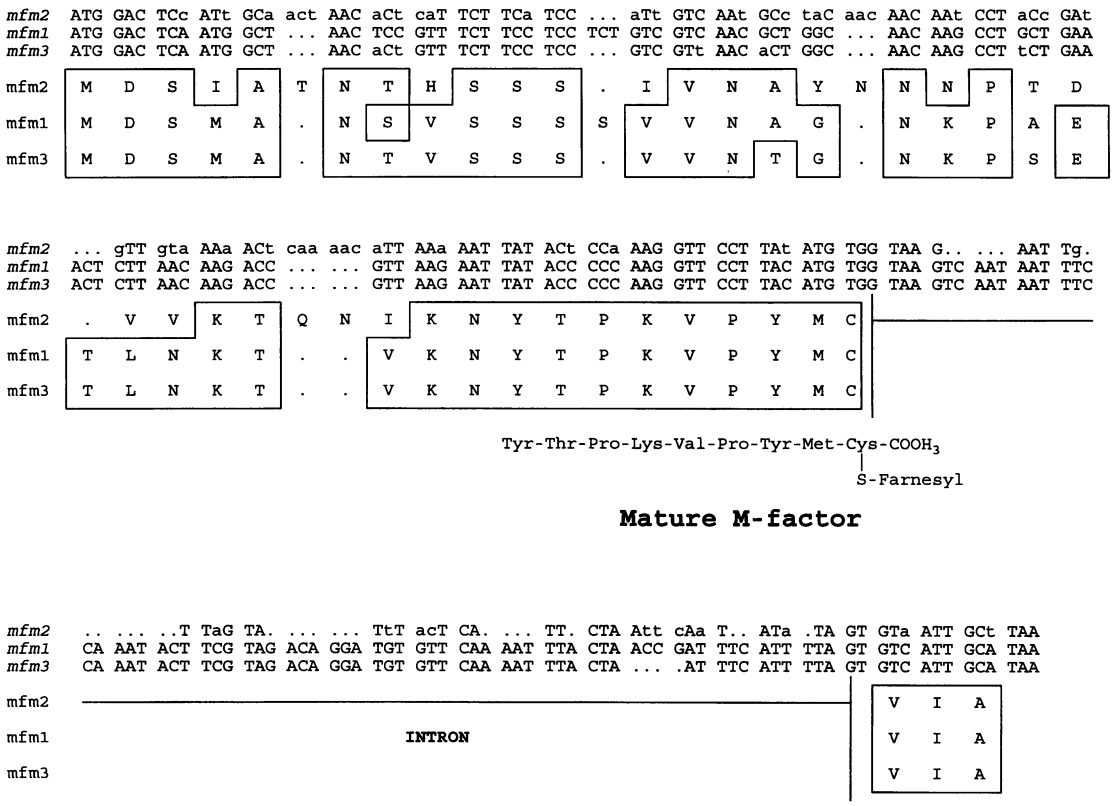


FIG. 3. Sequence alignment. The ORF and intron of *mfm1* are compared with the ORFs and introns of *mfm2* and *mfm3*, respectively. Lowercase letters indicate divergence from the *mfm1* sequence. Identical amino acids are boxed.

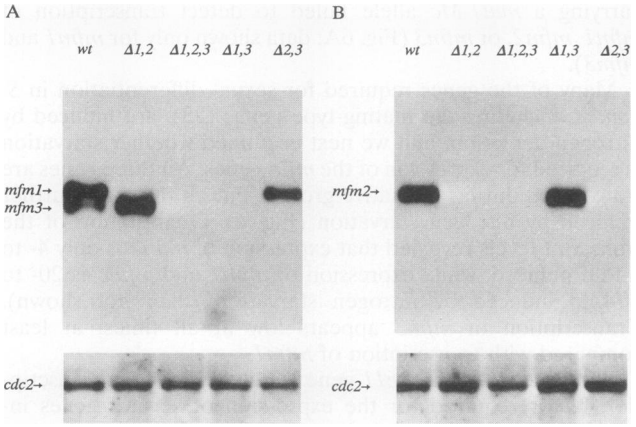


FIG. 4. Identification of the transcripts of the *mfm1*, *mfm2*, and *mfm3* genes by Northern blot analysis. The five homothallic strains EG282 (wild type [wt]), EG664 (Δ*mfm1*::*LEU2* Δ*mfm2*::*ura4*$^+$), EG773 (Δ*mfm1*::*LEU2* Δ*mfm2-D4* Δ*mfm3*::*ura4*$^+$), EG774 (Δ*mfm1*::*LEU2* Δ*mfm3*::*ura4*$^+$), and EG776 (Δ*mfm2-D4* Δ*mfm3*::*ura4*$^+$) were grown in nitrogen-depleted medium. (A) Expression of the 450-bp *mfm1* and 400-bp *mfm3* transcripts. The membrane was hybridized to an *mfm3* probe, which hybridizes to both the *mfm1* and *mfm3* transcripts. (B) Expression of the 450-bp *mfm2* transcript. The lower panel shows the same two membranes hybridized to a *cdc2*-specific probe, which here serves as an internal standard. Transcription of the *cdc2* gene is not affected by the development state of the cells (43).

fold induced by a pheromone signal (1, 55). A preliminary experiment suggested that a similar mechanism may exist in *S. pombe*, a homothallic h^{90} strain showing a higher level of *mfm3* transcription than a heterothallic h^- strain (data not shown). Since the P-factor has recently been isolated and characterized (26), we examined whether synthetic P-factor could induce transcription of the *mfm* genes. Wild-type M cells secrete a very potent P-factor-degrading protease, which is encoded by the *sxa2* gene (25). We therefore performed the induction experiments on h^- *sxa2* cells (Fig. 7). Addition of P-factor clearly induced the transcription of the *mfm* genes in cells starved of nitrogen, and quantitation of the transcript levels revealed a twofold induction of the expression of *mfm1* and *mfm2*, while the expression of *mfm3* was induced fourfold. Nutritional starvation was a prerequisite for the induction of the *mfm* genes by P-factor. When P-factor was added to cells growing exponentially in rich medium, there was no induction of the M-factor genes.

To demonstrate that pheromone induction also takes place in wild-type M cells, we next determined the level of *mfm* transcription in the h^{90} *mat1-Pc* mutant strain. This strain contains the same fraction of M cells as the h^{90} wild type (51), but because of the *mat1-Pc* mutation, the P cells do not secrete P-factor and consequently the M cells are not pheromone stimulated. Transcription is clearly reduced in the mutant compared with that in the wild type (Fig. 8; data shown only for *mfm1* and *mfm2*), confirming that the M-factor genes are induced by pheromone signalling.

Transcription of the M-factor genes requires an intact signalling pathway. The products of the *mam2*, *gpa1*, *ras1*,

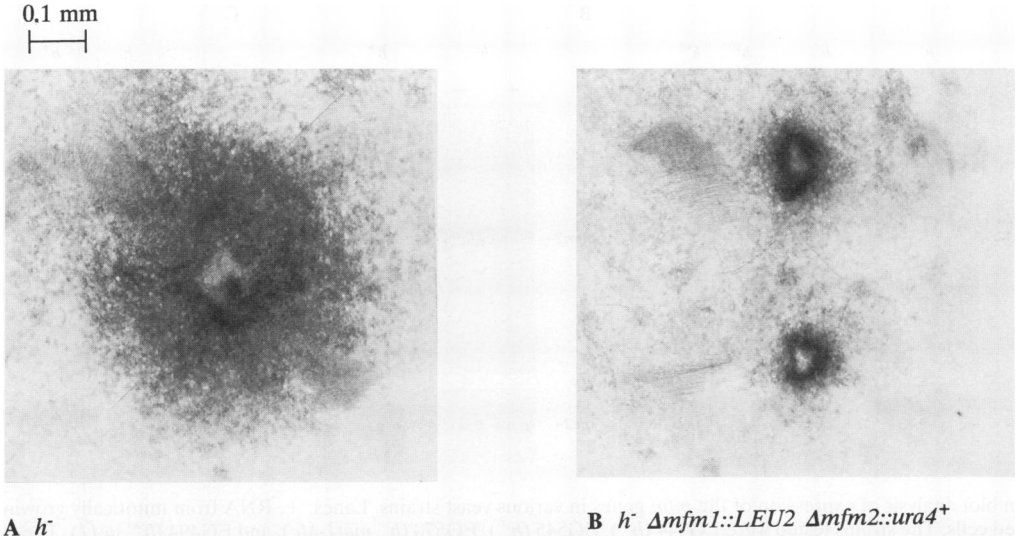


FIG. 5. M-factor secretion by a heterothallic *mfm1 mfm2* disrupted strain and a heterothallic wild-type strain. This halo assay is based on the induction of meiosis in the diploid strain *mat1-Mc⁻/mat1-P* (EG670) by exposure to external M-factor. M-factor-producing strains and the diploid tester strain were grown to a density of 10^6 cells per ml and mixed in a 100-fold excess of the tester strain. The mixture was spotted on MSA and incubated for 2 days. Prior to photography, the plates were exposed to iodine, which stains the haloes of azygotic asci that surround the M-factor-producing cells. (A) EG544; (B) EG667.

byr2, *byr1*, and *spk1* genes are believed to be components of the transduction machinery required for pheromone signalling in an M cell (29, 44, 46, 49, 56, 59). A homothallic h^{90} strain lacking any one of these functions could not therefore be stimulated by P-factor and would be expected to express the *mfm* genes at the same level as a homothallic h^{90} *mat1-Pc* strain, and so we monitored transcription of the M-factor genes in homothallic strains disrupted for either *mam2*, *gpa1*, *ras1*, *byr2*, *byr1*, or *spk1* (Fig. 8; data shown only for *mfm1* and *mfm2*). As expected, loss of the P-factor receptor (encoded by the *mam2* gene) reduced the level of transcription to that of the homothallic *mat1-Pc* strain. In contrast, disruption of the signalling pathway downstream of the receptor caused a further reduction in transcription of the *mfm* genes, the extent of the reduction depending on the mutation. Loss of *spk1*, for example, had little effect on transcription, whereas loss of *ras1* and *byr1* caused a more dramatic reduction in transcription. In general, the transcription of *mfm2* was less affected by the signalling mutations than the transcription of *mfm1* and *mfm3*.

TABLE 3. M-factor production of mutants defective in *mfm1*, *mfm2*, and *mfm3*

Heterothallic strain[a]	M-factor production[b]
Wild type	+++
Δ*mfm1*	++
Δ*mfm2*	++
Δ*mfm3*	++
Δ*mfm1* Δ*mfm2*	+
Δ*mfm1* Δ*mfm3*	+
Δ*mfm2* Δ*mfm3*	+±
Δ*mfm1* Δ*mfm2* Δ*mfm3*	−

[a] The strains tested were EG544, EG728, EG781, EG800, EG667, EG777, EG782, and EG783.

[b] Measured by the halo assay described in Materials and Methods. Symbols: +++, wild-type halo; ++, slightly reduced halo; +, small halo; +±, haloes spanning from slightly reduced to small; −, no halo on the *Mc⁻/P* diploid tester strain.

These results suggest that the components of the signal transduction pathway play at least two roles in controlling expression of the *mfm* genes. Not only do they transmit the P-factor signal required for pheromone-induced expression, but they also play a role in controlling expression in the absence of pheromone stimulation.

DISCUSSION

M-factor, the mating pheromone released by M cells of the fission yeast *S. pombe*, is a nonapeptide in which the C-terminal cysteine residue is S farnesylated and carboxyl methylated (7, 8, 60). Using an oligonucleotide based on the peptide backbone, we previously isolated two genes, *mfm1* and *mfm2*, with the potential to encode the pheromone (8). Our intention in this study was to further investigate these genes with respect to the production of M-factor, and we began by deleting the wild-type allele of each gene from the chromosome. To our surprise, a mutant lacking both *mfm1* and *mfm2* still produced M-factor and was able to mate. This prompted a search for further M-factor genes, and we now describe the isolation and characterization of a third *mfm* gene, *mfm3*. A homothallic strain lacking all three genes fails to produce M-factor and is unable to mate, a mating defect that is specific to the M cells. These observations demonstrate that *mfm1*, *mfm2*, and *mfm3* are the only functional M-factor genes and, furthermore, that M-factor is essential for mating.

The three *mfm* genes map to different regions in the genome and are unlinked (13). The three genes are similar, and each contains the same mature M-factor sequence as part of a larger precursor. Of particular note is the presence of an intron at the same position in each gene, and this feature, allied to the other similarities, strongly suggests that the copies have arisen by gene duplication and have then been dispersed throughout the genome. The *mfm1* and *mfm2* genes are 74% identical through the coding region, while *mfm2* and *mfm3* are 77% identical. There is striking homology between *mfm1* and *mfm3*; the coding regions are 94% identical, and the proposed precursors

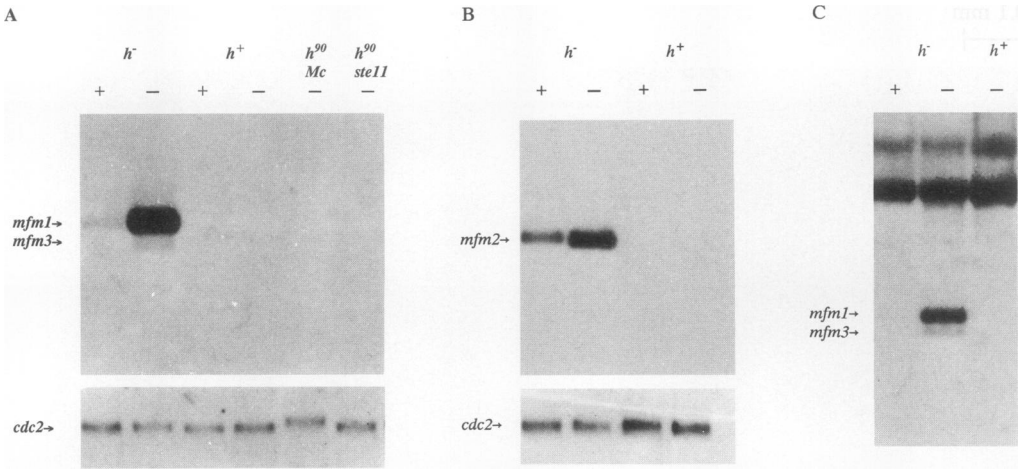


FIG. 6. Northern blot analysis of expression of the *mfm* genes in various yeast strains. Lanes: +, RNA from mitotically growing cells; −, RNA from nitrogen-starved cells. The strains tested were EG544 (h^-), EG545 (h^+), EG570 (h^- *mat1-Mc*), and EG494 (h^{90} *ste11*). The results for EG570 and EG494 are shown only for *mfm1* and *mfm3*. (A) Expression of the *mfm1* and *mfm3* transcripts. The membrane was hybridized to an *mfm1* probe, which hybridizes to both the *mfm1* and *mfm3* transcripts. The lower panel shows the same membrane hybridized to a *cdc2*-specific probe. (B) Expression of the *mfm2* transcript. The lower panel shows the membrane hybridized to a *cdc2* probe. (C) Expression of the *mfm1* and *mfm3* transcripts. The membrane was hybridized to an *mfm3* probe, which hybridizes to both the *mfm1* and *mfm3* transcripts. The two large bands represent unspecific hybridization to 18S and 26S rRNAs.

differ at only four positions, three of which are conserved changes. Even more surprising is the finding that the two introns in *mfm1* and *mfm3* are identical except for a small deletion in *mfm3*. These similarities could suggest that *mfm1* and *mfm3* have diverged only recently, although one cannot exclude other explanations. One possibility, for example, is that changes in these sequences are corrected by intergenic conver-

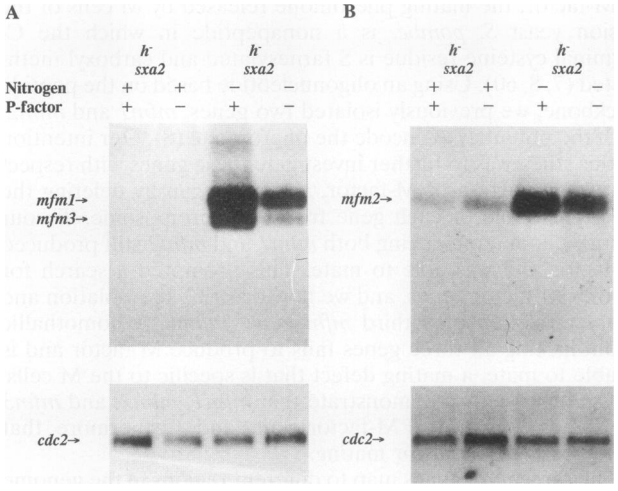


FIG. 7. P-factor-induced transcription of the M-factor genes. The h^- *sxa2* strain (EG699) was grown under four different conditions: nitrogen source plus P-factor present, nitrogen source present, nitrogen starvation in the presence of P-factor, and nitrogen starvation. Synthetic P-factor was added to a final concentration of 3 μg/ml. The ability to transcribe *mfm1*, *mfm2*, and *mfm3* was monitored by Northern analysis. (A) Expression of the *mfm1* and *mfm3* transcripts. The membrane was hybridized to an *mfm3* probe. (B) Expression of the *mfm2* transcript. The lower panel shows the same membranes hybridized to a *cdc2* probe.

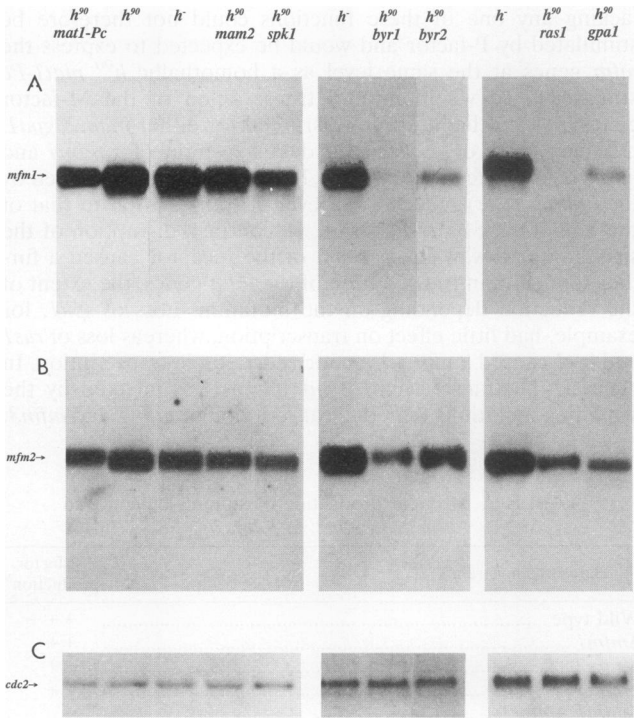


FIG. 8. Effect of interruption of the pheromone transduction pathway on *mfm* transcription. The strains tested were EG410 (h^{90} *mat1-Pc*), EG282 (h^{90}), EG544 (h^-), EG637 (h^{90} *mam2*), EG788 (h^{90} *spk1*), EG495 (h^{90} *byr1*), EG559 (h^{90} *byr2*), EG533 (h^{90} *ras1*), and EG710 (h^{90} *gpa1*). Expression of the M-factor genes was induced by nitrogen starvation. (A) Expression of *mfm1*; (B) the same membranes hybridized to an *mfm2*-specific probe; (C) the same membranes hybridized to a *cdc2*-specific probe.

sion similar to that responsible for the concerted evolution of the three unlinked serine tRNA genes in *S. pombe* (2).

Analysis of strains harboring different combinations of the three *mfm* genes revealed that each contributes to the production of M-factor and that each alone is capable of producing sufficient M-factor to sustain a relatively high mating efficiency. Why does *S. pombe* have three M-factor genes? The simplest explanation is that the genes are functionally redundant and having three copies merely ensures the production of sufficient M-factor when required. This is supported by the observation that a large decrease in M-factor production (Table 3) is accompanied by a small decrease in mating efficiency (Table 2), indicating that wild-type M cells normally produce an excess of M-factor. Redundancy among pheromone genes is not uncommon, and there are two copies of both the **a**-factor and α-factor genes of *S. cerevisiae* (6, 32, 40, 54). Furthermore, each α-factor gene contains tandem copies of the mature pheromone (34).

M-factor functions extracellularly by interacting with receptors on the surface of P cells, and we wondered whether the mating defect of the homothallic Δ*mfm1* Δ*mfm2* Δ*mfm3* triple disruptant could be rescued by providing exogenous M-factor. Addition of exogenous M-factor is known to induce many of the mating-related responses in P cells, including cell elongation (7), transcription of *mat1-Pm* (46), and a G_1 arrest of the cell cycle (9, 26). It did not however rescue the mating defect of the triple disruptant (Table 2), suggesting that M cells must secrete their own M-factor in order to be effective mating partners.

Further work is necessary to explain this requirement, but a similar situation exists in *S. cerevisiae*, in which the addition of pheromone is unable to rescue mutants defective in the production of either α-factor (32) or **a**-factor (40). From studies with *S. cerevisiae*, it appears that cell fusion is promoted by the high concentration of pheromone that is achieved only immediately adjacent to the source cells. This view is supported by triparental matings, which demonstrate that a tester strain presented with a choice of mating partners will choose the partner producing the higher amount of pheromone (27, 40). Further, indirect support comes from the fact that addition of exogenous pheromone will promote mating between pheromone-deficient mutants and strains that are supersensitive to the effects of pheromone (32, 40). Presumably in such cases, the effective concentration of pheromone in the medium is increased to more closely mimic that adjacent to the pheromone-producing cells. Alternative explanations are, of course, possible.

Transcription of the *mfm* genes is regulated at several levels. The first level of control is strictly mating type dependent, and the M-factor genes are transcribed only in M cells (Fig. 6). We find that *mat1-Mc*, which determines the mating type of M cells (28), is a positive regulator of the *mfm* genes (Fig. 6A). The *mat1-Mc* gene product contains a high-mobility-group (HMG) motif that is common to a diverse group of DNA-binding proteins (35, 53). It was recently shown that the *mat1-Mc* HMG box recognizes the AACAAAG heptamer sequence, a sequence also detected by the male determining factor SRY, another HMG box protein (11). Interestingly, this DNA motif is present in the promoters of all three *mfm* genes, positioned 140 to 160 bp upstream of the initiating codons (8) (data not shown). Hence, it is likely that *mat1-Mc* exerts a direct control on *mfm* transcription by binding to the DNA.

M cells transcribe the *mfm* genes at a basal level, regardless of nutritional conditions or extracellular stimuli, and yet we have been unable to isolate M-factor from cultures grown in the presence of nitrogen. Assuming that the *mfm* transcripts are translated under these conditions, a trivial explanation could be that the level of pheromone produced is below that which we can detect. This seems unlikely, however, because our isolation method includes steps designed to concentrate the M-factor several hundred-fold relative to the culture medium. A more likely explanation is that at least one of the other proteins required for the production of active M-factor, perhaps the enzymes responsible for the posttranslational modification of the precursor or the proposed membrane transporter (8), is not expressed under these conditions.

The basal level of *mfm* transcription is increased when the cells are transferred to nitrogen-free medium (Fig. 6), conditions which initiate sexual development in *S. pombe* (12, 14). This induction requires the Ste11 transcription factor (Fig. 6A) that controls the expression of a number of genes involved in sexual development (57). Like the *mat1-Mc* gene, *ste11* encodes a member of the family of HMG box proteins. While Ste11 may indirectly regulate *mfm* expression through its control of *mat1-Mc* (57), it must play some additional role, since a *ste11* strain harboring a plasmid in which the *mat1-Mc* gene is expressed under the control of the *nmt* promoter (37, 38) still fails to transcribe the *mfm* genes (29a).

A third level in the regulation of the M-factor genes is provided by exposure to P-factor, the pheromone released by P cells. When M cells are stimulated by P-factor, there is a twofold increase in transcription of *mfm1* and *mfm2* and a fourfold increase in transcription of *mfm3* (Fig. 7). This increase is not observed in M cells which lack the receptor for the P-factor (encoded by *mam2*) (Fig. 8). Even though we have not yet been able to convincingly demonstrate a corresponding increase in M-factor production, we believe that the pheromone-stimulated expression of the *mfm* genes is of physiological relevance and promotes mating between M cells and P cells. It may, for example, provide an essential boost in M-factor production in response to feedback from a responsive P cell, and this could further explain the failure of exogenous M-factor to rescue the mating defect of the Δ*mfm1* Δ*mfm2* Δ*mfm3* triple disruptant. A similar increase in **a**-factor production is observed in *S. cerevisiae* when **a** cells are stimulated by α-factor (55).

While we expected the pheromone-induced transcription of the *mfm* genes to require a functional signal transduction pathway, we also found that this signalling pathway played a regulatory role in unstimulated cells. Basal transcription of the *mfm* genes was reduced in homothallic strains lacking *gpa1*, *ras1* (*ste5*), *byr2* (*ste8*), *byr1* (*ste1,3*), or *spk1* (Fig. 8). The effect is not confined to homothallic (h^{90}) strains but is also observed in heterothallic minus (h^-) strains (data not shown). Again, a similar control exists in *S. cerevisiae*, in which the expression of mating-related genes is influenced by the *STE4*, *STE5*, *STE7*, and *STE11* gene products, each of which is a component of the signalling mechanism (17, 18, 23). The most likely explanation is that the signalling pathway operates at a low level even in the absence of stimulation by pheromone and that this is essential for expression of the *mfm* genes. We currently have no insights into how the signalling pathway regulates transcription of the pheromone genes, but it does not appear to exert an indirect effect via *mat1-Mc*, since transcription of this gene is unaffected by loss of *ras1* (43). The observation that *mfm* expression is differently affected in the various signalling mutants is not readily comprehensible. This may, however, indicate that we should not consider the pheromone transduction pathway a linear pathway; rather, some of the components may act in concert. Recently, it was found that the expression of *mam2* is similarly dependent on the components of the pheromone response pathway (64), and others showed that the expression

of *map3*, encoding the M-factor receptor, is dependent on *gpa1* (58). Hence, it seems that the signalling pathway also contributes to the specification of cell identity in an as yet unknown fashion.

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