Cell Fusion during Yeast Mating Requires High Levels of a-Factor Mating Pheromone

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Abstract. During conjugation, two yeast cells fuse to form a single zygote. Cell fusion requires extensive remodeling of the cell wall, both to form a seal between the two cells and to remove the intervening material. The two plasma membranes then fuse to produce a continuous cytoplasm. We report the characterization of two cell fusion defective (Fus^-) mutants, *fus5* and *fus8*, isolated previously in our laboratory. Fluorescence and electron microscopy demonstrated that the *fus5* and *fus8* mutant zygotes were defective for cell wall remodeling/removal but not plasma membrane fusion. Strikingly, *fus5* and *fus8* were **a** specific; both mutations caused the mutant phenotype when present in the *MAT***a** parent but not in the *MAT*α parent. Consistent with an **a**-specific defect, the *fus5* and *fus8* mutants produced less **a**-factor than the isogenic wild-type strain. *FUS5* and *FUS8* were determined to be allelic to *AXL1* and *RAM1*, respectively, two genes known to be required for biogenesis of **a**-factor. Several experiments demonstrated that the partial defect in **a**-factor production resulted in the Fus^- phenotype. First, overexpression of **a**-factor in the *fus* mutants suppressed the Fus^- defect. Second, matings to an *MAT*α partner supersensitive to mating pheromone (*sst2*Δ) suppressed the Fus^- defect in trans. Finally, the gene encoding **a**-factor, *MFA1*, was placed under the control of a repressible promoter; reduced levels of wild-type **a**-factor caused an identical cell fusion defect during mating. We conclude that high levels of pheromone are required as one component of the signal for prezygotes to initiate cell fusion.

THE mating pathway in the yeast *Saccharomyces cerevisiae* is an excellent system to study basic cellular processes such as signal transduction and cell fusion (for reviews see Cross, 1988; Konopka and Fields, 1992; Sprague and Thorner, 1992; Herskowitz, 1995). Each haploid cell secretes a mating type–specific pheromone (**a**-factor or α-factor) and expresses a surface receptor that is able to bind the pheromone secreted by the opposite cell type. Binding of the pheromone to the receptor causes the dissociation of a heterotrimeric G-protein, leading to activation of a mitogen-activated protein (MAP)[1] kinase signal transduction pathway that is identical in both cell types (Bender and Sprague, 1986, 1989). Stimulation by pheromone leads to arrest of the cell cycle in G1 and the transcriptional induction of several genes required for efficient mating (e.g., *STE2*, *STE3*, *MFA1*, *MFA2*, *STE6*, *FUS1*, and *FUS2*; for review see Sprague and Thorner, 1992). At higher pheromone concentrations, cells initiate directional cell growth, resulting in the development of a mating projection. The orientation of cell growth responds to the gradient of pheromone such that the mating projection points toward the nearby partner (Jackson and Hartwell, 1990*a*,*b*; Madden and Snyder, 1992; Segall, 1993; Dorer et al., 1995; Valtz et al., 1995). After contact between partner cells is established (generally at the shmoo tip), cell fusion produces the zygote. Subsequently, the two nuclei are brought together via microtubule-dependent movement and the nuclear membranes fuse at the spindle pole body to form a single diploid nucleus (Rose, 1991, 1996).

In contrast to the general understanding about the pathways of signal transduction and nuclear fusion in conjugation, little is known about the mechanism and regulation of cell fusion. To fuse, two mating cells must first undergo extensive remodeling of the cell wall to form a seal resistant to osmotic pressure, then the intervening cell wall is broken down, and finally the two plasma membranes must fuse. The structural machinery that mediates cell wall degradation at the site of cell contact and the fusion machinery that mediates the plasma membrane fusion process remain to be identified. Three genes, *FUS1*, *FUS2*, and *FUS3* (McCaffrey et al., 1987; Trueheart et al., 1987; Trueheart and Fink, 1989; Elion et al., 1990, 1995; Berlin et al., 1991)

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1. *Abbreviations used in this paper*: 5-FOA, 5-fluoro-orotic acid; DAPI, 4′,6′-diamidino-2-phenylindole; MAP, mitogen-activated protein; YEPD, yeast extract/peptone/glucose.

have been identified in which mutations result in cell fusion defective zygotes. Fusion defective zygotes are recognized microscopically by a remnant phase-dense plate at the intersection of the joined cells and the presence of two unfused nuclei. Fus1p is an *O*-glycosylated type I membrane protein localized to the shmoo projection (Trueheart et al., 1987; Trueheart and Fink, 1989). A carboxy-terminal Src homology 3 domain in Fus1p may be important for interactions with cytoskeletal structures (Amberg et al., 1995). Fus2p is also tightly associated with membranes and localizes to punctate intracellular structures under the surface of the shmoo projection (Elion et al., 1995). Although the biochemical role of Fus1p and Fus2p remains obscure, the pheromone-dependent expression and subcellular localization of the two proteins suggest a direct role for Fus1p and Fus2p in cell fusion. *FUS3* encodes a MAP kinase that has several functions in the pheromone signal transduction pathway (Elion et al., 1990). Fus3p activates Ste12p, a transcriptional activator of the pheromone response, and Far1p, an inhibitor of the Cdc28–Clnp complex necessary to promote the pheromone-induced G1 arrest (Elion et al., 1990; Fujimura, 1990; Peter et al., 1993; Tyers and Futcher, 1993; Peter and Herskowitz, 1994; Herskowitz, 1995). The role of Fus3p in cell fusion is unclear and could reflect either activation of a cell fusion gene via the pheromone response pathway or inactivation of a cell fusion inhibitor during the mitotic cell cycle. The *fus1*, *fus2*, and *fus3* mutants show no cell-type specificity, and their defects are most severe when both mating partners are mutant (McCaffrey et al., 1987; Trueheart et al., 1987; Elion et al., 1995).

In principle, activation of some parts of the cell fusion machinery should be hazardous during vegetative growth because it would make cells potentially sensitive to hypotonic conditions. It seems likely, therefore, that cell fusion would be subject to regulatory mechanisms that restrict it temporally and spatially during mating. Indeed, although the addition of α-factor to *MAT***a** cells induces many, if not all, of the initial steps in mating, such cells do not become osmotically sensitive. Therefore, some aspect of cell fusion must be triggered by the presence of the partner cell, once contact between the two mating cells is achieved. The identity of the signal, the signal reception, and the immediate responses required for cell fusion are not known.

A genetic screen for cell and nuclear fusion mutants re-

Table I. Yeast Strains Used in This Study

Strain	Genotype	Plasmid	Source
MS2073	*matΔ::LEU2 HIS3::pMR1811 ura3-52 trp1-Δ1 leu2-3,112*		*
MS2104	*matΔ::LEU2 HIS3::pMR1811 ura3-52 trp1-Δ1 leu2-3,112*	B1311	*
MS2326	*matΔ::LEU2 HIS3::pMR1811 ura3-52 trp1-Δ1 leu2-3,112 fus5-424*	B1311	*
MS2328	*matΔ::LEU2 HIS3::pMR1811 ura3-52 trp1-Δ1 leu2-3,112 fus5-424*		*
MS2741	*matΔ::LEU2 HIS3::pMR1811 ura3-52 trp1-Δ1 leu2-3,112 fus8-1325*	B1311	*
MS2742	*matΔ::LEU2 HIS3::pMR1811 ura3-52 trp1-Δ1 leu2-3,112 fus8-1325*		*
MS14	*MATα ura3-52 ade2-101*		‡
MY3371	*MAT***a** *ura3-52 leu2-Δ1hap2 mal*		‡
MY3666	*MAT***a** *leu2 ura3-52 fus5-424*		‡
MS3963	*MAT***a** *ura3-52 leu2-3,112 ade2-101 his3-Δ200 fus5-Δ::LEU2*		‡
SM1581	*MAT***a** *leu2 ura3 trp1 his4 can1*	pSM219	§
MS18	*MATα ura3-52 trp1-Δ1*		‡
SM1086	*MATα his6 met1 can1 cuh2 rme sst2-1*		§
MS600	*MAT***a** *ura3-52 leu2-3,112 skd2-1*	pHS1	‖
MS4222	*MATα ura3-52 ade2-101 sst2Δ*		‡
MY4203	*MAT***a** *ura3-52 leu2-Δ1 hap2 mal*	pRS426	‡
MY4204	*MAT***a** *ura3-52 leu2-Δ1 hap2 mal*	pSM219	‡
MY4205	*MAT***a** *ura3-52 leu2-Δ1 fus5-424*	pRS426	‡
MY4206	*MAT***a** *ura3-52 leu2-Δ1 fus5-424*	pSM219	‡
MS4279	*MAT***a** *ura3-52 leu2-Δ1 fus8-1325*	pRS426	‡
MS4280	*MAT***a** *ura3-52 leu2-Δ1 fus8-1325*	pSM219	‡
MS4281	*MAT***a** *ura3-52 leu2-3,112 ade2-101 his3-Δ200 fus5-Δ::LEU2*	pRS426	‡
MS4282	*MAT***a** *ura3-52 leu2-3,112 ade2-101 his3-Δ200 fus5-Δ::LEU2*	pSM219	‡
MS4236	*MAT***a** *leu2-3,112 trp1-Δ1 ura3-52 his3-Δ200*	pRS416	‡
MS4235	*MAT***a** *leu2-3,112 trp1-Δ1 ura3-52 his3-Δ200*	pSM233	‡
MS4234	*MAT***a** *leu2-3,112 trp1-Δ1 ura3-52 his3-Δ200*	pSM1023	‡
MS2288	*MAT***a** *leu2-3,112 trp1-Δ1 ura3-52 his3-Δ200*		‡
MS4233	*MAT***a** *leu2-3,112 trp1-Δ1 ura3-52 his3-Δ200 mfa1Δ mfa2Δ*	pRS416	‡
MS4232	*MAT***a** *leu2-3,112 trp1-Δ1 ura3-52 his3-Δ200 mfa1Δ mfa2Δ*	pSM233	‡
MS4231	*MAT***a** *leu2-3,112 trp1-Δ1 ura3-52 his3-Δ200 mfa1Δ mfa2Δ*	pSM1023	‡
XBH16-15A	*MATα ade2-1 his leu2-3,112 trp1-289 ura3-52 kex2-1*		¶
A2S3	*MATα ade6 his6 leu1 met1 trp5-1 ste13-1 rme*		¶
XT1172-S245c	*MATα ade6 his6 leu1 met1 trp5-1 rme*		¶
MS518	*MATα ade2-101 ura3-52 skd2-1*		‖

*Kurihara et al., 1994;
‡Rose laboratory;
§Michaelis laboratory;
‖Scidmore, 1993;
¶Chan et al., 1983.

cently conducted in our laboratory yielded seven new cell fusion defective mutations, defining alleles of four new genes required for cell fusion, *FUS5*, *FUS6*, *FUS7*, and *FUS8*, as well as the previously characterized gene *FUS2* (Kurihara et al., 1994). In this paper, we describe the characterization of the cell fusion defect of *fus5* and *fus8* mutants and identify the *FUS5* and *FUS8* genes as being two previously characterized genes involved in the biogenesis of **a**-factor. Based upon these and additional results, we propose a model in which high levels of pheromone are required to trigger cell fusion after the two mating cells are in close contact.

Materials and Methods

Microbial Techniques, General Methods, and Strains

Yeast media and genetic techniques were essentially as described previously (Rose et al., 1990). Yeast and *Escherichia coli* plasmid DNA minipreps were performed as described elsewhere (Rose et al., 1990). Yeast transformations were done by the lithium acetate method (Ito et al., 1983). The ends of the 5-kb insert in plasmid pMR2652 were sequenced using Sequenase (United States Biochemical Corp., Cleveland, OH) and the T3 and T7 primers following the manufacturers instructions. Isolation of yeast DNA for Southern blotting analysis to confirm generation of deletion alleles of *FUS5* and *MFA1* was prepared by the method of Hoffman and Winston (1987). Southern blots were performed as described elsewhere (Rose et al., 1990). The strains used in this study are listed in Table I. Unless stated otherwise, all strains are isogenic to S288C.

Strain Construction and Plasmids

To generate an *axl1*Δ allele, plasmid pMR3128 (see Fig. 5) was constructed by cloning the 1041-bp SalI/BamHI and the 1,100-bp XbaI/SalI restriction fragments from pMR2652 into the pRS405 YIp-*LEU2* vector (Sikorski and Hieter, 1989) cut with XbaI/BamHI. To introduce the deletion allele into yeast, pMR3128 was linearized with SalI before transformation of MS1554.

Plasmid pBC14 (from J. Trueheart; Cadus Pharmaceutical, Tarrytown, NY), a YIp5-based plasmid with an internal HpaI-HpaI fragment deleted from the *SST2* gene was used to generate a *sst2*Δ allele. To integrate the deletion, pBC14 was linearized with NheI before transformation of MS14. Loop-outs of pBC14 were selected on 5-fluoro-orotic acid (5-FOA) media. Candidate transformants were tested by PCR amplification and confirmed by their increased sensitivity to **a**-factor in a standard halo assay.

Plasmids pDH6 and pDH9 (from George Sprague, University of Oregon, Eugene, OR) were used to generate *mfa1*Δ *mfa2*Δ double deletion strain. Plasmid pDH9, carrying the *mfa2*Δ allele, was linearized with HindIII and used to transform MS2288. Loop-outs of pDH9 were selected by plating on 5-FOA media. Potential positives were screened by Southern blot hybridization of genomic DNA as described in Rose et al. (1990). One of the positives carrying *mfa2*Δ was used as a recipient in the transformation with pDH6, containing the *mfa1*Δ allele, linearized with SphI. After selection for pDH6 loop-outs by growth on 5-FOA medium, several colonies were tested for **a**-factor production and mating sterility.

Plasmid pSM1023 carrying P_{MET}-*MFA1* was generated by cloning the XbaI/EcoRI fragment containing the *MFA1* coding sequence from pSM118 (Chen et al., 1997), into p416MET25 (Mumberg et al., 1994).

Plasmids pB1311, containing the *MAT*α locus, and pHS1, carrying the *RAM1/DPR1* gene, were obtained from Jim Broach (Princeton University, Princeton, NJ). Plasmids pSM51 (Sapperstein et al., 1994), pSM192 (Berkower and Michaelis, 1991), pSM219 (Chen et al., 1997), and pSM233 (Chen et al., 1997) containing *STE14*, *STE6*, *MFA1*, and *MFA1*, respectively, were described previously. Vector control plasmids used in this study were pRS416 and pRS426 (Sikorski and Hieter, 1989).

Mating Assays

Limited plate matings were performed as described in Kurihara et al. (1994). Briefly, patches of wild-type and mutant strains were replica printed onto prewarmed plates containing lawns of wild-type or mutant strains. Yeast extract/peptone/glucose (YEPD) medium was used for most experiments. For experiments in which the level of **a**-factor was regulated by P_{MET}, synthetic dextrose minimal medium plates containing adenine, tryptophan, histidine, and leucine were used supplemented with either 0, 1.5, or 3.0 mM of methionine. The mating plates were incubated at 30°C for 3 h, followed by replica printing to appropriate media to select diploids. Halo assays were performed by replica printing patches of wild-type or mutant strains onto YEPD plates containing a lawn of a strain of the opposite mating type, supersensitive to the pheromone (SM1086 or MS4222), and incubating the plate for 1 or 2 d at 23°C.

Filter matings for the microscopic analysis of the zygotes were performed as described in (Kurihara et al., 1994). Liquid cultures of **a** and α cells were grown in YEPD or in synthetic media lacking uracil (if selecting for a plasmid) to equivalent cell densities in early exponential phase. 1 ml of each culture was filtered onto a 0.45-μm nitrocellulose filter disc (Millipore Corp., Milford, MA). The filters were placed cell-side up on a prewarmed YEPD plate for most experiments or on a synthetic dextrose minimal medium plate supplemented with adenine, tryptophan, histidine, and leucine and either 0, 1.5, or 3.0 mM methionine for experiments in which the level of **a**-factor was varied. Matings were done for 3 h at 30°C. The cells were then rinsed off the filter with 1 ml of cold PBS, pH 7.4, into a microfuge tube, centrifuged briefly, and washed twice in cold PBS. The mating mixtures were fixed in methanol/acetic acid (3:1) for 60 min on ice and then washed several times in PBS. Finally, the mating mixtures were stained with 4′,6′-diamidino-2-phenylindole (DAPI) (1 μg/ml in PBS) for 5 min and washed once with PBS. The mating mixtures were kept at 4°C for up to 1 wk before examination. Samples were lightly sonicated before scoring to break up aggregates. The mating mixtures were then examined by differential interference and fluorescence microscopy using a microscope (Axiophot; Carl Zeiss, Inc., Thornwood, NY). For experiments in which the plasma membrane was stained with FM4-64 (Molecular Probes, Eugene, OR), the mating mixtures were resuspended in ice-cold H_2O. The dye was added to 33 μM final concentration just before fluorescence microscopic examination using the standard rhodamine filter set (Carl Zeiss, Inc.).

Electron Microscopy of Zygotes

To produce sufficient numbers of cells for electron microscopy, the limited filter mating protocol was scaled up 20-fold. The mating mixtures were fixed with FIX (40 mM potassium phosphate, pH 7.4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.2 mM sorbitol, 2% fresh glutaraldehyde) for 30 min at room temperature. After washing three times in 50 mM potassium phosphate, pH 7.4, the samples were incubated in 4% potassium permanganate at 4°C for 4 to 6 h. The cells were washed four times with distilled H_2O and then resuspended in 1 ml of 1% sodium periodate (Sigma Chemical Co., St. Louis, MO) for 15 min at room temperature. The samples were washed once with 50 mM potassium phosphate and then resuspended in 50 mM ammonium phosphate for 15 min at room temperature. After two washes with distilled H_2O, the cells were resuspended in 2% filtered uranyl acetate and incubated at 4°C overnight with mixing. Cell dehydration was done by a series of washes in ethanol (50% ethanol and 70% ethanol for 5 min, twice; 95% ethanol for 5 min; 100% ethanol for 5 min, three times). The samples were embedded in LR White resin (Polysciences, Inc., Warrington, PA). The sections were cut to 70–90 nm and stained with Reynolds lead citrate (Reynolds, 1963).

Cloning of FUS5 and Identification of FUS8

The mating defect of *fus5-424* was used to clone *FUS5*. A yeast centromere–based (YCp50) genomic library (Rose et al., 1987) was transformed into an *MAT***a** *fus5-424* strain (MS2328). 15,000 Ura^+ transformants were screened for restoration of mating ability with a *MAT*α *fus5-424* strain MS2326. Five candidate plasmids were identified that suppressed the mating defect. After reisolation of the plasmids in *E. coli*, they were all found to share DNA fragments in common (judged by restriction enzyme analysis). Retransformation of MS2328 confirmed that all of the plasmids complemented the mating defects. The smallest of the plasmids contained a 5-kb insert. Examination of partial DNA sequence from the ends of the insert and consultation of GenBank showed that the complementing DNA contained only one entire open reading frame of 3.6 kb corresponding to *AXL1*.

An *axl1*Δ*::LEU2* allele was generated in an *MAT***a** strain as described above (MS3963). This strain was used to generate a mating diploid by mating to a *mat*Δ *fus5-424* strain (MS2326) containing the *URA3*-marked plasmid, pB1311, that carries the *MAT*α gene. The *MAT*α gene was subsequently segregated away by growing the diploid on 5-FOA media and

the *fus5-424/axl1Δ* mating diploid was tested for complementation of the cell fusion phenotype in regular mating filter assays. For the linkage analysis between *axl1Δ::LEU2* and *fus5-424*, the diploid strain MS3963 × MS2326 was sporulated. After tetrad dissection, plasmid pB1311 containing the *MATα* gene was segregated away by growing the spore colonies on 5-FOA media. This resulted in all four spore colonies being phenotypically **a** mating type. The plate mating phenotype of the spore colonies was then tested by mating to a wild-type *MATα* strain MS23. In all 23 tetrads analyzed, the segregation pattern was 4:0 (mating defective/wild-type), indicating that *FUS5* and *AXL1* are less than 2.1 cM apart.

fus8-1325 was previously thought to be a recessive allele of *FUS5/AXL1* (Kurihara et al., 1994). However, none of the plasmids carrying *FUS5/AXL1* were able to suppress the mating defect of a *fus8-1325* strain (MS2742). Because *fus8-1325* exhibited an **a**-specific mating defect, plasmids pHS1, pSM51, and pSM192 containing *RAM1/DPR1*, *STE14*, and *STE6*, respectively, were introduced into a *fus8-1325* strain MS2742 and tested for the ability to suppress the mating defect. Only transformants containing the *RAM1/DPR1* plasmid mated like a wild-type control when mated to MS16. To test if *fus8-1325* was an allele of *RAM1*, a *matΔ fus8-1325* strain (MS2741) containing the plasmid pB1311 was mated to MS600, which carries a temperature-sensitive allele of *RAM1* (*skd2-1*) covered by a *URA3*-marked plasmid (pHS1) containing *RAM1*. Subsequently, pB1311 and pHS1 plasmids were segregated away by growing the diploid on 5-FOA media. The *fus8-1325/skd2-1* mating diploid was then tested for complementation of the cell fusion phenotype in regular mating filter assays. These mutations failed to complement each other since the *fus8-1325/skd2-1* strain showed a cell fusion defect identical to that of the *fus8-1325*. To perform linkage analysis, the MS2741 × MS600 diploids were sporulated. After tetrad dissection, pB1311 and pHS1 plasmids were lost by growth on 5-FOA media resulting in all four spore colonies being phenotypically **a** mating type. The plate mating phenotype of the spore colonies was then tested by mating to a wild-type *MATα* strain MS23. In nine tetrads analyzed, the segregation pattern was 4:0 (mating defective/wild-type) and 2:2 for the Ts$^-$ phenotype of *skd2-1*, indicating that *FUS8* and *RAM1* are less than 5.5 cM apart.

a-Factor Quantitation by Immunoprecipitation

Metabolic labeling of the cells with ^{35}S-Cys and **a**-factor immunoprecipitation using anti-**a**-factor antiserum were done as described in Sapperstein et al. (1994). The samples were analyzed by SDS-PAGE and quantified by Phosphorimager analysis.

Results

The fus5 and fus8 Mutants Are Defective in Cell Fusion

The *fus5-424*, *fus5-1829*, and *fus8-1325* mutations were identified as causing cell fusion defects in a screen for mating defective mutants (Kurihara et al., 1994). Diploid formation in matings between **a** and α mutant parents was reduced by 30 to 300-fold compared to wild type (Kurihara et al., 1994). In addition, the zygotes produced by these mutants exhibited a pronounced septum between the two joined mutant cells, reminiscent of previously characterized *fus* mutants. Fig. 1 illustrates the morphology of the zygotes that resulted from a *MAT***a** *fus5-424* × *MATα* wild-type mating. *A–I* are DAPI-Nomarski double images of fixed zygotes. Three major classes of zygotes were observed: wild type, partial Fus$^-$, and full Fus$^-$ zygotes. In contrast to wild type (Fig. 1, *A*, *D*, and *G*), full Fus$^-$ zygotes (Fig. 1, *C*, *F*, and *I*) showed a prominent septum across the entire zygote and retained two unfused nuclei. Partial Fus$^-$ zygotes (Fig. 1, *B*, *E*, and *H*) had a partial septum and fused nuclei. Live zygotes (Fig. 1, *J–O*) stained with the membrane-specific styryl dye FM4-64 (Vida and Emr, 1995) indicated the presence of plasma membrane all across the zygote in full Fus$^-$ and plasma membrane remnant in partial Fus$^-$ zygotes.

To analyze further the cell fusion block displayed by the *fus* mutants and investigate which steps in cell fusion were affected (i.e., cell wall remodeling, breakdown, or membrane fusion), electron microscopic analysis of zygotes was performed (Fig. 2). Similar to the classes observed by light microscopy, we identified three major classes of zygotes: wild-type, partial, and full Fus$^-$ zygotes. Complete cell wall removal and successful plasma membrane fusion occurred in the wild-type zygotes (Fig. 2, *A* and *D*). However, both the partial and full Fus$^-$ types clearly showed the presence of residual plasma membrane and cell wall material at the region of cell fusion (Fig. 2, *B*, *E*, *C*, and *F*). The correlation between the electron and light microscopic images suggested that the septa observed by Nomarski were indeed cell wall remnants.

Full Fus$^-$ zygotes retained cell wall material from both mating cells across the width of the zygote (Fig. 2, *C*, *F*, *G*, and *H*). Several observations suggested that these cell pairs constitute zygotes instead of two juxtaposed cells. First, there was an obvious region of close contact between the two mating cells. Second, there was cell wall remodeling at the periphery of the region of cell contact to produce a smooth contour. Third, some thinning of the cell wall at the region of cell contact was often observed, suggesting that some cell wall degradation had occurred. Fourth, we frequently observed the presence of clustered 100-nm vesicles near the zone of cell contact (Fig. 2, *G* and *H*). Clustered 100-nm vesicles are seen at the point of cell fusion in early wild-type zygotes and may be involved in cell fusion (Byers and Goetsch, 1975).

Unlike the full Fus$^-$ zygotes, the partial Fus$^-$ class of zygotes (Fig. 2, *B*, *E*, and *I*) always had a region in which the cell wall between the cells was broken down and the plasma membranes had fused. In these zygotes, cytoplasmic mixing and in some cases nuclear fusion had occurred. In cases where the cell wall had partially broken down, we did not see the presence of juxtaposed unfused plasma membrane. Therefore, we concluded that the cell fusion defect in *fus5* and *fus8* mutants is at the level of cell wall removal rather than at the plasma membrane fusion step.

Several features of the Fus$^-$ zygotes indicate that they are truly aberrant and not simply kinetically delayed wild-type zygotes (Gammie, A.E., V. Brizzio, and M.D. Rose, manuscript in preparation). First, the very large region of apposition between the two mating cells in Fus$^-$ zygotes suggests that they have been in close contact without fusing for an extended period. Second, there are characteristic cell wall remnants in the zone of cell fusion of partial Fus$^-$ zygotes (Fig. 2, *B*, *E*, and *I*). In addition, the partial Fus$^-$ zygotes can produce buds with the remnant cell wall still present. Third, both parents in full Fus$^-$ zygotes are able to reenter the cell cycle and produce haploid buds. None of these features is ever seen in mature zygotes from a wild-type mating. While we can't exclude the possibility that some of the full Fus$^-$ zygotes eventually become partial Fus$^-$ zygotes, it is clear that many full Fus$^-$ zygotes never undergo cell fusion before reentering the cell cycle.

The Cell Fusion Defect in fus5 and fus8 Mutants Is Specific to MATa Cells

The mutants *fus5-424* and *fus8-1325* were isolated in a

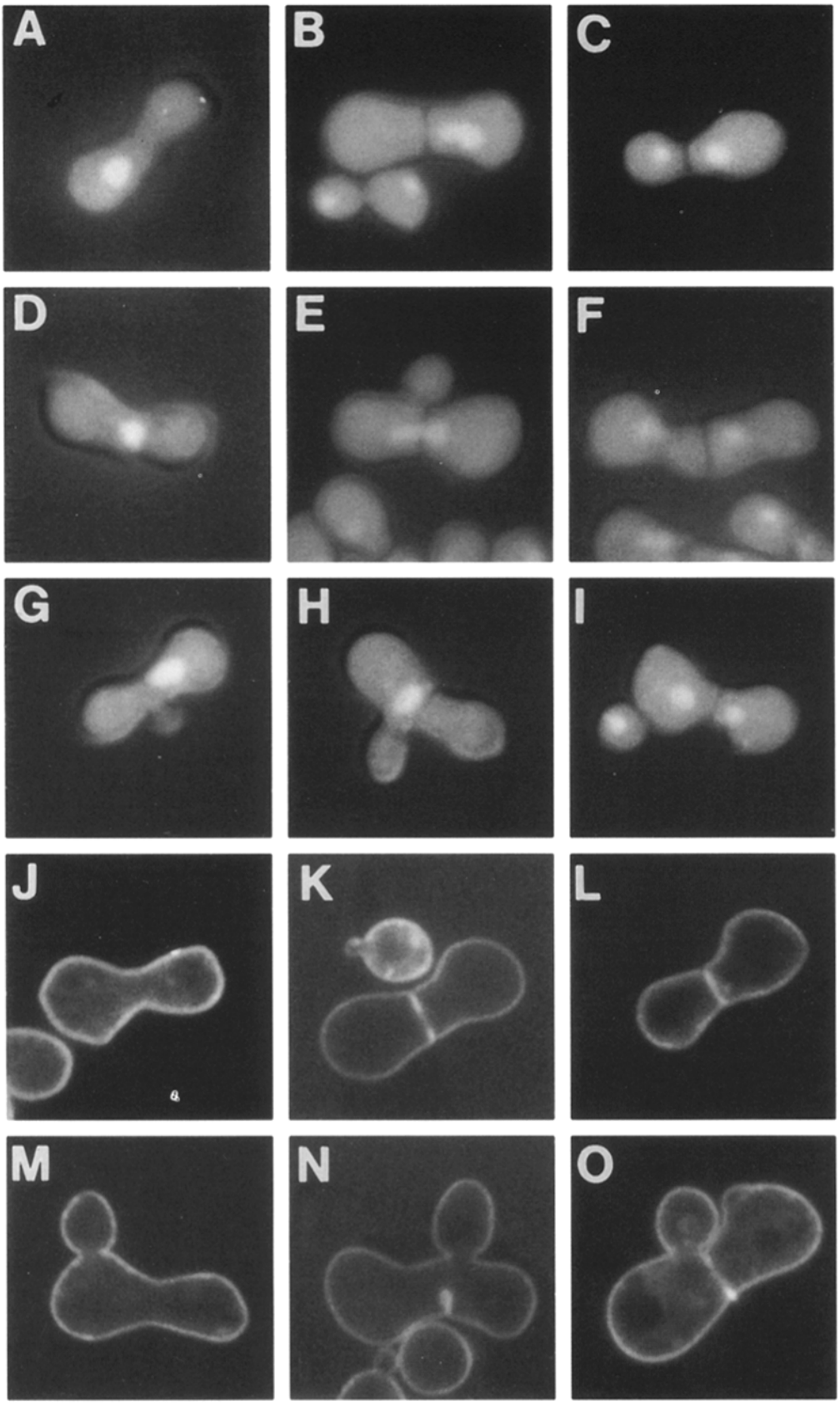

Figure 1. Phenotype of cell fusion defective zygotes. (*A–I*). DAPI-Nomarski double images of fixed zygotes from a filter mating between MY3666 (**a** *fus5-424*) and MS14 (α wild-type). (*J–O*) Live stained zygotes from the same mating using a lipophilic styryl dye FM4-64 (Vida and Emr, 1995). *A*, *D*, *G*, *J*, and *M* are examples of wild-type zygotes. *B*, *E*, *H*, *K*, and *N* are examples of partial Fus$^-$ zygotes. *C*, *F*, *I*, *L*, and *O* are examples of full Fus$^-$ zygotes.

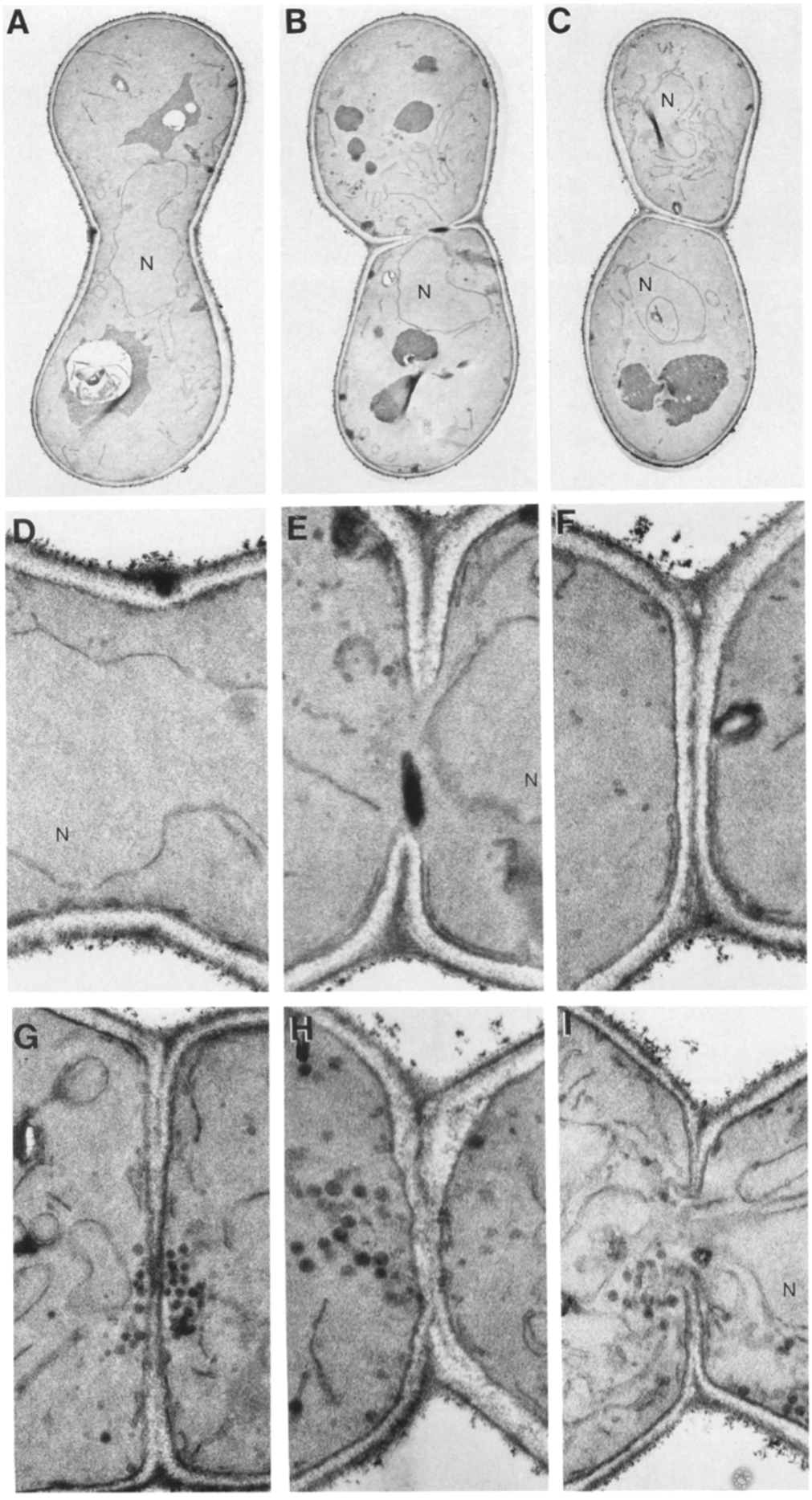

Figure 2. Electron microscopy analysis of cell fusion defective zygotes. (*A–C*) Images of wild-type, partial Fus⁻, and full Fus⁻ zygotes respectively from MS3963 (**a** *fus5*Δ) × MS14 (α wild-type) mating. The relevant cell fusion region from each zygote is enlarged in *D–F*. (*G–I*) Other examples of aberrant zygotes showing incomplete cell wall removal and the presence of 100-nm vesicles (*dark dots*) at the region of cell fusion. Similar results were obtained when zygotes from a mating involving *fus8-1325* × wild-type were analyzed (data not shown). *N*, nucleus.

screen for bilateral mating mutants (Kurihara et al., 1994). However, in that work only the karyogamy mutants were directly tested to show that they were truly bilateral. To determine if the cell fusion mutants were bilateral or showed cell-type specificity, we performed limited plate mating assays in which mutant strains, *MAT***a** or *MAT*α, were mated to lawns of a wild-type strain of the opposite mating type (Fig. 3 *a*). The number of diploid colonies arising from *MAT***a** *fus5-424* and *MAT***a** *fus8-1325* mated to a *MAT*α wild-type strain were reduced compared to the *MAT***a** wild type. However, *MAT*α *fus5-424* and *MAT*α *fus8-1325* mate like the wild-type control when mated to a wild-type **a** strain (Fig. 3 *a*). Microscopic examination of the zygotes that resulted from filter matings was performed (Table II). Large numbers of full Fus⁻ zygotes (47–57%) and partial Fus⁻ zygotes (39–29%) were observed only in matings involving the mutant **a** parent (Table II). Moreover, matings between **a** and α mutant parents (*MAT***a** *fus5-424* × *MAT*α *fus5-424*) did not result in a more severe defect than the defect shown by *MAT***a** *fus5-424* × *MAT*α wild-type strain (Table II). Since the cell fusion defect is displayed only when *fus5-424* or *fus8-*

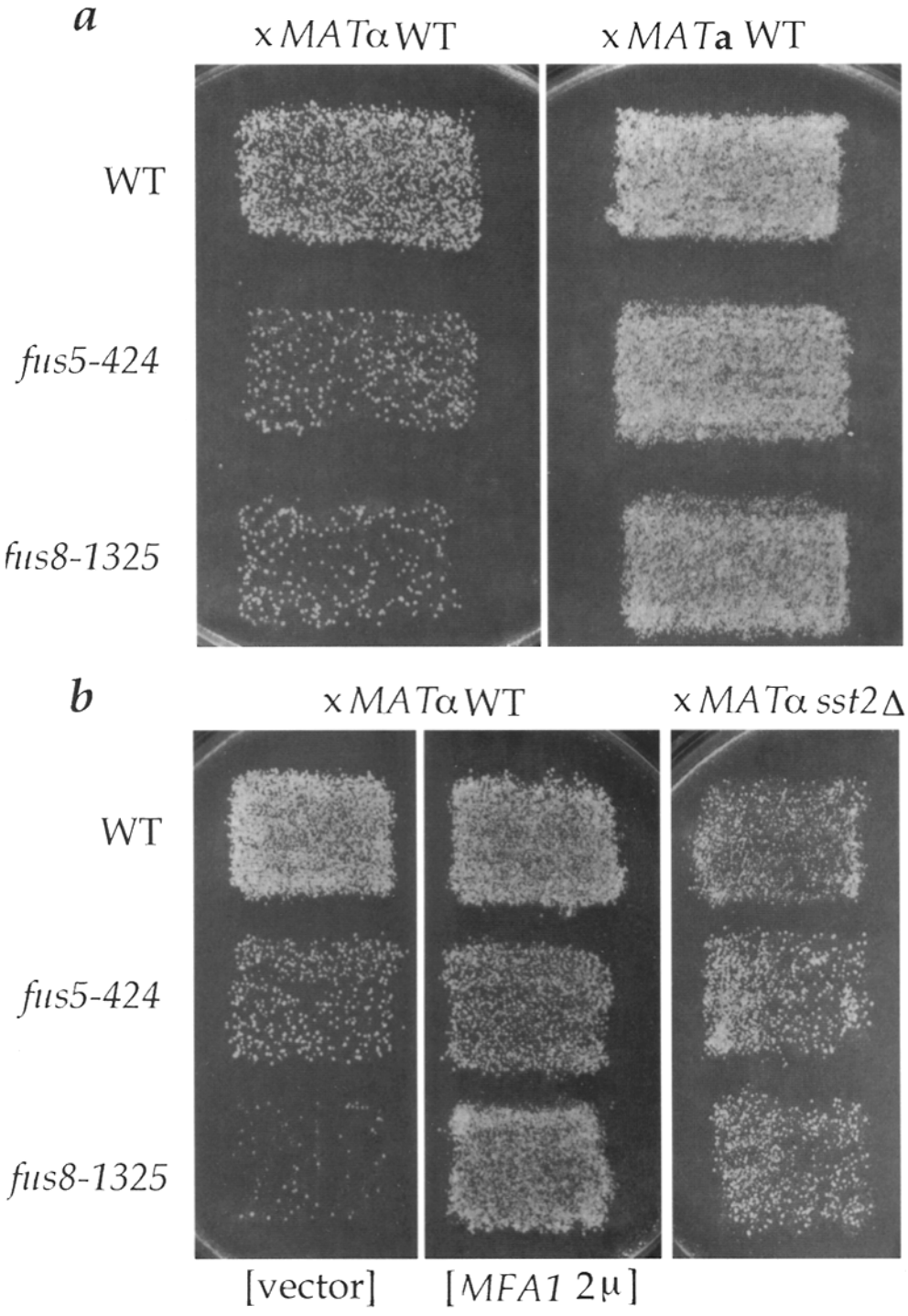

Figure 3. Limited plate mating assays. (*a*) Cell type specificity of the *fus5-424* and *fus8-1325* mating defect. Patches of *mat*Δ strains: wild type (MS2073) (*WT*), *fus5-424* (MS2328), and *fus8-1325* (MS2742) (*left*). Patches of *mat*Δ transformed with *MAT*α plasmid (pB1311): wild type (MS2104) (*WT*), *fus5-424* (MS2326), and *fus8-1325* (MS2741) (*right*) were mated against MS2104 and MS2073 respectively for 3 h at 30°C in limited plate mating assays. (*b*) Suppression by *MFA1* 2μ plasmid and mating to *MAT*α *sst2*Δ. Patches of wild type, *fus5-424*, and *fus8-1325* transformed with the vector, pRS426 (MY4203, MY4205, and MS4279, *left*), or transformed with the *MFA1* 2μ plasmid, pSM219 (MY4204, MY4206, and MS4280, *middle*), were mated against the *MAT*α wild type (MS14). The right panel represents limited plate mating assays between MY4203, MY4205, MS4279, and *MAT*α *sst2*Δ strain (MS4222) isogenic with MS14.

Table II. Cell Type Specificity of the fus5-424 and fus8-1325 Cell Fusion Defect

	Percentage of wild type	Percentage of partial Fus⁻	Percentage of full Fus⁻	Percentage of zygotes formed*
× *MAT*α wild type				
wild type	91	8	1	27
fus5-424	14	39	47	14
fus8-1325	14	29	57	9
× *MAT***a** wild type				
wild type	91	8	1	27
fus5-424	88	9	3	28
fus8-1325	89	7	4	32
× *MAT***a** *fus5-424*				
fus5-424	16	30	54	12

Zygotes from filter matings between MS2104 (wild-type *MAT*α) and the *mat*Δ strains: wild type (MS2073), *fus5-424* (MS2328), *fus8-1325* (MS2742) (*upper*) or MS2073 (wild-type *mat*Δ) and the *mat*Δ transformed with *MAT*α plasmid (pB1311), wild type (MS2104), *fus5-424* (MS2326), and *fus8-1325* (MS2741) (*middle*), were analyzed microscopically. The last row represents the *fus5-424* (MS2326) × *fus5-424* (MS2328) mating. At least 200 zygotes were analyzed in each experiment. The numbers represent the percentages of wild-type, partial Fus⁻ and full Fus⁻ zygotes.
*Number of mating pairs formed/total cells × 100, where the total number of cells was at least 500.

1325 mutations are present in the *MAT***a** parent, we concluded that these mutants were **a** specific. The *fus5* and *fus8* mutants, therefore, differ in two ways from previously characterized mutants: *fus1*, *fus2*, and *fus3* mutants show no cell type specificity and both parents need to be mutant to display a severe Fus⁻ phenotype. In addition to the reduction in wild-type zygotes produced by *fus5-424* and *fus8-1325* mutants, the total number of mating pairs produced was also reduced (Table II). Therefore, the mating defect observed in plate matings of *fus5* and *fus8* is a combination of two factors: inability to efficiently undergo cell fusion and a reduction in the ability to form mating pairs.

We next determined whether the **a**-specific defect was related to pheromone response or production. First, we tested whether *fus5-424* and *fus8-1325* responded to pheromone normally. As judged by growth inhibition by α-factor in disc assays, and by projection formation upon α-factor addition to liquid cultures, the mutants exhibited wild-type sensitivity to α-factor (data not shown). Next, **a**-factor and α-factor production were assessed by growth inhibition of pheromone supersensitive strains (halo assays). Production of α-factor appeared to be normal when tested on a lawn of sensitive *MAT***a** cells (data not shown). However, the halos of growth inhibition produced by the *MAT***a** *fus5-424* and the *MAT***a** *fus8-1325* patches on the *MAT*α lawn were much smaller than the halo produced by the wild-type control (Fig. 4). The differences in halo sizes were not a consequence of reduced growth rates since the mutants did not show a significant growth defect with respect to the wild-type isogenic strain (data not shown). Therefore we concluded that both *fus5-424* and *fus8-1325* affect **a**-factor production.

FUS5 and FUS8 are Allelic to AXL1 and RAM1, Genes Required for a-Factor Biogenesis

FUS5 was cloned by complementation of the *fus5-424* mating defect. Five candidate plasmids were isolated, the smallest of which contained only one entire open reading frame corresponding to *AXL1* (Fig. 5). A *MAT***a** strain carrying a deletion in *AXL1* (see Materials and Methods), exhibited an **a**-specific unilateral cell fusion defect identical to that of *fus5-424* (Table III). Furthermore, *fus5-424* and *axl1*Δ failed to complement for the mating defect. Linkage analysis (see Materials and Methods) confirmed that *FUS5* is allelic to *AXL1*.

Axl1p is a bifunctional protein that is required for the axial budding pattern in haploid cells and for **a**-factor processing (Fujita et al., 1994; Adames et al., 1995). It has homology to several proteases including human and *Drosophila* insulin-degrading enzyme and *E. coli* Ptr (Fujita et al., 1994). Interestingly, the protease activity is needed for NH_2 terminus processing of the **a**-factor precursor but not during bud site selection (Adames et al., 1995). In the absence of Axl1p, some processing still occurs because of the presence of an *AXL1*-related gene, *STE23* (Adames et al., 1995). The double mutant *axl1*Δ *ste23*Δ strain produces no mature **a**-factor (Adames et al., 1995).

The inability to produce wild-type levels of **a**-factor by *fus8-1325* in the halo assay suggested that *fus8-1325* might also be a mutation in one of the genes involved in the biogenesis of **a**-factor. To determine if the *fus8-1325* was a mutation in any of the known genes that are involved in **a**-factor biogenesis, plasmids containing *RAM1/DPR1*, *STE14*, or *STE6* were tested for the ability to suppress the mating defect of *fus8-1325*. A plasmid containing *RAM1/DPR1* was able to suppress the defect, suggesting that *fus8-1325* was an allele of *RAM1/DPR1*. *RAM1/DPR1* encodes the β-subunit of the farnesyl transferase required at the first step in pro-**a**-factor modification (Powers et al., 1986). To demonstrate that *FUS8* was identical to *RAM1*, we tested complementation and linkage between *fus8-1325* and *skd2-1*, an allele of *RAM1* (Scidmore, 1993). Results from these tests confirmed that *FUS8* is allelic to *RAM1* (see Materials and Methods).

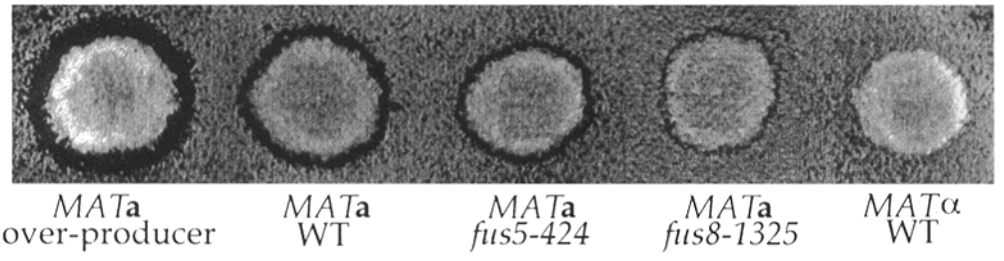

Figure 4. **a**-factor pheromone production. Patches of wild type (MS2073), *fus5-424* (MS2328), *fus8-1325* (MS2742), **a**-factor overproducer (SM1581), and *MAT*α control (MS18) were tested for ability to inhibit the growth of an **a**-factor supersensitive strain (SM1086) in a standard halo assay. *WT*, wild type.

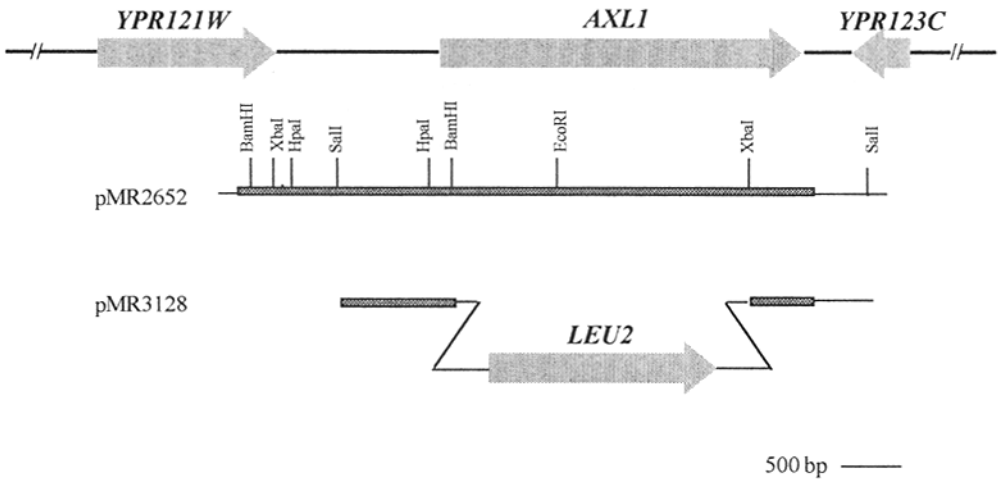

Figure 5. Restriction map of *FUS5/AXL1* and deletion plasmid. Shown in the figure is the insert (*shadowed box*) of the smallest of the positive clones pMR2652 able to complement the *fus5-424* mating defect. Plasmid pMR3128 was used to generate the *fus5/axl1*Δ::*LEU2* allele by one step gene replacement.

Suppression by Overexpression of MFA1 or Mating to a Supersensitive Partner

The identification of mutations in genes affecting the biogenesis of **a**-factor raised two possible models for the defect in cell fusion. The first was that reduced levels of **a**-factor lead to the defect, and the second was that *RAM1* and *AXL1* might have a second function in the cell fusion pathway. To address this issue, we first investigated whether overexpression of *MFA1*, which encodes **a**-factor in *fus5* and *fus8* mutants, would suppress the cell fusion defect. Accordingly, the mutants were transformed with a high copy number plasmid pSM219 (*MFA1* 2 μ) or a control plasmid pRS426. Overexpression of *MFA1* in *MAT***a** *fus5-424* and in *MAT***a** *fus8-1325* strains resulted in a partial rescue of the mating defect observed by plate mating assays (Fig. 3 *b*). Microscopic examination showed that overexpression of *MFA1* also substantially rescued the cell fusion defect of the *fus* mutants (Table III). These results indicated that increasing the amount of **a**-factor made by *fus5* and *fus8* mutants resulted both in an increase in the number of mating pairs and, more importantly, in a substantial suppression of the Fus⁻ phenotype.

Table III. Suppression of the Cell Fusion Defect by MFA1 Overexpression and by Mating to an sstΔ α Partner

	Plasmid	Percentage of wild type	Percentage of partial Fus⁻	Percentage of full Fus⁻	Percentage of zygotes formed*
× *MATα* wild type					
wild type	[vector]	88	12	4	36
fus5-424	[vector]	14	29	57	14
fus8-1325	[vector]	14	36	50	4
fus5Δ	[vector]	15	35	50	9
wild type	[MFA1 2μ]	77	13	10	35
fus5-424	[MFA1 2μ]	41	38	21	15
fus8-1325	[MFA1 2μ]	56	37	7	19
fus5Δ	[MFA1 2μ]	47	39	14	19
× *MATα ss2Δ*					
wild type	[vector]	67	15	18	8
fus5-424	[vector]	64	13	23	9
fus8-1325	[vector]	65	16	19	10
fus5Δ	[vector]	64	23	13	7

Zygotes from filter matings between MS14 (*MATα* wild type) and wild type, *fus5-424*, *fus8-1325*, and *fus5Δ* transfromed with the vector, pRS426 (MY4203, MY4205, MS4279, and MS4281), or transformed with the *MFA1* 2μ plasmid, pSM219 (MY4204, MY4206, MS4280, and MS4282), were analyzed microscopically (*upper* and *middle*). The lower group also shows filter matings between an *MATα sst2Δ* strain (MS4222, isogenic with MS14) and MY4203, MY4205, MS4279, and MS4281. At least 200 fixed zygotes from each of the above matings were analyzed. The numbers represent the percentages of wild-type, partial Fus⁻, and full Fus⁻ zygotes.

*Number of mating pairs formed/total cells × 100, where the total number of cells was at least 500.

Next, we tested whether mating to an α partner that can respond to very low levels of **a**-factor (*MATα* supersensitive strain) can also suppress the Fus⁻ phenotype of the **a**-specific *fus* mutants. Both plate mating assays and microscopic analysis of the zygotes demonstrated that matings between the *MATα sst2Δ* strain and *MAT***a** *fus5* or *MAT***a** *fus8* mutants behaved just like the wild-type control (Fig. 3 *b* and Table III). Therefore, a *MATα sst2Δ* partner strain was able to suppress the Fus⁻ defect of the **a**-specific *fus* mutants.

Taken together, the suppression by increased levels of **a**-factor or by mating to a supersensitive partner demonstrate that **a**-factor production is compromised in the *fus5-424*, *fus5Δ*, and *fus8-1325* mutant strains. These results suggest that reduced levels of **a**-factor may account for the observed Fus⁻ phenotype.

Lowering the Levels of a-Factor Results in the Formation of Fus⁻ Zygotes

The cell fusion defect of mutants defective in the **a**-factor biogenesis pathway suggested an important role for **a**-factor in the cell fusion process, beyond that required for the initial steps in mating pair formation. One possible model is that high levels of **a**-factor are needed to produce a normal cell fusion event. It has been proposed that one way to order events during mating could be through different cellular responses being differentially sensitive to levels of pheromone (Cross, 1988). Indeed, it was shown that low levels of pheromone are sufficient to induce the cell surface agglutinins and cause cell cycle arrest, whereas higher levels are required to produce morphological alterations, e.g., development of the shmoo projection (Moore, 1983). Perhaps an even greater amount of pheromone is necessary to provide an additional signal for cell fusion. In this context, *fus5* and *fus8* can produce enough **a**-factor so that the initial steps in mating occur (cell cycle arrest, shmoo formation, mating pair formation) but cannot reach the

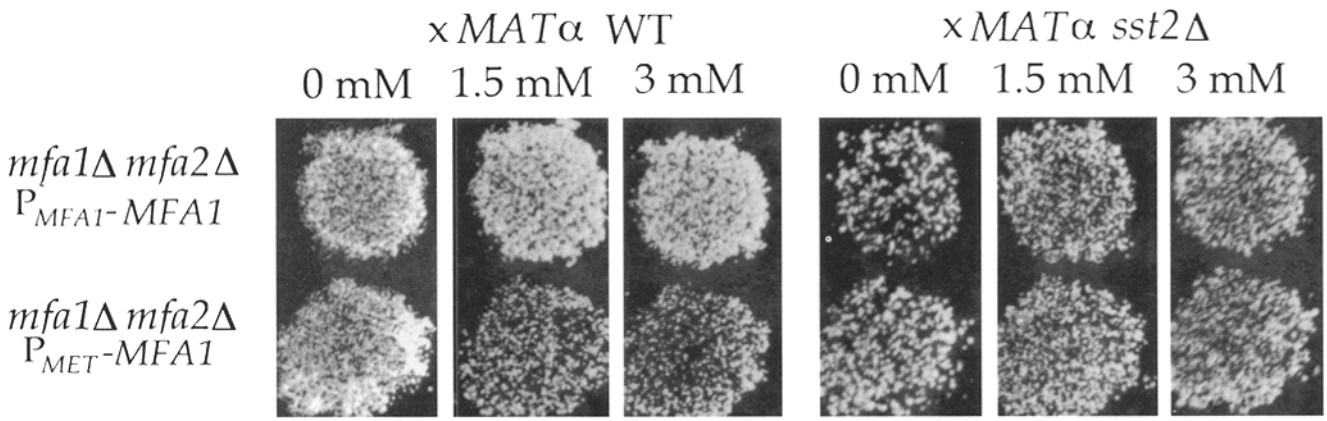

Figure 6. Mating ability and pheromone production of *mfa1Δ mfa2Δ* [P_{MET}-*MFA1*] in the presence of methionine. Patches of an *mfa1Δ mfa2Δ* strain transformed with the P_{MFA1}-*MFA1* plasmid, pSM233 (strain MS4232), or with the P_{MET}-*MFA1* plasmid, pSM1023 (strain MS4231), were mated with the wild-type strain, MS14 (*left*), or an *sst2Δ* strain, MS4222 (*right*), in the presence of 0, 1.5, and 3 mM of methionine.

levels required to elicit the cell fusion response in the partner α cells. Alternatively, a particular form of **a**-factor or an **a**-factor–like pheromone may provide a specific signal for cell fusion. Inability to generate a sufficient level of this putative factor by *fus5* and *fus8* could explain the observed phenotype.

To test if the level of **a**-factor production is key for regulating the cell fusion event, we placed the *MFA1* gene under the control of the methionine promoter (P_{MET}). A plasmid containing the P_{MET}-*MFA1* (pSM1023) was transformed into a strain background in which both genes coding for **a**-factor (*MFA1* and *MFA2*) were deleted. In this system, we can reduce the level of *MFA1* expression by adding increasing amounts of methionine. To quantify the repression of *MFA1* by methionine, **a**-factor immunoprecipitation experiments were performed using anti-**a**-factor antiserum. In the absence of methionine, *mfa1Δ mfa2Δ* [P_{MET}-*MFA1*] produced about 50% of the amount of **a**-factor produced by the *mfa1Δ mfa2Δ* [P_{MFA1}-*MFA1*] control. Addition of 0.5 or 2 mM of methionine resulted in a drop in **a**-factor production to 14.5 and 12.9%, respectively.

Fig. 6 shows limited plate mating assays between *mfa1Δ mfa2Δ* transformed with P_{MET}-*MFA1* or P_{MFA1}-*MFA1* and a wild-type *MATα* strain, performed in the presence of 0, 1.5, or 3 mM of methionine. In the absence of methionine, the mating of *mfa1Δ mfa2Δ* [P_{MET}-*MFA1*] was similar to the control, *mfa1Δ mfa2Δ* [P_{MFA1}-*MFA1*], as well as the wild-type *MFA1 MFA2* strain (Table IV and data not shown). However, increasing the methionine concentration to 1.5 or 3 mM resulted in a reduction in the mating ability of *mfa1Δ mfa2Δ* [P_{MET}-*MFA1*] compared to the wild-type controls. At all methionine concentrations tested, *mfa1Δ mfa2Δ* [P_{MET}-*MFA1*] mated much better than the negative control, *mfa1Δ mfa2Δ* transformed with the vector (Table IV and data not shown), consistent with the observation that some **a**-factor was being produced under repressing conditions.

To look specifically at the cell fusion phenotype, we performed microscopic analysis of zygotes. In the wild-type control matings, regardless of the methionine concentration present in the medium, the zygotes had wild-type morphology (98%) (Table IV). Similarly, 98% of the zygotes in the *mfa1Δ mfa2Δ* [P_{MFA1}-*MFA1*] matings, at all methionine concentrations tested, were wild type. No zygotes were found in matings with the negative control *mfa1Δ mfa2Δ* [vector]. In the absence of methionine, the *mfa1Δ mfa2Δ* [P_{MET}-*MFA1*] strain behaved like the wild-type controls (Table IV). Thus, 50% reduction in pheromone levels was not sufficient to produce a cell fusion defect. In contrast, the addition of 1.5 or 3 mM of methionine to the mating media resulted in a dramatic accumulation of both full Fus^- and partial Fus^- zygotes (Table IV). In addition to a reduction in the proportion of zygotes with a wild-type appearance, the presence of methionine also resulted in a reduction in the amount of mating pairs compared to wild type. Therefore, down regulation of *MFA1* expression in *mfa1Δ mfa2Δ* [P_{MET}-*MFA1*] resulted in a reduction in the mating ability because of an accumulation of Fus^- zygotes and a decreased ability to form mating pairs. Finally, just like *fus5* and *fus8* mutants, matings of *mfa1Δ mfa2Δ* [P_{MET}-*MFA1*] to a *MATα sst2Δ* strain resulted in suppression of the cell fusion defect (Table IV). Thus, by decreasing the **a**-factor levels in an otherwise wild-type background, we have mimicked the **a**-specific defect of the *fus5*/*axl1* and *fus8*/*ram1*.

Strains that Produce Lower Levels of α-Factor also Show a Cell Fusion Defect

To investigate if the dependency of cell fusion is specific to **a**-factor alone, several *MATα* strains that produce lower levels of α-factor were tested for their cell fusion phenotype. Mature α-factor is produced by processing of precursors (encoded by *MFα1* and *MFα2*) as they transit the secretory pathway (Sprague and Thorner, 1992). Kex2p and Ste13p are the KR-endopeptidase and dipeptidyl-aminopeptidase, respectively, required for the processing of α-factor (Julius et al., 1983, 1984). Strains XBH16-15A (*MATα kex2-1*), and A2S3 (*MATα ste13-1*) were previously shown to produce lower levels of α-factor and have a mating defect (Chan et al., 1983). However, the nature of the mating defect was never explored. Microscopic exami-

Table IV. Cell Fusion Phenotype of mfa1Δ mfa2Δ [P_{MET}-MFA1] in the Presence of Methionine

		[MET]	Percentage of wild type	Percentage of partial Fus⁻	Percentage of full Fus⁻	Percentage of zygotes formed*
× *MATα* wild type						
wild type‡		all [MET]	98 ± 1	2 ± 1	0	35 ± 4
mfa1Δ mfa2Δ	[vector]	all [MET]	0	0	0	0
mfa1Δ mfa2Δ	[P_{MFA1}-*MFA1*]	all [MET]§	98 ± 1	2 ± 1	0	26 ± 4
mfa1Δ mfa2Δ	[P_{MFA1}-*MFA1*]	0 mM	98	2	0	17
		1.5 mM	46	15	39	2
		3.0 mM	29	27	44	3
× *MATα sst2Δ*						
wild type‡		all [MET]	78 ± 6	11 ± 3	11 ± 4	8 ± 2
mfa1Δ mfa2Δ	[vector]	all [MET]	0	0	0	0
mfa1Δ mfa2Δ	[P_{MFA1}-*MFA1*]	all [MET]§	79 ± 10	10 ± 7	11 ± 5	9 ± 3
mfa1Δ mfa2Δ	[P_{MET1}-*MFA1*]	0 mM	89	5	6	6
		1.5 mM	91	5	4	4
		3.0 mM	81	9	10	10

Zygotes from filter matings performed in the presence of 0, 1.5, and 3 mM of methionine, between MS14 (*MATα* wild type) and wild type or *mfa1Δ mfa2Δ* strains transformed either with the vector control, pRS416 (MS4236 and MS4233), P_{MFA1}-*MFA1*, pSM233 (MS4235 and MS4232) or P_{MET}-*MFA1*, pSM1023 (MS4234 and MS4231), were analyzed microscopically (*upper*). The same strains under the same conditions were also mated with MS4222 (*sst2Δ*) (*lower*). Between 110 and 157 zygotes were counted in each mating against MS14 and between 54 and 135 zygotes were counted for each mating against MS4222.

*Number of mating pairs formed/total cells × 100, where the total number of cells was at least 500.

‡Average of the following controls: wild type [vector], wild type [P_{MFA1}-*MFA1*], and wild type [P_{MET}-*MFA1*] at all methionine concentrations.

§Average of *mfa1Δ mfa2Δ* [P_{MFA1}-*MFA1*] matings done at all methionine concentrations.

nation of zygotes from a *MATα kex2-1* × *MAT***a** wild-type mating showed a substantial percentage of both partial Fus⁻ (37%) and full Fus⁻ zygotes (43%) (Table V). In addition, a *MATα ste13-1* × *MAT***a** wild-type mating also resulted in a mild cell fusion defect (26% partial Fus⁻ and 7% full Fus⁻), compared to the wild-type control (Table V).

We also tested another strain, MS518 (*MATα skd2-1*) with reduced expression of α-factor (Scidmore, 1993). Unlike *fus8-1325*, the *skd2-1* mutation behaves like a null allele of *RAM1* and causes complete sterility when present in a *MAT***a** strain (Scidmore, 1993). The *MATα skd2-1* strain shows reduced expression of α-factor, which is correlated with a weak mating defect (Scidmore, 1993). A mating between *MATα skd2-1* and *MAT***a** wild-type performed at 30°C showed a large number of partial Fus⁻ (15%) and full Fus⁻ (45%) (Table V). Taken together, these data demonstrated that strains that produce lower levels of α-factor (*kex2-1*, *ste13-1*, and *skd2-1*) have a cell fusion defect. Therefore, we suggest that high concentrations of α-factor may also be needed to trigger the cell fusion response in **a** cells.

Table V. Cell Fusion Defect of kex2-1, ste13-1, and skd2-1 Strains

	Percentage of wild type	Percentage of partial Fus⁻	Percentage of full Fus⁻	Percentage of zygotes formed*
× *MAT***a** wild type				
wild type	95	4	1	51
kex2-1	20	37	43	2
ste13-1	67	26	7	3
wild type	88	9	3	43
skd2-1	40	15	45	12

Zygotes from filter matings between MY3371 (*MAT***a** wild type) and XT1172-S245c (*MATα* wild type) or XBH16-15A (*MATα kex2-1*) or A2S3 (*MATα ste13-1*) were analyzed microscopically. Strain XT1172-S245c is isogenic to A2S3 and closely related to XBH16-15A. The bottom part of the table shows the analysis of MY3371 (*MAT***a** wild type) and MS14 (*MATα* wild type) or MS518(*MATα skd2-1*). Between 94 and 200 zygotes were analyzed in each experiment. The numbers represent the percentages of wild-type, partial Fus⁻, and full Fus⁻ zygotes.

*Number of mating pairs formed/total cells × 100, where the total number of cells was at least 500.

Discussion

Cell fusion in *Saccharomyces cerevisiae* is a relatively unexplored event of the mating pathway. In an effort to understand and learn more about the process of cell fusion, we characterized two mating defective mutants, *fus5* and *fus8*, that show pronounced cell fusion defects only when the mutation was in the *MAT***a** parent. The *fus5* and *fus8* mutant zygotes retain substantial cell wall and plasma membrane in the region of cell fusion. Electron microscopy indicated that the primary defect appeared to be cell wall breakdown rather than plasma membrane fusion.

Five lines of evidence support the conclusion that the cell fusion defect of the **a**-specific *fus* mutants was due to insufficient production of **a**-factor. First, both cell fusion defective mutants *fus5* and *fus8* produced reduced amounts of active **a**-factor compared to wild-type. Second, the *FUS5* and *FUS8* genes are identical to *AXL1* and *RAM1/DPR1*, respectively, two genes known to be involved in the **a**-factor biogenesis pathway. Interestingly, an allele of *STE6*, the **a**-factor transporter (*ste6-cef1*) has been reported to result in a strong **a**-specific unilateral cell fusion defect (Elia and Marsh, 1996). Moreover, several mutant alleles of *STE6* that reduce pheromone export also result in an **a**-specific unilateral cell fusion defect (Nijbroek, G., and S. Michaelis, unpublished observations). Third, the cell fusion defect was partially rescued by introducing a 2μ *MFA1* plasmid into the *fus* mutants. Fourth, a *MATα sst2Δ* strain that is more sensitive to **a**-factor suppressed the Fus⁻ phenotype, in trans. Fifth, reduced levels of wild-type **a**-factor from a repressible promoter produced a cell fusion defect identical to that observed in the *fus5* and *fus8*

mutants. Taking all of these results together, we conclude that high levels of **a**-factor are required for cell fusion.

To determine whether the requirement for high levels of pheromone is specific to **a**-factor, we also investigated the phenotype of *MAT*α strains that produce reduced amounts of α-factor. We showed that strains carrying the *kex2-1*, *ste13-1*, and *skd2-1* mutations, previously known to secrete lower levels of α-factor (Chan et al., 1983; Julius et al., 1983, 1984; Scidmore, 1993), have appreciable cell fusion defects. Interestingly, mutations in *SPT3*, which result in reduced levels of *MFα1*, *MFA1*, and *MFA2* expression, have been reported to cause a defect in mating, perhaps because of a defect in cell fusion (Hirschhorn and Winston, 1988). Therefore, based on these observations, we suggest a more general model for cell fusion in which a high threshold of pheromone (**a**-factor and α-factor) is needed to signal the partner cell to undergo cell fusion after cell contact has been achieved.

Many of the initial steps in mating (including cell cycle arrest, shmoo development, and transcription of mating-specific genes) can be triggered by simple addition of pheromone to a culture of haploid cells. However, even in the presence of high isotropic concentrations of pheromone in the culture media, haploid cells do not undergo the cell wall breakdown characteristic of cell fusion. Inappropriate activation of the pathway would be likely to cause the cells to become inviable in low osmolarity media. Therefore it is reasonable to expect that some additional signal, possibly involving contact with the partner cell, is required to activate the cell fusion machinery. Nevertheless, our results indicate that a high level of pheromone is a necessary part of the signal for triggering cell fusion.

Several aspects of the mating pathway suggest that pheromone signaling in the presence of a mating partner is higher or different from that achieved by the isotropic presence of pheromone in the culture medium (for review see Sprague and Thorner, 1992). First, in response to pheromone, the secretory machinery becomes polarized such that pheromone secretion should be maximal from the shmoo projection (Field and Schekman, 1980; Adams and Pringle, 1984; Hasek et al., 1987; Read et al., 1992). Second, preexisting pheromone receptors are cleared from the cell surface and the new pheromone-induced receptors would become concentrated at the shmoo projection. Accordingly, the shmoo projections can be considered as specialized organelles that would be highly enriched for signaling and signal reception. The close contact between two mating cells should therefore create an environment of very high local concentration of pheromone and pheromone receptors. In contrast, the rest of the cell surface should be impoverished for both receptors and pheromone. In this context, the cell fusion zone in the prezygote should experience the strongest pheromone signal and represents the high end of the gradient of signaling along the length of the cell. Based on these considerations and our data, we propose that the strength of the pheromone signal is one component of the mechanism by which two partner cells communicate to begin cell fusion.

Several other observations suggest that the strength of signaling cannot be sufficient for initiating the cell fusion pathway. Saturation of the pheromone receptors with isotropic ligand does not cause a large number of cells to progress into the cell fusion pathway and lyse (Dorer et al., 1995). Under these conditions, the cells can still mate, albeit at greatly reduced efficiency (Dorer et al., 1995). One explanation is that in such cells there is an inefficient secondary pathway that allows for initiation of cell fusion independent of the pheromone signaling pathway. An alternative explanation is that a small fraction of cells initiate cell fusion without a partner and lyse. However, such cells could be rescued in the assay conditions of Dorer et al. (1995), where they are surrounded by cells of the opposite mating type. Finally, it is possible that the very steep gradient of signaling required for efficient cell fusion is only achievable in the context of a prezygote.

It remains to be determined how the pheromone signal may act as part of a specific cell fusion signal. In one model, the intracellular response pathway may have several built-in thresholds for differential responses to different levels of signal. Consistent with this idea, G1-arrest requires a lower level of pheromone than shmoo formation (Moore, 1983). Such differential responses could arise from signals branching off the pheromone response pathway at different levels of the signaling cascade (e.g., cell cycle arrest may require only activation of the Fus3p kinase, whereas shmoo formation would require Ste12p to activate the synthesis of new components). Alternatively, different targets for Ste12p transcriptional activation may require different levels of activation. Finally, it is not yet known whether the signal for cell fusion (e.g., high levels of pheromone) is received and transduced using the regular MAP kinase pheromone pathway. It remains possible that the signal diverges at the level of the receptors or the trimeric G-proteins.

Some clues about the cell fusion mechanism come from the analysis of wild-type Fus$^-$ zygotes by electron microscopy and the characterization of cell fusion defective mutants (this study, our unpublished observations, and Byers and Goetsch, 1975). Close examination of the region of cell fusion by electron microscopy showed the presence of numerous 100-nm vesicles. These vesicles were clustered on either side of the intervening cell wall. It is tempting to speculate that these vesicles are crucial in the cell fusion process. On the one hand, they might have a secretory origin and could carry hydrolytic enzymes and other products to degrade the cell wall and promote the fusion of the two plasma membranes. Alternatively, they could have an endocytic origin and be responsible for taking up degraded cell wall material. However, it is interesting that mutants defective in tropomyosin accumulate similar vesicles and also show a defect in cell fusion (Liu and Bretscher, 1992). Two proteins known to be required for cell fusion are Fus1p and Fus2p. Fus1p is an integral membrane protein that localizes to the tip of the shmoo projection (Trueheart et al., 1987; Trueheart and Fink, 1989). Fus2p localizes to punctate structures (possibly specialized vesicles) that accumulate progressively within the neck of the shmoo and near the plasma membrane of the projection tip (Elion et al., 1995). In zygotes, Fus2p localizes to the interface between partner cells that have undergone cell fusion but not nuclear fusion, and Fus2p is no longer detected in zygotes that have completed nuclear fusion (Elion et al., 1995). One possibility is that reception of the signal results in the regulated and localized release of the 100-nm vesicles at

the point of cell fusion. Release of vesicles would then deliver proteins required for cell wall hydrolysis and membrane fusion. It is tempting to assign to Fus1p a docking function and Fus2p a role as part of the cargo of the vesicles. In addition to these mating-specific components, proteins constitutively required for cell polarization should be part of the mechanism for cell fusion.

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