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The a-Factor Pheromone of *Saccharomyces cerevisiae* Is Essential for Mating

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The *Saccharomyces cerevisiae* pheromone a-factor is produced by a cells and interacts with α cells to cause cell cycle arrest and other physiological responses associated with mating. Two a-factor structural genes, *MFA1* and *MFA2*, have been previously cloned with synthetic probes based on the a-factor amino acid sequence (A. Brake, C. Brenner, R. Najarian, P. Laybourn, and J. Merryweather, cited in M.-J. Gething [ed.], *Protein transport and secretion*, 1985). We have examined the function of these genes in a-factor production and mating by construction and analysis of chromosomal null mutations. *mfa1* and *mfa2* single mutants each exhibited approximately half the wild-type level of a-factor activity and were proficient in mating, whereas the *mfa1 mfa2* double mutant produced no a-factor and was unable to mate. These results demonstrate that both genes are functional, that each gene makes an equivalent contribution to the a-factor activity and mating capacity of a cells, and that a-factor plays an essential role in mating. Strikingly, exogenous a-factor did not alleviate the mating defect of the double mutant, suggesting that an a cell must be producing a-factor to be an effective mating partner.

Cell-cell communication mediated by signaling molecules, such as hormones, neuropeptides, and growth factors, plays a crucial role in a variety of processes. The yeast *Saccharomyces cerevisiae* can exist in two haploid cell types, **a** and α, which are distinguished by their ability to produce and respond to such extracellular signaling molecules (for a review, see reference 17). Cells of the **a** mating type secrete **a**-factor, whereas cells of the α mating type secrete α-factor. By interaction with surface receptors on cells of the opposite mating type, these pheromones cause a set of responses, including cell cycle arrest at G_1, morphological alteration, induction of cell surface agglutinins, and stimulation of transcription of certain genes (for reviews, see references 33, 44, and 49). Similar physiological responses occur not only when cells are exposed to purified pheromones, but also during mating when **a** and α cells conjugate to form **a**/α diploids. These observations, together with the finding that many mutants unable to mate are defective in pheromone synthesis or response, suggest that the **a**- and α-factor pheromones play a key role in mating (16, 29, 30).

α-Factor is encoded by two similar structural genes, *MFα1* and *MFα2* (27, 28, 42). The mature pheromone is an extracellular oligopeptide, 13 residues long, which is derived from a precursor by multiple proteolytic processing steps (for a review, see reference 12). The observation that mutants defective in these processing steps are unable to mate, i.e., are sterile, supports the view that α-factor is required for mating. The demonstration that *mfα1 mfα2* null mutants are also unable to mate provides direct proof of an essential role for α-factor in mating (27).

Much less is known about the biosynthesis and function of **a**-factor. Indeed, the precise structure of the active pheromone has not yet been determined. Characterization of peptides purified from **a** cell culture supernatants indicates that there are two active species of **a**-factor, each 12 amino acids long, which differ from one another at a single residue (3, 4, 6) (see Fig. 1). Surprisingly, corresponding synthetically derived peptides have been shown to exhibit only scant biological activity (12; J. Becker and F. Naider, personal communication). Several lines of evidence suggest these synthetic peptides are not fully active because the carboxy-terminal cysteine residue of native **a**-factor may contain a lipid modification critical for activity (3, 36).

Making use of the peptide sequence data, Brake and co-workers (cited in reference 13) designed oligonucleotide probes and isolated two segments of DNA encoding presumptive **a**-factor precursors (see Fig. 1). The corresponding genes are designated *MFA1* and *MFA2*. The detection of increased levels of **a**-factor activity in **a** cells transformed with high-copy-number plasmids carrying *MFA1* or *MFA2* suggested that both of these genes are expressed. However, the involvement of these genes in mating by **a** cells was not examined.

A critical role for **a**-factor in mating has been inferred from the behavior of three mutants, *ste6* (51; J. D. Rine, Ph.D. thesis, University of Oregon, Eugene, Oregon, 1979), *ste14* (L. C. Blair, Ph.D. thesis, University of Oregon, Eugene, 1979), and *ram* (formerly designated *ste16*) (36, 52), which are defective in **a**-factor production. The *ste6*, *ste14*, and *ste16* mutations confer sterility specifically to **a**, but not α, cells. Because of possible pleiotropic effects (known to be the case for *ram* [36]), the inability of these **a**-factor-defective mutants to mate supports but does not prove the hypothesis that **a**-factor, like α-factor, is essential for mating.

In this study, we examined whether the *MFA1* and *MFA2* genes identified by hybridization methods are functional and whether **a**-factor is essential for mating. We describe the cloning of the two presumptive **a**-factor structural genes and

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

Strain	Genotype	Source or reference
EG123	*MAT***a** *trp1 leu2 ura3 his4 can1*	41
246.1.1	*MAT*α isogenic with EG123	41
23α182	*mat*α*2* isogenic with EG123	K. Tatchell
SM1225	*MAT***a** *mfa1-Δ1::LEU2* isogenic with EG123	This study
SM1227	*MAT***a** *mfa2-Δ1::URA3* isogenic with EG123	This study
SM1229	*MAT***a** *mfa1-Δ1::LEU2 mfa2-Δ1::URA3* isogenic with EG123	This study
SM1359	*MAT***a** *TRP1* isogenic with EG123	This study
H1788	*MAT***a**/*MAT*α diploid isogenic with EG123	This laboratory
AM1266-3c	*MAT***a** *rme1::LEU2* isogenic with EG123	A. Mitchell
AM1247-8c	*MAT*α *hmr*α *trp1 leu2 ura 3 lys1 can1*	A. Mitchell
RC757	*MAT*α *sst2-1 met1 his6 can1 cyh2*	8

the use of the cloned genes to produce *mfa1* and *mfa2* null mutants. We show that *mfa1* or *mfa2* single mutants produce ample **a**-factor and are mating proficient, whereas the *mfa1 mfa2* double mutant produces no **a**-factor and exhibits an absolute mating defect. Thus, both genes are functional, and at least one **a**-factor gene must be present for **a** cells to be capable of mating. We also show that the *MFA1* and *MFA2* genes are transcribed at similar levels, are expressed only in **a** cells, and are repressed by the α2 product of *MAT*α.

MATERIALS AND METHODS

Strains, media, genetic methods, and transformation. Yeast strains are shown in Table 1. Strain SM1359 is a Trp^+ derivative of EG123 produced by the one-step gene replacement method of Rothstein (37) with plasmid pUC18-*TRP1* (kindly provided by B. Patterson) as the source of *TRP1* DNA.

Complete medium (YEPD) and synthetic minimal medium (SD) were prepared as previously described (19). Where necessary, SD medium was supplemented with the appropriate nutrients (40). Drop-out media SD-Leu, SD-Ura, and SD-Trp were used to maintain plasmids selectively. Genetic manipulations, such as sporulation and tetrad analysis, were performed as previously described (40).

Yeast transformation was performed by the lithium acetate method (20) with 1 to 10 μg of DNA, with 30 μg of sonicated calf thymus DNA as the carrier. For constructing gene replacements, digestion of 10 μg of CsCl-purified plasmid DNA was carried out in a 10-μl total volume, terminated by heating to 65° C for 10 min, and used directly for transformation without further treatment. Transformants were selected on the appropriate drop-out media.

Standard methods (31) were used for transformation of bacteria. *Escherichia coli* MH1 (14) was used to propagate plasmids.

Oligonucleotide hybridization probes. Oligonucleotides were synthesized by J. Barnett and C. Craik on a Beckman One Plus DNA synthesizer by using solid-phase phosphoramidite chemistry and were purified by electrophoresis on a nondenaturing polyacrylamide gel. The following 30-mer oligonucleotides were used as probes (Fig. 1): SM01 (*MFA1*), 5′-TATATTATCAAAGGTGTCTTCTGGGACC CA-3′; SM02 (*MFA1*), 3′-TGCTGGGTCCCAGAAGACA CCTTTGATAAT-5′; and SM03 (*MFA2*), 3′-GGCGGGATC CCAGAAGAGGCCCTTGATTAT-5′. SM01 (corresponding to codons 22 through 31 of *MFA1*) has the same sequence as *MFA1* mRNA. SM02 (corresponding to codons 23 through 32 of *MFA1*) and SM03 (corresponding to codons 25 through 34 of *MFA2*) are complementary to *MFA1* and *MFA2* mRNA, respectively.

Isolation of plasmids carrying *MFA1* or *MFA2*. A genomic library, containing yeast DNA partially digested with *Sau*3A and cloned into the vector YCp50, was kindly provided by M. Rose, J. Thomas, and P. Novick. Plasmid DNA was transformed into *E. coli*, and transformants were subjected to colony hybridization (31). Oligonucleotide probes SM01 and SM03 were used for identification of *MFA1* and *MFA2* clones, respectively, with the hybridization conditions described below. Positive clones were obtained at a frequency of 1 per 1,000 colonies screened. Two such plasmids, pSM10 (*MFA1*) and pSM23 (*MFA2*), were used in subsequent analyses.

Plasmid constructions. The cloned DNA segments used in this study are shown in Fig. 2 and 3. In vitro manipulations were done by standard procedures as described by Maniatis et al. (31). Plasmid constructions were performed essentially

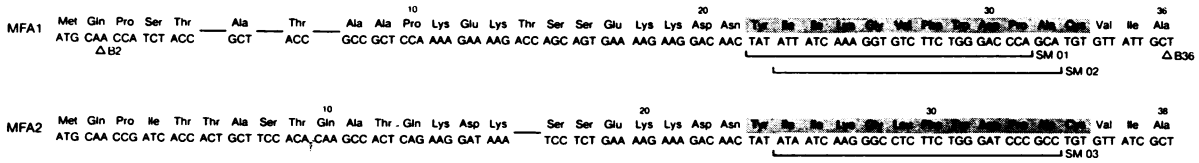

FIG. 1. Structure of the *MFA1* and *MFA2* coding regions. The nucleotide sequence and predicted amino acid sequence of the **a**-factor precursors, as determined by Brake and co-workers (cited in reference 13), are shown. Codons in *MFA1* and *MFA2* are aligned to reflect similar or identical amino acids. Dashes indicate codons not present in the other gene. Codons are numbered with respect to the ATG initiation codon for that gene. The shaded area encompasses the 12 amino acid residues found in mature **a**-factor (3). Oligonucleotide SM01 contains the indicated bases (brackets). Oligonucleotides SM02 and SM03 contain the bases complementary to those indicated (see Materials and Methods). The positions where the nucleotides GGATCC were inserted to create *Bam*HI sites B2 and B36 (see Materials and Methods) are indicated (△).

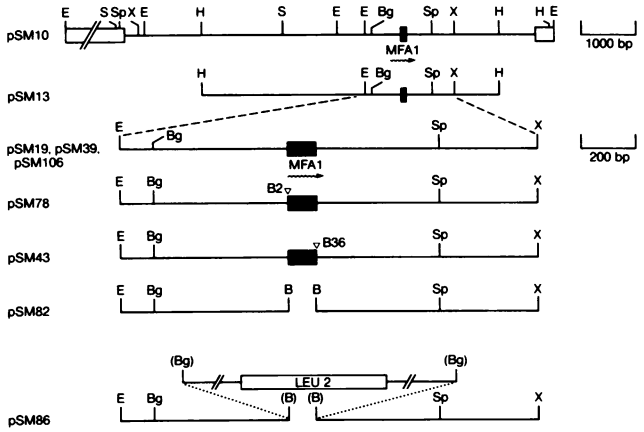

FIG. 2. Cloned segments containing the *MFA1* gene. Only the yeast DNA segments (——) in each plasmid (named at left) are shown, except for pSM10, in which vector sequences are indicated (▭). Construction of the plasmids is described in Materials and Methods. The position of the *MFA1* gene (▬) and its direction of transcription (〰▸) are indicated. The position of insertion of nucleotides GGATCC to create *Bam*HI sites B2 and B36 (see Fig. 1 and Materials and Methods) is indicated (▽). The blank space in pSM82 and pSM86 denotes that the corresponding sequences were deleted. The 3.0-kb *Bgl*II fragment containing *LEU2* is not drawn to scale, and restriction sites contained within it are not shown. The dotted lines show where this *Bgl*II fragment was joined to *Bam*HI sites, resulting in inactivation of these sites as indicated by parentheses. The *Bgl*II-*Xba*I fragment of pSM39 which carries the wild-type *MFA1* gene is 1.5 kb in length, and the *Bgl*II-*Xba*I fragment in pSM86 which carries the *mfa1-Δ1::LEU2* substitution mutation used for gene replacement is 4.6 kb in length. Scale markers refer to plasmids on the adjacent line and below. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; Sp, *Sph*I; X, *Xba*I. bp, Base pairs.

as described by Struhl (47). After restriction digestion, fragments to be cloned were separated by electrophoresis in low-melting-temperature agarose (SeaPlaque from FMC Corp., Marine Colloids Div., Rockland, Maine) and were visualized by staining in ethidium bromide (1 μg/ml). Gel bands were excised, and subsequent ligations and transformations were performed without removal of agarose or ethidium bromide. For constructions in which the vector could recircularize, calf intestinal alkaline phosphatase was added to restriction digests 10 min before electrophoresis and required no further inactivation. Enzymes were obtained from standard sources and were used as recommended by the manufacturer.

To construct a high-copy-number plasmid carrying the *MFA1* gene, the 5.5-kilobase (kb) *Hin*dIII fragment from pSM10 was inserted into YEp13 (*LEU2*, 2μm DNA) (7) that had been cleaved with *Hin*dIII, yielding pSM13. The M13 derivative pSM19, used as a substrate for mutagenesis of *MFA1*, was constructed by inserting the 1.6-kb *Eco*RI-*Xba*I fragment from pSM10 into the *Eco*RI and *Xba*I polylinker sites in M13mp18. Oligonucleotide-directed mutagenesis (described below) was used to incorporate *Bam*HI sites B2 and B36 at codon 2 or 36 of *MFA1* (Fig. 1), and the *Eco*RI-*Xba*I fragments bearing the mutated genes were transferred from pSM19 into pUC18 to yield plasmids pSM78 and pSM43, respectively. A deletion of *MFA1* was produced by replacing the *Eco*RI-*Bam*HI fragment of pSM43 with the *Eco*RI-*Bam*HI fragment from pSM78. The resulting plasmid, pSM82, contains a 102-base-pair deletion (designated *mfa1-Δ1*) extending from the second codon through the final codon of *MFA1*, which encompasses virtually all of the a-factor coding sequence. To genetically mark the deleted *MFA1* gene, the 3.0-kb *LEU2*-bearing *Bgl*II fragment from YEp13 was cloned into the *Bam*HI site of pSM82. The resulting plasmid, pSM86, was used as the source of DNA for producing the chromosomal substitution mutation *mfa1-Δ1::LEU2*. pSM39, used as a hybridization probe for *MFA1*, was constructed by inserting the 1.6-kb *Eco*RI-*Xba*I fragment from pSM10 between the *Eco*RI and *Xba*I sites in pUC18. To produce an autonomously replicating plasmid containing both the *MFA1* gene and a selectable marker, the *Eco*RI fragment from pUC18-*TRP1*, which includes both *TRP1* and *ARS1*, was inserted into the *Eco*RI site of pSM39, yielding pSM106.

To construct a high-copy-number plasmid containing *MFA2*, the 1.75-kb *Hin*dIII fragment from pSM23 was inserted between the *Hin*dIII sites of YEp13, resulting in pSM25. This same *Hin*dIII fragment was also subcloned into pRM3-1, a pUC18 derivative in which the polylinker *Sma*I site had been converted to a *Hin*dIII site (kindly provided by R. McKown), to yield plasmid pSM29, which was used as a hybridization probe for *MFA2*. pSM33 was constructed by replacing the *Eco*RI insert of pUC4-K (Pharmacia) with the 1.6-kb *Eco*RI fragment from pSM23 and was the parental plasmid for creating a deletion of *MFA2*. To provide a convenient source of *URA3* DNA, pSM32 was constructed by filling in the ends of the 1.1-kb *Hin*dIII fragment from YIp30 (clone 6 [1]) and inserting it into the *Hin*cII polylinker site of the pUC18. To construct an *mfa2* substitution mutation, the *Bam*HI-*Sph*I *URA3*-bearing fragment from pSM32 was ligated to pSM33, which had been digested with *Bam*HI and *Sph*I to drop out the *MFA2* region. The resulting plasmid, pSM35, bears a 0.75-kb deletion encompassing the entire *MFA2* coding region replaced by a 1.15-kb segment

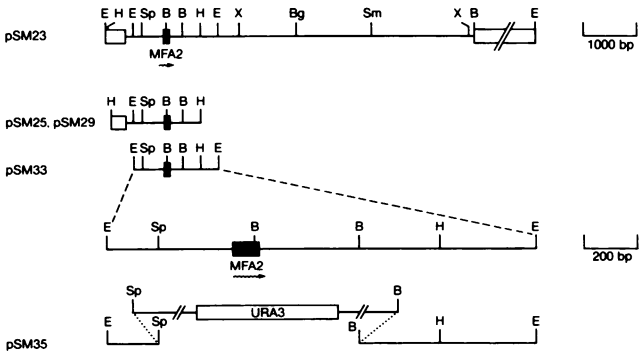

FIG. 3. Cloned segments containing the *MFA2* gene. Only the yeast DNA segments (——) in each plasmid (named at left) are shown, except for pSM23, pSM25, and pSM29, in which vector sequences are indicated (▭). Construction of the plasmids is described in Materials and Methods. The position of the *MFA2* gene (▬) and its direction of transcription (〰▸) are indicated. The blank space in pSM35 indicates that the corresponding sequences were deleted. The 1.15-kb *Sph*I-*Bam*HI fragment containing *URA3* is not drawn to scale, and restriction sites contained within it are not shown. The position where this fragment was inserted is indicated by dotted lines. The *Eco*RI fragment of pSM33 which contains the wild-type *MFA2* gene is 1.6 kb in length, and the *Eco*RI fragment of pSM35 which carries the *mfa2-Δ1::URA3* substitution mutation used for gene replacement is 2.0 kb in length. Scale markers refer to plasmids on the adjacent line and below. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; Sm, *Sma*I; Sp, *Sph*I; X, *Xba*I. Not all restriction sites are shown in pSM23 for *Eco*RI, *Hin*dIII, and *Sph*I. bp, Base pairs.

containing *URA3*. This plasmid was used for chromosomal gene replacement to produce the *mfa2-Δ1::URA3* mutant.

Preparation of probes and hybridization conditions. Oligonucleotides were end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (31). When oligonucleotides were used as probes, hybridizations were performed for 12 h at 25°C in a solution containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 30% formamide, 0.1% sodium dodecyl sulfate (SDS), 5× Denhardt solution, and 100 μg of denatured calf thymus DNA per ml. Filters were washed first for 30 min at 25°C and then for 10 min at 53°C in 1× SSC–0.1% SDS. Under these conditions, there was no detectable cross-hybridization between the *MFA2* probe SM03 and the *MFA1* gene or between the *MFA1* probes SM01 and SM02 and the *MFA2* gene. Plasmid DNA was labeled by nick translation with [α-^{32}P]dCTP (31). Hybridizations with plasmid probes were carried out for 14 h at 42°C in a solution containing 6× SSC, 50% formamide, 0.5% SDS, 5× Denhardt solution, and 100 μg of denatured calf thymus DNA per ml. Filters were washed at 55°C for 1.5 h in 0.2× SSC–0.1% SDS.

Southern and Northern analyses of DNA and RNA. Chromosomal yeast DNA was prepared by the method of Denis and Young (9). For Southern analysis, chromosomal or plasmid DNA was fractionated by electrophoresis in 0.9% agarose and transferred to nitrocellulose paper (31). Hybridization conditions are described above. Washed filters were autoradiographed for 24 h at −70°C with Kodak XAR-2 film and a Du Pont 1 Lightning-Plus intensifying screen.

Total yeast RNA was prepared as described by Jensen et al. (21). For Northern analysis, 20 μg of total RNA per lane was fractionated by electrophoresis on a 1.5% agarose-formaldehyde gel and transferred to a nitrocellulose filter as previously described (11). Hybridization, washing, and autoradiography of RNA filters were the same as for DNA filters.

Oligonucleotide-directed mutagenesis. For insertion of *Bam*HI sites into the *MFA1* gene, oligonucleotide-directed mutagenesis was performed by the double-primer method described by Zoller and Smith (53) with the parental bacteriophage pSM19. The mutagenic oligonucleotides SM07 (5′-AGCGGTAGATGGTGGATCCTGCATTTCTAT-3′) and SM04 (5′-TACGCAGAAACTAGGATCCGCAAA AACACA-3′) contain *Bam*HI sites (underlined) and were synthesized as described above. These oligonucleotides were used to introduce *Bam*HI sites B2 and B36 into *MFA1* at codon 2 and at the carboxy-terminal codon, 36, respectively (Fig. 1). After plaque lifts and hybridization, mutants were identified by a discriminating wash at 55°C for 3 min in 6× SSC. Phages from plaques yielding DNA which remained hybridized at this temperature were purified twice and reprobed to ensure purity. Insertion of *Bam*HI sites at the predicted locations was confirmed by restriction digestion analysis.

a-Factor assays. a-Factor activity produced by growing cells was assayed as described by Wilson (51). Patches of **a** strains to be tested were replicated onto YEPD plates spread with 10^6 cells of the α tester strain RC757 and were incubated at 30°C for 24 h. The *sst2* mutation in RC757 renders it supersensitive to growth inhibition by a-factor (8). For a strain producing a-factor, the patch is surrounded by a clear zone (or halo) where growth of the lawn has been inhibited. The width of the clear zone provides a qualitative measure of a-factor activity.

A second, more quantitative assay was used to measure a-factor activity in cell-free culture supernatants. After growth of cultures in YEPD to saturation (2.4×10^8 cells per ml for all strains tested here), cells were removed by centrifugation, bovine serum albumin (final concentration, 0.1 mg/ml) was added (46), and all remaining cells were removed by filtration with a 0.45-μm-pore-size filter (Millipore Corp., Bedford, Mass.). The supernatant was diluted in twofold increments into YEPD plus 0.1 mg of bovine serum albumin per ml. Aliquots (3 μl) of this dilution series were spotted onto a YEPD plate spread with RC757 cells and incubated as above. The highest dilution of supernatant producing a zone of growth inhibition indicates the level of a-factor present.

Concentration of a cell culture supernatant. A cell-free culture supernatant was prepared as described above from the **a** strain EG123 and was concentrated 50-fold by ultrafiltration with a YM-30 membrane (Amicon Corp., Danvers, Mass.) which retains a-factor (4, 46). The titer of a-factor activity was determined by the quantitative assay (see above) and was shown to be 50-fold higher in the concentrated preparation than in the unconcentrated culture supernatant.

Mating assays. Strains to be assayed were grown to stationary phase overnight. Cells were washed, resuspended in YEPD, and, when necessary, diluted into YEPD. Various numbers of **a** cells (10^2 to 10^7) in 0.2 ml of YEPD were spread on SD plates, together with 10^8 α cells in 0.1 ml of YEPD. For triparental matings, 10^8 **a** helper cells were added together with the **a** cells being tested. Appropriate nutritional supplements were provided for the particular pair of **a** and α strains being evaluated such that diploids, but not haploids, could produce colonies. Where indicated, a concentrated culture supernatant preparation containing a-factor (see above) was added directly to the mating plates. The efficiency of mating is calculated as the ratio of the number of diploids formed (after incubation at 30°C for 2 days) to the number of **a** cells plated (assayed on YEPD).

Other physiological tests. Agglutination was assayed as previously described (16) by mixing exponentially growing cultures of **a** and α strains and visually monitoring the formation of large aggregates 60 min after gentle shaking at 30°C. Strain EMS63 (16), which constitutively produces α agglutinins, was used as the α tester. Response of **a** cell populations to various amounts of α-factor (Sigma Chemical Co., St. Louis, Mo.) was measured by determination of the percentage of morphologically altered cells (shmoos) appearing 90 min after the addition of pheromone. Response to α-factor by single **a** cells was assessed by the confrontation assay (10). **a** cells were placed in close proximity to a population of α-factor-producing cells (strain 246.1.1). The **a** cells were examined microscopically over a period of 6 h to detect arrest of budding and morphological alteration.

Zygote formation was monitored microscopically after **a** and α cell mixtures had been incubated on YEPD medium for 6 h at 30°C, either with or without exogenous a-factor (0.1 ml of 50-fold concentrated culture supernatant per plate).

RESULTS

Cloning the two a-factor genes, *MFA1* and *MFA2*. To isolate the two a-factor genes, we used oligonucleotide SM01, which is homologous to *MFA1*, and oligonucleotide SM03, which is homologous to *MFA2*, as hybridization probes (Fig. 1 and Materials and Methods). A library of yeast genomic DNA in *E. coli* was screened by colony hybridization with each oligonucleotide, and plasmids

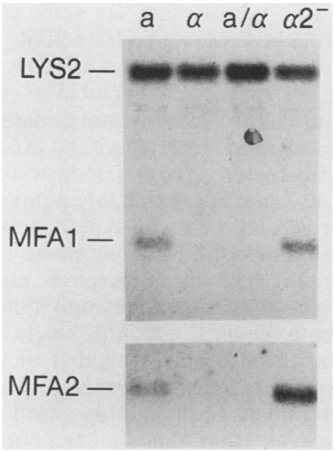

FIG. 4. Regulation of *MFA1* and *MFA2* mRNA synthesis by the mating type locus. Total RNA was isolated from the following isogenic strains: EG123 (*MAT***a**), 246.1.1 (*MAT*α), H1788 (*MAT***a**/*MAT*α), and 23α182 (*matα2*). The relevant genotype of each strain is shown above each lane. RNA was fractionated by formaldehyde-agarose electrophoresis, transferred to a nitrocellulose filter, and probed with a mixture of nick-translated pBR328-LYS2 (11) plasmid DNA to detect *LYS2* RNA and end-labeled oligonucleotide SM02 to detect *MFA1* RNA (top gel) or end-labeled oligonucleotide SM03 to detect *MFA2* RNA (bottom gel). The positions of these RNA transcripts are indicated.

pSM10 containing *MFA1* and pSM23 containing *MFA2* were obtained. Restriction maps of these plasmids are shown in Fig. 2 and 3. The *MFA1* and *MFA2* genes were localized on the cloned DNA by Southern analysis of restriction digests of the plasmids with the appropriate oligonucleotide as a hybridization probe. *MFA1* lies on a 1.6-kb *Exo*RI-*Xba*I fragment and *MFA2* lies on a 1.6-kb *Eco*RI-*Eco*RI fragment, in agreement with Brake and co-workers (cited in reference 13).

Because no **a**-factor mutants existed at the outset of this study, we could not directly determine whether the cloned *MFA1* and *MFA2* segments contained intact, functional coding sequences by complementation analysis. Therefore, we examined whether high levels of expression of these cloned segments would result in increased **a**-factor activity. Restriction fragments bearing the *MFA1* and *MFA2* genes were cloned into the high-copy-number vector YEp13 to construct the plasmids pSM13 and pSM25 (Fig. 2 and 3), and yeast **a** cell transformants containing these plasmids were examined for **a**-factor activity. With both plasmids, increased **a**-factor activity was apparent by two different criteria. In patch tests, larger **a**-factor haloes were observed for the transformants compared with nontransformed strains (as has also been observed by Brake and co-workers, cited in reference 13). Quantitation of **a**-factor activity (see Materials and Methods) in culture supernatants of transformants also demonstrated increased **a**-factor activity, twofold for *MFA1* and fourfold for *MFA2* (data not shown). Therefore, these cloned segments contain active **a**-factor genes. The definitive test of whether the *MFA1* and *MFA2* genes are responsible for all or part of the **a**-factor activity produced by **a** cells is described below.

Regulation of *MFA1* and *MFA2* by *MAT*α. **a** cells produce **a**-factor, whereas α cells and **a**/α diploids do not (5, 50). Cell type specificity is determined by the *MAT* locus, which encodes regulators that control expression of genes specific to the **a** and α cell types (for reviews, see references 17, 18, and 44). It has been demonstrated that three other genes (*STE6* [51], *BAR1* [26], and *STE2* [15, 35]) expressed solely in **a** cells are transcriptionally repressed by the α2 product of *MAT*α. In **a** cells or *matα2* mutants where α2 protein is absent, these genes are transcribed, whereas in α cells and **a**/α diploids where α2 protein is present, their transcription is repressed.

The coding sequences of *MFA1* and *MFA2* are preceded by a potential α2 binding site (22; Brake and co-workers, cited in reference 13). To determine whether transcription of the **a**-factor genes is repressed in an α2-dependent fashion, we assayed *MFA1* and *MFA2* mRNA production in **a**, α, and **a**/α strains and in a *matα2* mutant. As has been previously observed (11; Brake and co-workers, cited in reference 13), Northern blot analysis of total yeast RNA indicated that both **a**-factor genes were transcribed in **a** cells but not in α cells or in **a**/α diploids (Fig. 4). The two genes expressed equivalent amounts of mRNA. Furthermore, we observed that *MFA1* and *MFA2* were transcribed in a *matα2* mutant. These results demonstrate that transcription of *MFA1* and *MFA2* is cell type specific and is negatively regulated by the α2 repressor, likely by direct interaction of the α2 protein with these genes.

Construction of *mfa1* and *mfa2* null mutations. To ascertain the functions of the *MFA1* and *MFA2* genes, we constructed substitution alleles for both genes and then introduced these mutations into the chromosome by the single-step gene replacement technique (37). For *MFA1*, a nearly precise deletion of the coding region was produced in several steps by using oligonucleotide-directed mutagenesis. The deletion was genetically marked by introduction of the *LEU2* gene to produce the substitution mutation *mfa1-Δ1::LEU2*. For *MFA2*, 700 base pairs encompassing the entire coding region and some additional flanking sequences were removed, using available restriction sites. This deletion was genetically marked by introduction of the *URA3* gene to produce the substitution allele *mfa2-Δ1::URA3*. These constructions are shown in Fig. 2 and 3 and are described in detail in Materials and Methods.

Replacement of the resident chromosomal *MFA1* and *MFA2* genes was performed in two sequential steps in a diploid. pSM35, bearing the *mfa2-Δ1::URA3* substitution mutation, was digested with *Eco*RI and transformed into strain H1788, selecting for Ura$^+$ transformants. Southern hybridization analysis of DNA from one such diploid transformant (data not shown) exhibited the pattern expected for a strain heterozygous for the *MFA2* substitution. Subsequently, the *mfa1-Δ1::LEU2* mutation was introduced into the singly disrupted diploid strain by transformation with pSM86 that had been digested with *Bgl*II and *Xba*I, followed by selection for Leu$^+$ transformants. These transformants were anticipated to be diploids heterozygous for the **a**-factor gene replacements (i.e., *mfa1-Δ1::LEU2/MFA1* and *mfa2-Δ1::URA3/MFA2*).

One such Leu$^+$ Ura$^+$ diploid transformant was sporulated, and product tetrads were analyzed to obtain both single and double null mutants. Nearly all tetrads (18 of 21) produced four viable spores, indicating that the **a**-factor genes are not essential. The substitution markers *LEU2* and *URA3* each segregated 2:2 in all 18 tetrads analyzed and were unlinked to each other, which indicates that *MFA1* and *MFA2* are not genetically linked. The genotypes of representative segregants (SM1225, SM1227, and SM1229) containing either the single mutations *mfa1-Δ1::LEU2* and *mfa2-Δ1::URA3* or the double mutation *mfa1-Δ1::LEU2*

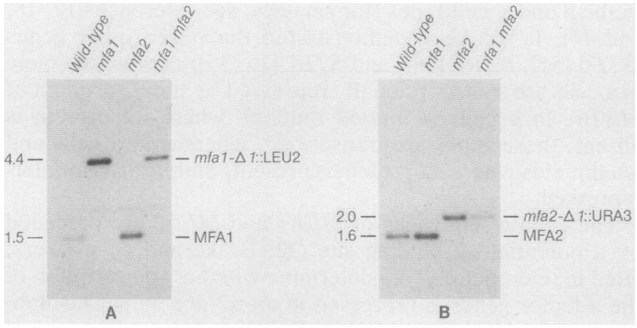

FIG. 5. Southern hybridization analysis of *mfa1* and *mfa2* substitution mutations in single and double mutants. Genomic DNA was isolated from the following strains: EG123 (wild type), SM1225 (*mfa1*), SM1227 (*mfa2*), and SM1229 (*mfa1 mfa2*). DNA was digested with *Bgl*II and *Xba*I (A) and *Eco*RI (B). DNA fragments were electrophoresed in a 1% agarose gel and transferred to a nitrocellulose filter. Filters were probed with nick-translated plasmids pSM39, which contains the *MFA1* gene (A), and pSM29, which contains the *MFA2* gene (B). The size (left) and genotype (right) of the hybridizing fragments are indicated.

mfa2-Δ1::URA3, respectively, were confirmed by Southern hybridization analysis (Fig. 5). For simplicity, we will hereafter refer to *mfa1-Δ1::LEU2* as *mfa1* and to *mfa2-Δ1::URA3* as *mfa2*.

Effect of *mfa1* and *mfa2* null mutations on a-factor production. a-Factor production in the single and double null mutants was measured by using the qualitative and quantitative assays described in Materials and Methods. The qualitative patch tests revealed that *mfa1* and *mfa2* single mutants produced a-factor haloes indistinguishable from those of the wild type (Fig. 6A). In contrast, the *mfa1 mfa2* double mutant produced no discernible halo. To quantitate these observations, we used the endpoint dilution assay to measure a-factor activity in culture supernatants of the various mutants. This test (Fig. 6B) indicated a twofold decrease in a-factor production in the single mutants as compared with wild-type strains. (In some trials, the *mfa1* strain exhibited slightly more a-factor activity than the *mfa2* strain.) With the double mutant, no a-factor was detected. Furthermore, a-factor was undetectable even after 50-fold concentration of culture supernatants (data not shown). These results demonstrate that *MFA1* and *MFA2* are the sole genes encoding a-factor. They demonstrate further that *MFA1* and *MFA2* make approximately equivalent contributions to a-factor activity in a wild-type a strain.

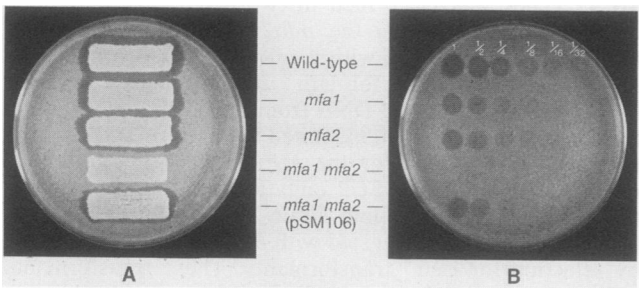

FIG. 6. a-Factor production in single and double mutants. Strains tested are EG123 (wild type), SM1225 (*mfa1*), SM1227 (*mfa2*), SM1229 (*mfa1 mfa2*), and SM1229(pSM106). Plasmid pSM106 contains the *MFA1* gene. (A) Patches of these a strains were replicated onto a lawn of the α *sst2* strain RC757. Secretion of a-factor by the cells in patches prevents growth of the surrounding lawn, resulting in a clear zone or halo. (B) Culture supernatants from the same a strains were prepared and diluted as described in Materials and Methods. Aliquots (3 μl) from the dilution series were spotted onto a lawn of RC757. Dilutions are indicated above; 1 refers to undiluted supernatant, 1/2 refers to twofold-diluted supernatant, 1/4 refers to fourfold-diluted supernatant, etc. Dilution endpoints are 1/16 for the wild type, 1/8 for *mfa1*, 1/8 for *mfa2*, and 1/4 for *mfa1 mfa2* (pSM106). No a-factor was detected for *mfa1 mfa2*.

Effect of *mfa1* and *mfa2* null mutations on mating. To examine the requirement for a-factor in mating, we performed mating tests with the various null mutants (Table 2). We found that both the *mfa1* and *mfa2* single mutants mated with an efficiency comparable with that of the wild type. Thus, their twofold decrease in a-factor activity had little impact on mating under the conditions used. Furthermore, these results suggest that the similar, but not identical a-factor species encoded by *MFA1* and *MFA2*,are similar in promoting mating. In striking contrast to either single mutant, the *mfa1 mfa2* double mutant had a profound mating defect; it mated with an efficiency at least 10^6-fold less than that of the wild type (Table 2). No diploids were detected. This result demonstrates that a-factor is essential for mating.

If the mating defect of the double mutant were due solely to its inability to produce a-factor, introduction of a plasmid bearing an active a-factor gene should reverse both the a-factor production and the mating defects of the double mutant. Therefore, we transformed the *mfa1 mfa2* mutant with pSM106, which carries *MFA1* and *TRP1*. We found that Trp^+ transformants simultaneously regained both the ability to produce a-factor (Fig. 6) and the ability to mate with α cells (data not shown). This complementation test also directly demonstrates that the cloned *MFA1* gene is functional.

We have also shown that the sterile phenotype conferred by the *mfa1 mfa2* mutations is specific to a cells. We observed that α *mfa1 mfa2* segregants obtained from sporulation of the diploid heterozygous for both mutations mated efficiently (data not shown). Furthermore, the *mfa1 mfa2* mutant was able to mate as an α cell after undergoing spontaneous mating type interconversion from a to α (data not shown). Thus, the *mfa1 mfa2* mutations do not result in a nonspecific mating defect, but rather confer sterility to a cells by eliminating a-factor production.

Inability of exogenous a-factor to alleviate the mating defect of the *mfa1 mfa2* mutant. The experiments described above demonstrate that a-factor is essential for mating. To analyze

TABLE 2. Mating efficiency of mutants defective in *MFA1* and *MFA2*

Strain[a]	Mating efficiency[b,c]
Wild type	3.5×10^{-1}
mfa1	2.0×10^{-1}
mfa2	2.8×10^{-1}
mfa1 mfa2	$<1.0 \times 10^{-7}$

[a] The a strains tested were EG123 (wild type), SM1225 (*mfa1*), SM1227 (*mfa2*), and SM1229 (*mfa1 mfa2*).

[b] The α strain used as a mating partner was AM1247-8c. To score mating, cells were plated on SD medium supplemented with leucine, uracil, and tryptophan.

[c] The mating procedure and calculation of mating efficiency are described in Materials and Methods. The reported values represent the mean of four separate trials. The standard deviations for these measurements were 1.4×10^{-1} for the wild type, 0.6×10^{-1} for *mfa1*, and 1.0×10^{-1} for *mfa2*. Mating efficiencies were linear over a range of dilutions (10^0 to 10^{-5}) of a cells tested. 1.0×10^{-7} represents the lowest mating efficiency detectable in this test.

TABLE 3. Inability of a mating-proficient **a** strain to promote mating of the *mfa1 mfa2* mutant

Strain 1[a]	Strain 2[b]	Mating efficiency[c,d]	
		Strain 1	Strain 2
Wild type	−	4.4×10^{-1}	
Wild type	+	4.8×10^{-1}	7.0×10^{-1}
mfa1 mfa2	−	$<1.0 \times 10^{-7}$	
mfa1 mfa2	+	$<1.0 \times 10^{-7}$	5.0×10^{-1}

[a] Strains tested were AM1266-3c (wild type) and SM1229 (*mfa1 mfa2*).
[b] The presence (+) or absence (−) of helper (strain 2) is indicated. Strain 2 is SM1359.
[c] The α strain used as a mating partner was AM1247-8c. Cells were plated on SD medium containing uracil and tryptophan to score mating of strain 1 and on SD medium containing uracil and leucine to score mating of strain 2.
[d] See footnote *c*, Table 2.

further the nature of the mating defect in cells unable to produce **a**-factor, we asked whether the inability of the *mfa1 mfa2* mutant to mate could be overcome by the addition of exogenous pheromone. Mating tests were performed with culture supernatants containing **a**-factor. We were unable to detect mating between the *mfa1 mfa2* mutant and an α tester strain. Even highly concentrated supernatant preparations (up to 50-fold concentrated) were unable to reverse the mating defect of the mutant.

In another test, **a**-factor was supplied by a helper **a** strain. We carried out triparental matings, in which the mating capacity of the *mfa1 mfa2* mutant to α tester cells was examined in the presence of wild-type **a** helper cells. The mating efficiencies of the mutant and the helper **a** cells in this mixture could be scored independently because each **a** strain carried distinguishing nutritional markers. We found that the mating defect of the *mfa1 mfa2* mutant was not alleviated in the presence of helper **a** cells (Table 3). Thus, although the helper cells themselves produce **a**-factor and participate in mating, mutant cells in this same mixture remained unable to mate. We have obtained similar results with a variety of **a** helper and α tester strains (data not shown).

Thus, exogenous **a**-factor, either added directly or provided by other **a** cells, cannot reverse the mating defect of the *mfa1 mfa2* null mutant. These results suggest that **a** cells must be actively producing **a**-factor to mate with wild-type α cells.

Mating of the *mfa1 mfa2* mutant to an α *sst2* strain. In the experiments described above, we examined mating of the *mfa1 mfa2* mutant to a wild-type α strain. We also tested the mating of this mutant to an α *sst2* strain. The *sst2* mutation was initially identified because it confers heightened sensitivity to pheromone (8). Further characterization has suggested this pheromone sensitivity may reflect an alteration in the mating response pathway (9a). In quantitative mating experiments (Table 4), we observed that the *mfa1 mfa2* mutant exhibited a low, but detectable, level of mating to an α *sst2* partner, in contrast to its failure to exhibit any capacity to mate with a wild-type α partner (Table 2). This mating was stimulated nearly 100-fold with exogenous **a**-factor.

Ability of the *mfa1 mfa2* mutant to agglutinate and respond to α-factor. To determine whether aspects of the mating behavior of **a** cells other than **a**-factor production are affected in the *mfa1 mfa2* mutant, we examined other processes associated with mating, such as ability to respond to α-factor and ability to agglutinate with α cells. Response of the mutant to α-factor was measured in two ways. In one test, the confrontation assay was used to determine the response of single **a** cells after exposure to α-factor. Arrest of budding and appearance of morphologically altered cells (shmoos) were identical for mutant and wild-type cells. In a second test, various concentrations of purified α-factor were added to an exponentially growing population of **a** cells and the percentage of shmoos was monitored. The dose-response curves of the mutant and wild-type cells were identical (>95% shmoos at 10^{-5} α-factor, 80% shmoos at 10^{-6} M α-factor, and 2% shmoos at 10^{-7} α-factor). The capacity of the mutant cells to agglutinate with α cells was also tested and found to be indistinguishable from that of wild-type **a** cells (data not shown). These tests show that the *mfa1 mfa2* mutant responds normally to α-factor and is not defective in agglutination. Thus, although the *mfa1 mfa2* mutant is impaired in mating, it can carry out a variety of responses associated with mating.

Zygote formation between **a** and α cells was examined by microscopic inspection of mating mixtures; under conditions in which 10% of wild-type **a** and α cells formed zygotes, no zygotes were observed with *mfa1 mfa2* mutants in mixtures containing thousands of **a** and α cells. The same result was observed when the mating mixtures were supplemented with exogenous **a**-factor. Thus, mating of the mutant is blocked at or before the step of zygote formation.

DISCUSSION

Two active a-factor structural genes. Purification of **a**-factor has led to the identification of two related peptide species, each 12 residues long, which differ from one another at a single amino acid (3, 4, 6) (Fig. 1). Using oligonucleotides predicted from the peptide sequences, Brake and co-workers (cited in reference 13) cloned and sequenced two genes, *MFA1* and *MFA2*, which encode putative **a**-factor precursors. To directly assess the function of *MFA1* and *MFA2* in **a**-factor production and mating, we cloned these genes and created single and double null mutants. The null alleles we constructed contain deletions of the entire coding region of the **a**-factor precursors substituted by selectable markers and were used to replace the chromosomal *MFA1* and *MFA2* genes.

We found that the *mfa1 mfa2* double mutant produced no detectable **a**-factor and was unable to mate with wild-type α cells. This result established that *MFA1* and *MFA2* are the sole structural genes for **a**-factor and that **a**-factor is essential for mating. Our analysis also revealed that *MFA1* and *MFA2* are functionally equivalent genes; they were transcribed at similar levels, and each gene was responsible for approxi-

TABLE 4. Ability of exogenous **a**-factor to promote mating of the *mfa1 mfa2* mutant to an α *sst* partner

Strain[a]	**a**-Factor[b]	Mating efficiency[c,d]
Wild type	−	3.4×10^{-1}
Wild type	+	5.0×10^{-1}
mfa1 mfa2	−	2.3×10^{-6}
mfa1 mfa2	+	1.4×10^{-4}

[a] The **a** strains tested were EG123 (wild type) and SM1229 (*mfa1 mfa2*).
[b] A cell culture supernatant containing **a**-factor was concentrated 50-fold as described in Materials and Methods. A plus sign indicates that 0.2 ml of this preparation was spread on plates before addition of cells; a minus sign indicates that no addition was made.
[c] The *sst2* strain used as a mating partner was RC757. Cells were plated on SD medium.
[d] See footnote *c*, Table 2.

mately half the wild-type level of **a**-factor activity. Mutants defective in *mfa1* or *mfa2* mated as efficiently as a wild-type **a** strain. The different species of **a**-factor encoded by *MFA1* and *MFA2* thus appear to have similar activities. The production of active pheromone by a two-gene family explains the previous failure to isolate **a**-specific sterile mutants which contain mutations in the **a**-factor structural genes (52).

α-Factor is also encoded by two genes, *MFα1* and *MFα2*. Kurjan (27) has shown that an α strain deleted for both genes is unable to mate, establishing that α-factor, like **a**-factor, is essential for mating. The *MFα1* and *MFα2* genes make highly disparate contributions to α-factor activity and mating capacity, unlike the more nearly equivalent contributions to **a**-factor activity and mating provided by the *MFA1* and *MFA2* genes. It is not understood why the pheromone genes are duplicated. However, redundancy of functional genes is not uncommon in *S. cerevisiae* and has been observed, for example, for histones H2A and H2B (24, 38), H3 and H4 (43), α-tubulin (39), *RAS* proteins (23, 48), and the *HMG1*- and *HMG2*-encoded enzymes (2).

Mating defect of the *mfa1 mfa2* double mutant. The mating defect of the *mfa1 mfa2* mutant is severe; its mating efficiency was reduced at least 10^6-fold from that of a wild-type **a** strain. Indeed, we have never recovered a diploid from matings between the double mutant and a wild-type α partner. Thus, **a**-factor appears to be absolutely required for mating. As expected (because only **a** cells express the **a**-factor genes and produce **a**-factor), the mating defect conferred by the *mfa1 mfa2* mutations was specific to **a** cells. α cells harboring the *mfa1 mfa2* mutations mate normally. The sole role of the *MFA1* and *MFA2* genes appears to be in production of **a**-factor, since we have shown that the double mutant is unimpaired in other **a** cell functions, such as response to α-factor and agglutination.

Inability of exogenous pheromone to alleviate the mating defect of the *mfa1 mfa2* mutant. Given the view that **a**-factor is an extracellular signaling molecule which interacts with a receptor on α cells to promote mating, it might be expected that the mating defect of the *mfa1 mfa2* mutant would be alleviated by providing exogenous **a**-factor. We tested this expectation in two ways and found that in neither case did exogenous **a**-factor help the *mfa1 mfa2* mutant become proficient in mating. In one test, we supplemented a mating mixture with a culture supernatant containing **a**-factor, and in another test, we performed triparental matings in which **a**-factor was supplied by wild-type **a** cells. In the latter case, the wild-type **a** partner mated efficiently; therefore, the observed mating defect of the *mfa1 mfa2* mutant cannot be due to the lack of extracellular **a**-factor in the mating mixture. Thus, we conclude that **a** cells must be actively producing **a**-factor to be effective mating partners. Similarly, Kurjan (27) has found that the mating defect of an *mfα1 mfα2* mutant is not alleviated with exogenous α-factor.

Several explanations can be considered to account for the inability of exogenous **a**-factor to promote mating of the *mfa1 mfa2* mutant. In one view, some form of **a**-factor or its precursor may remain physically associated with the cell producing it. (It has been suggested that **a**-factor contains a lipid which could anchor it to the cell surface [3, 36].) Cell-bound **a**-factor could provide a mating signal to α cells that is distinct from the signal to arrest growth or cause morphological alteration, since these latter responses can be triggered by purified **a**-factor which is not cell associated (4). A well-established example of a signaling process involving a cell-bound molecule is the antigen-mediated stimulation of T cells, which occurs only when antigens are presented together with cell surface components encoded by the major histocompatibility complex (25). Another role for cell-bound **a**-factor might be to promote cell fusion during mating. Cell-associated **a**-factor does not appear to be necessary for agglutination, since we found that the *mfa1 mfa2* mutant is not defective in agglutination.

In another view, **a**-factor could play a solely extracellular role, but to function fully, it would be required at an extremely high local concentration, achieved only immediately adjacent to cells which are producing **a**-factor (and unattainable by addition of exogenous **a**-factor). Different levels of pheromone are known to elicit different aspects of the mating response. Low levels of pheromone are sufficient to induce cell surface agglutinins and cause cell cycle arrest, whereas higher levels are required to produce morphological alterations (5, 34). Perhaps an even greater amount of **a**-factor is necessary to provide an additional signal critical for mating. This hypothesized signal might be required to ensure that **a** and α cells are correctly positioned for fusion. Sufficiently high levels of pheromone may be found only at, or near, sites on the **a** cell surface where **a**-factor is newly secreted, after which rapid dilution by the culture medium or degradation might occur. In this regard, it is interesting to note that **a** cells secrete a protease (encoded by the *BAR* gene) which degrades α-factor (32, 45). High levels of exogenous α-factor can promote at least some mating between an α *mfα1 mfα2* mutant and an **a** *bar* partner which lacks the ability to degrade α-factor (27).

Although we were unable to detect mating of the *mfa1 mfa2* mutant with a wild-type α partner, we have shown that it does exhibit a low level of mating with an α *sst2* partner and that this mating is considerably stimulated by exogenous **a**-factor. Similarly, exogenous α-factor stimulates mating between an α-factor null mutant and an **a** *sst2* partner (27). The ability of exogenous pheromone to promote some mating between pheromone-deficient strains and *sst2* partners, but not wild-type partners, may reflect the heightened sensitivity of *sst2* mutants to mating pheromones.

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