Abstract. Though early stages of yeast conjugation are well-mimicked by treatment with pheromones, the final degradation of the cell wall and membrane fusion of mating that leads to cytoplasmic mixing may require separate signals. Mutations that blocked cell fusion during mating in Saccharomyces cerevisiae were identified in a multipartite screen. The three tightest mutations proved to be partial-function alleles of the ABC-transporter gene STE6 required for transport of α-factor. The ste6(cef1-1) allele was recovered and sequenced. The ste6(cef1-1) allele contained a stop codon predicted to truncate Ste6 at amino acid residue 862 (of 1290). The ste6(cef) mutations reduced, but did not eliminate, expression of α-factor. Light and electron microscopy revealed that unlike ste6 null mutations which block mating before the formation of mating pairs, the ste6(cef) (cell fusion) alleles permitted early steps in mating to proceed normally but blocked at a late stage in conjugation where mating partners were encased by a single cell wall and separated by only a thin layer of cell wall material we term the fusion wall. Morphologically the prezygotes appeared symmetrical with successful cell wall fusion at the periphery of the region of cell contact. Responses to α-factor were efficiently induced in partner cells under mating conditions as expected given the symmetric appearance of the prezygotes. A strain expressing a ste6(K1093A) mutation that conferred export of a twofold to fourfold higher level of α-factor than ste6(cef) did not accumulate prezygotes during mating which could indicate a tight threshold of α-factor signaling required for mating. However, mating to an sst2 partner which has a greatly increased sensitivity to α-factor did not suppress the fusion defect of a ste6(cef) strain. Overexpression of the structural gene for α-factor also did not suppress the fusion defect. It is possible that α-factor or STE6 play more complex roles in cell fusion.

Yeast cells of α and α mating type produce and respond to mating pheromones during the initiation of mating processes that lead to cell-to-cell fusion (reviewed in references 25, 28, 40). Pheromone treatment of non-mating cells leads to cell cycle arrest but not to the cell lysis that might be expected with loss of cell wall or membrane integrity (20) suggesting that the end stages of cell fusion may require special signals. Though the process of pheromone response in yeast leading to cell cycle arrest and gene expression has been well-characterized, the details of the cell fusion event remain obscure. The FUS1 and FUS2 genes play distinct but as yet undefined roles in this process (29, 43). The FUS3 gene encodes a MAP kinase that plays a central role in control of transcriptional and cell cycle aspects of pheromone response. The specific role of FUS3 in cell fusion is unclear (9). Yeast sterols also play a role in cell fusion. Ergosterol is specifically required for high-efficiency fusion during mating (42). Additional fus genes have been described but not characterized (19).

The Ste6 protein is a member of the ABC-cassette transporter family related to the mammalian multidrug resistance (mdr) protein and the cystic fibrosis CFTR protein (16, 23, 31). The farnesylated and methylated peptide pheromone α-factor is exported directly from the cell by the Ste6 transporter and is required for mating (18, 30, 32). The modified α-factor peptide activates the G protein-coupled α-factor receptor to activate responses required for mating. Deletion of the STE6 gene blocks mating before committed cell–cell interaction. Though members of the ABC transporter family were initially thought to be involved solely in transport, it is now known that they can play additional roles as receptors, mediators of cell adhesion or apoptotic cell engulfment, regulators of exocytosis and as receptors, or ion channels (8, 11, 13, 22, additional roles reviewed in reference 14).

We report here the isolation of mutant yeast strains that carry alleles of STE6 that permit initiation of mating but that lead to an accumulation of conjugating cell pairs with an intact fusion wall preventing cytoplasmic mixing. We provide evidence that STE6 or α-factor might play a special role in efficient completion of a late stage of cell fusion distinct from roles in activating pheromone responses.
**Materials and Methods**

**Strains and Media**

Strains and plasmids are listed in Table I. Standard YPD rich medium and SD selective media have been described. Synthetic α-factor peptide was purchased from Sigma Chemical Co. (St. Louis, MO).

**Mutant Screening Procedure**

Strain LM23-3az was mutagenized with EMS (34, 38) with a survival rate of 32% and accumulation of ~0.1% red Ade colonies. Five independent mutagenesis reactions were conducted, and ~80,700 colonies were screened (outlined in Fig. 1). Both sterile and partially sterile mutants were identified by replicating mutagenized colonies from YPD master plates onto lawns of IH1701 mating tester spread on SD minimal medium supplemented with 0.6% YPD and incubated at 30°C. Under these conditions wild-type colonies produce abundant prototrophic diploid papillae whereas sterile mutants produce a reduced number (27). Only the mutants with a fairly strong mating defect have been characterized. Mating-defective mutant strains were tested for their ability to respond to synthetic α-factor using a quantitative growth inhibition assay and a filter assay for induction of the pheromone inducible reporter FUS1-lacZ (26). Strains with obviously reduced sensitivity to α-factor were eliminated. Strains with mutations in the HIS2 or ADE5 loci, which would appear sterile in our screen, were eliminated by auxotrophy tests and by testing mating to another tester strain with different auxotrophies. The ability to export α-factor was assayed by patching mutant strains onto a lawn of strain RC884, a er2, which is sensitive to α-factor. Strain producing obviously reduced zones of growth arrest were eliminated. Mutant and control strains were tested for agglutination activity by incubation with a tester strain in 13 mm tubes followed by visual inspection of pellets (21). None of our mutant strains were defective in agglutination. Mutant strains were incubated with or without synthetic α-factor (0.1 μg/ml) in liquid medium for 3 h and observed microscopically. Strains with abnormal morphologies without α-factor exposure, or that did not undergo a normal-appearing morphological transition (shmooing) after exposure to α-factor were eliminated. The remaining mutant strains were tested for their ability to complete the mating process. Strains were mixed in 10-fold excess with a wild-type MAT α mating partner, FC139, and filtered onto a 0.45-μm pore filter. The filter was incubated on YPD medium for 4 h, then the mating mixture was observed microscopically. Strains which accumulated prezygote forms when mated to a wild-type partner were retained. Strains in which normal-appearing zygote structures predominated were eliminated.

**Mating Assays**

Matings for determination of mating frequency were performed by filtering 10^6 MAT α partner cells with 10^7 MAT α partner cells and incubating on YPD medium for 4 h at 30°C (39). Cells were washed from filters and dilutions were plated on selective medium to determine diploid formation. Control filters with only the MAT α partner were used to normalize mating frequencies.

**Electron Microscopy**

Wild-type (parent) or Cef strains were mated on nitrocellulose filters to a wild-type partner (FC139) as described above for 3.5 to 4 h. Cells were then fixed with 2.5% gluteraldehyde, 0.1 M cacodylate and embedded in Spurr resin. Slices (~70-nm thickness) were stained with uranyl acetate and lead citrate and viewed with a JEOL 1200EX transmission electron microscope (3, 43).

**Cloning CEF1**

We first demonstrated that the cef1-1 mutation was recessive. We mated the cef1 strain LE4-89 to strain FC139 to yield a MAT α/MAT α CEF1/cef1 strain. This was converted to a MAT α/MAT α strain by introducing a

---

**Table I. Yeast Strains and Plasmids Used in This Study**

| Strain          | Genotype                              | Source    |
|-----------------|---------------------------------------|-----------|
| LM23-3az (parent strain) | MATα STE6 (CEF1+) bar1 [FUS1-lacZ::URA3] leu2 ura3 his4 lys5 met1 | This work |
| LE4-89          | MATα ste6 (cefl)-1 bar1 [FUS1-lacZ::URA3] leu2 ura3 his4 lys5 met1 | This work |
| LE4-75          | MATα ste6 (cefl)-2 bar1 [FUS1-lacZ::URA3] leu2 ura3 his4 lys5 met1 | This work |
| LE4-65          | MATα ste6 (cefl)-3 bar1 [FUS1-lacZ::URA3] leu2 ura3 his4 lys5 met1 | This work |
| LE4-89a         | Isogenic to LE4-89 but cured of [FUS1-lacZ::URA3] | This work |
| LE4-75a         | Isogenic to LE4-75 but cured of [FUS1-lacZ::URA3] | This work |
| LE4-65a         | Isogenic to LE4-65 but cured of [FUS1-lacZ::URA3] | This work |
| LE1-5           | MATα fus3 bar1 [FUS1-lacZ::URA3] leu2 ura3 his4 lys5 met1 | This work |
| IH1701          | MATα his2 ade6 | I. Herskowitz |
| FC139           | MATα HMR α HML α ura3-52 met1 lys5 | 26        |
| RC884           | MATα sst2-3 his6 leu1 met1 trp5 ura3 can1 cyh2 rme | 5         |
| LM104           | Isogenic to LM23-3az but cured of [FUS1-lacZ::URA3] | This work |
| LM104           | Isogenic to LM104 but ade6::URA3 | This work |
| LM112           | Isogenic to LM104 but ade6 | This work |
| LM23-11aza      | MATα bar1 STE2::mtrn1 TRP1-1.4 leu2 ura3-52 lys5 met1 FUS1-lacZ | 26        |
| SM1229          | MATα mfu1-D1::LEU2 mfu2-D1::URA3 trp1 leu2 ura3 his4 can1 | 24        |

**Plasmids**

- pC2-1: YCP50 genomic library clone containing STE6
- pLE89G1: ste6 (cefl1) allele on a CEN-based plasmid
- pLE131: ste6 (cefl1) in YEp13
- pSM192: STE6 in pRS316
- pSM322: STE6 in pRS315
- pSM401: ste6 (K1093A) in pRS315
- pSL324: FUS1 in YEp13
- pSB257: FUS2 in YEp24
- pLE426: MFA2 in pRS426
pGAL-HO plasmid and growing the strain briefly on galactose medium. The pGAL-HO plasmid (URA3) was then cured by growth on 5-fluoroorotic acid medium (34). The resulting MATa/MATa CFE1/cefl strain mated efficiently as a MATa strain indicating that cefl was recessive. The cefl gene was cloned by complementation of the mating defect of the cefl strain, LE4-89, using a CEN-based genomic library (33). A plasmid, pC2-1 containing the STE6 gene, was recovered when retransformed into the original LE4-89 strain was able to restore mating.

**STE6 Disruption and Tagging.** STE6 disruption construct was prepared by PCR. 3.6 kb of STE6 coding region was replaced with the URA3 gene leaving ~0.6 to 0.7 kb of flanking STE6 region DNA. Transformation of the construct into the STE6+/ura3 yeast strain LM104 led to a defect in α-factor export. Presence of the deletion/replacement in LM10 was confirmed by Southern blot analysis (34, 36). To tag the STE6 gene for genetic analysis an integrating plasmid with STE6 region DNA was constructed. A 3.6-kb endonuclease BamHI fragment of wild-type STE6 region DNA was cloned into the integrating vector YIp5 yielding plasmid pBY4.0. Integration was targeted to the STE6 gene of FC139 by digesting the plasmid DNA with endonuclease Spel and selecting for Ura+ transformants (34). Alternatively a two-step “pop-in, pop-out” method was used to create an unmarked deletion strain, LM112 (1). Deletions were confirmed using Southern blot analysis (34, 36).

**Recovery and Mapping of the ste6(cef-1) Allele of LE4-89.** An in vivo recombination-based recovery approach was used to recover a ste6(cef) allele (35). The STE6 CEN-based plasmid pSM322 (1) (provided by S. Michaelis) was linearized by digestion with restriction endonucleases Stul and NcoI which cut this plasmid only at sites 111 and 3129 within the STE6 coding region (numbering begins at the start codon of STE6). A 9-kb segment of DNA containing the gapped plasmid was gel-purified and transformed into ste6(cef) mutant yeast strains using a standard lithium acetate protocol (34). Plasmids were recovered from Leu+ transformants (13) into Escherichia coli and analyzed to ensure that they contained DNA corresponding to a complete STE6 gene. Plasmids were transformed into a wildtype URA3 strain, LM110, to determine whether they contained mutant or wild-type alleles of STE6. Restriction fragment exchange was performed on residues 1000-3129 of the STE6/ste6(cef-1) coding region (contained in a BamHI-NcoI restriction fragment).

**Construction of a Plasmid for Overexpression of α-factor**

A 1.75-kb HindIII fragment of DNA from pSM23 containing the MFA2 gene was subcloned into the 2μ-based plasmid pRS426 containing the selectable URA3 gene.

**Quantitation of Activity of Partially Purified α-factor Preparations**

Partially purified α-factor was prepared from strains by a modification of the method of Strazdis and MacKay (41). Amberlite XAD-2 resin beads (10 ml; Sigma) were added to 100 ml of log-phase culture in SD-based medium and incubated 24 h at 30°C with vigorous shaking. After incubation, the resin beads were washed repeatedly with dH2O until washes were clear, followed by a wash with 30 ml 40% methanol. The α-factor activity was eluted with 30 ml of isopropanol. Isopropanol was removed by rotary evaporation and the crude pheromone dissolved in 200 μl of DMSO for an effective 500× concentration from starting culture volume. Aliquots of independent preparations were subjected to serial 1:4 dilutions with water containing 1 mg/ml bovine serum albumin as a carrier. Diluted samples (10 μl) were spotted on lawns of RC884 (MAT α stu1) on YPD medium containing 0.05% Triton X-100 (see results section). Plates were photographed after an incubation of ~48 h.

**Results**

**Mutants Defective in Cell Fusion**

Many mutants of S. cerevisiae defective in mating are known. Most of these are defective in the pheromone response pathway that leads to cell cycle arrest and specific gene transcription or are unable to make or secrete mating pheromones (25, 28, 40). To identify mutants specifically defective in the cell fusion step of conjugation, but functional for other steps we used a multi-tiered screen summarized in Fig. 1 (see Materials and Methods for details). In brief, 68,700 mutagenized colonies were first screened to identify 189 strains with greatly reduced mating. Additional mutants exhibiting more modest mating defects were also observed. Strains unable to make α-factor or to respond to α-factor were eliminated. Strains were assayed for the ability to undergo characteristic changes in cell morphology (shmooing) in response to synthetic α-factor. Strains with abnormal morphology in the absence of α-factor or that failed to undergo the usual morphological changes in response to α-factor or that failed to agglutinate were eliminated. Finally, mating mixtures of mutant strains mixed with a wild-type MATα strain (FC139) were observed microscopically to identify strains that initiated but failed to complete cell fusion. Most sterile strains do not interact at all under these conditions. Wild-type strains form zygotes in which cells have fused, but intermediates in the mating process must be very transient since they are only rarely observed. We looked for strains accumulating prezygote forms in which the two mating cells were partly fused but retained a partition between the partners. Strains that appeared to undergo normal cell fusion were

![Figure 1. Schematic diagram of the multi-tiered screen for mutants blocked in the cell fusion step of mating.](image-url)
discarded. From this screen we identified three mutants with a significant mating defect that were blocked at the cell fusion stage of mating. We term the phenotype of our mutants Cef- (cell fusion) to distinguish them from the previously identified Fus- mutants that only produce a substantial mating defect when both partners carry a mutation.

**MAT a-Specificity of the cef1 Defect**

Mutant strain LE4-89 (cef1-1) was crossed to a wild-type MAT α strain, FC139 (mating frequency of the LE4-89 strain was low but detectable, permitting this cross). Tetrad analysis indicated that the mating defect was caused by a single mutation because ~50% of the MAT α progeny were sterile. However, none of the MAT α progeny were sterile, suggesting that cef1 was MAT a-specific. The cef1 MAT a progeny also had a partial defect in α-factor production whereas the MAT α progeny had no defect in α-factor production. We outcrossed a MAT α strain from a tetrad in which none of the progeny exhibited a mating defect and recovered sterile MAT a progeny. Thus cef1 exhibited a MAT a-specific mating defect such as that observed with mutants defective in STE6, STE3, STE14, etc.

The **cef Mutations in LE4-89, LE4-75, and LE4-65 Mutants Are Allelic with STE6**

The mutation in LE4-89 (cef1-1) was recessive since a MAT a/MAT a + /cef1 diploid did not exhibit a mating defect (see Materials and Methods). The CEF1 gene was cloned by complementation of the cef1 mating defect using a low-copy CEN-based genomic clone, pC2-1 mated at nearly the frequency in quantitative assays to less than 0.02% of the parental frequency (Table II, lines 1 and 2) and dramatically increased the proportion of prezygotes observed in mating mixtures from <1% of mating pairs to 71% (Table III, lines 1 and 2). The cef1-1 strain, LE4-89, with the CEN-based genomic clone, pC2-1 mated at nearly the frequency of the parent strain (Table II, line 2). The clone pC2-1 complemented not only the cef1 defect but also the independent mutations in the mutants LE4-75, and LE4-65 (Table II, lines 3 and 4). The active region of the complementing clone was mapped using deletion and subcloning analysis. Sequence analysis of the functional portion of the CEF1 gene indicated identity with the STE6 gene (18, 30). A CEN-based STE6 plasmid, pSM192, complemented the mating defects of the LE4-89, LE4-75, and LE4-65 Cef- mutants (Table II, lines 1–4). Also, there was no observable accumulation of prezygotes when these transformants were mated to wild-type indicating that the cell fusion defect was also complemented by the STE6 plasmid (data not shown). The three cef1 strains do not contain null alleles of STE6 since they mated at a frequency almost 1,000-fold higher than a ste6 null strain derived from our parent strain by gene disruption (Table II, line 5).

The cef1-1 mutation was mapped relative to STE6. A STE6::URA3 construct was integrated into a MAT α strain and crossed to ura3 derivatives of our Cef- mutants. Tetrad analysis indicated tight linkage between STE6::URA3 and the cef mutations in LE4-89, LE4-75, and LE4-65 (not shown). Thus cef1-1 is both allelic to STE6 and complemented by STE6. We refer to the cef alleles of STE6 as ste6(cef). The most carefully characterized allele is that of LE4-89 which we refer to as ste6(cef1-1).

**Recovery and Mapping of the ste6(cef1-1) Mutation**

To isolate a cef mutant allele of STE6 we used a gapped gene recovery scheme. The ste6(cef) strain LE4-89 was transformed with the STE6 plasmid pSM322 digested with the restriction endonucleases StuI and NcoI which cut only within the coding region of the STE6 gene, generating a 3-kb gap. Recovered plasmids were tested for their ability to confer a Cef- phenotype on a ste6 null strain. The ste6(cef1-1) allele was recovered frequently, suggesting that it might lie within the gapped region of the plasmid. The other ste6(cef) mutations were not efficiently recovered, suggesting that they might lie outside of this region. To further map the ste6(cef1-1) mutation, a 2.1-kb BamHI–NcoI restriction fragment of DNA from the plasmid pLE89-G1 carrying ste6(cef1-1) was used to replace the equivalent segment of wild-type STE6 in the plasmid pSM322. The resulting plasmid, pLE131, conferred the ste6(cef1-1) fusion defect when transformed into the ste6 null strain, LM110 (Table III, line 2). Thus the ste6(cef1-1) mutation lies within codons 333–1043 of the 1290 codon STE6 gene. The region of the STE6 gene containing the ste6(cef1-1) mutation encodes hydroporphic membrane-spanning regions as well as an ABC-cassette domain.

**Sequence Analysis of ste6(cef1-1)**

The region of STE6 containing the ste6(cef) mutation was sequenced using spaced primers and automated DNA sequencing. A silent polymorphism was detected at base 1851 of the coding sequence where both the mutant STE6 allele and wild-type STE6 recovered from our parent strain contained C rather than A that has been reported (30). A substitution mutation (G2585A) leading to creation of an amber stop codon was found in codon 862 which normally encodes Trp. This stop codon is predicted to lead to a truncated product lacking several transmembrane domains and one of the nucleotide binding domains.
### Table III. Prezygote Accumulation in Mating Cell Pairs

| MAT a Strain | MAT a partner | Prezygotes | Zygotes | Prezygotes | Pairs/10^6 CFU |
|--------------|---------------|------------|---------|------------|----------------|
| 1. LM23-3az  | STE6          | no         | 1       | 334        | <1             | 120            |
| 2. LE4-89    | STE6          | 1          | 32      | 71         | 75             |
| 3. LM110     | STE6          | 107        | 99      | 52         | 412            |
| 4. LM112     | STE6          | 1          | 330     | <1         | 166            |
| 5. LM112     | STE6          | 25         | 16      | 61         | n.d.           |
| 6. LM110     | STE6          | 0          | 0       | –          | <1             |
| 7. LM23-3az  | SST2          | 1          | 645     | <1         | 231            |
| 8. LE4-89    | STE6          | 251        | 158     | 61         | 108            |
| 9. LM110     | STE6          | 346        | 266     | 57         | 204            |
| 10. LM110    | STE6          | 0          | 0       | –          | <1             |

* See Materials and Methods for mating conditions.

1. MAT a partner and relevant genotype. All are isogenic with LM23-3az.
2. MAT a partner: SST2*, FC139, ss2*, RC084.
3. Dilutions of mating preparations used for microscopic observation were plated on YPD medium to determine CFU. Because mating induces cell clumping these numbers should by interpreted only as a guide of ability of cells to form pairs.

of Ste6. A silent second mutation was found in codon 615 (A1843T). The presence of a stop codon potentially truncating a large portion of the transporter was unexpected in ste6(cef) since it did not act like a null mutation. Based on previous studies of STE6 it seemed unlikely that the transporter could function without all of the membrane-spanning domains intact (1). Our strain contained an amber trp1 mutation and was Trp" making the possibility that it contained an amber suppressor unlikely though not impossible. The finding that translation proceeds efficiently through other STE6 nonsense mutations provides a possible mechanism for synthesis of full-length Ste6 (12). It is unclear if the phenotype of ste6(cef) derives solely from underexpression, or from expression of a mixture of truncated and functional forms of the transporter.

**Expression of ste6(cef-1) in a Δste6 Strain**

Though the ste6(cef-1) mutation segregated in crosses as a single, MAT a-specific mutation, we further examined the phenotype conferred by a ste6(cef-1) plasmid on a Δste6::URA3 strain to rule out a role for secondary mutations in the ste6(cef) phenotype. A Δste6::URA3 strain, LM110, carrying the 2μ-based ste6(cef-1) plasmid, pLE131, exhibited a cell fusion defect (Table III, line 3). Thus the ste6(cef-1) mutation is both necessary and sufficient to produce the cell fusion defect. The ste6(cef-1) plasmid conferred an ~6-fold increase in mating relative to the parent ste6(cef1-1) strain, LE4-89, but still accumulated more than 50% prezygotes in mating mixtures (Table III).

**Light Microscopy of Prezygotes**

To characterize the prezygotes that accumulated in mating mixtures of [ste6(cef)] × [wild type] we examined them by Nomarski optics. In parental strain crosses prezygotes were too rare to characterize. Zygotes exhibited a typical morphology in which cells appeared to have formed a seamless zygote wall surrounding the cytoplasm of the two fused cells (Fig. 2 A). In contrast, in ste6(cef) crosses the zygote wall often appeared normal but a septum we term the fusion wall remained, separating the cells and preventing cytoplasmic mixing (plasmogamy). The original ste6(cef1-1) strain and a strain with ste6(cef1-1) expressed from a plasmid produced prezygotes of similar appearance (Fig. 2, B and C). In some cases one cell appeared to be pushing into the other as has been reported as a normal mating intermediate for Hansenula wingei (6). The ste6(cef) prezygotes appeared somewhat more uniformly arrested at a later stage of fusion than prezygotes of previously published fus strains. As a comparison we examined a mutant (LE1-5) that arose in our screen that was complemented by a CEN-based plasmid bearing FUS3. FUS3 was originally identified for its role in cell fusion (9). The prezygotes of the presumptive fus3 mutant in our strain background closely resembled published photomicrographs of prezygotes of fus1, fus2, and fus3 strains. Unlike ste6(cef) prezygotes the cells of the mating pairs of the presumptive fus3 strain often were deformed and met at odd angles (Fig. 2 D). However, ~10% of fus prezygotes are arrested with a morphology similar to that of the ste6(cef) mutants (data not shown). The other cef mutants we have examined thus far in our collection, as well as fus2 null mutations introduced into our background, produce the broader mixture of prezygote forms characteristic of fus1, fus2, and fus3 (9, 10, 29, 42, 43, data not shown). Only ste6(cef) mutants produce uniform prezygotes of the ste6(cef) type that have...
progressed to a late stage of fusion. The significance of this difference is unclear but might indicate that the fis mutants act in a process required throughout fusion, whereas STE6 acts in a process required slightly later.

**Electron Microscopy Analysis of the Cell Fusion Defect Induced by ste6(cef)**

The cell fusion defect of the ste6(cef1-1) mutant LE4-89 was further characterized using electron microscopy. Wild-type zygotes exhibited a seamlessly fused cell wall surrounding the new diploid and an absence of a fusion wall separating the original haploid partners (Fig. 3). The [ste6(cef1-1) × wild-type] prezygotes accumulated in a late stage in the zygote formation process (Fig. 3). The ste6(cef1-1) prezygotes had a morphological form appropriate for a diploid zygote except for a thin septum separating the conjugating pair. The outer layer of the cell wall of the two cells was contiguous in most ste6(cef1-1) prezygotes suggesting that early stages of the cell wall fusion program were normal and that the prezygotes had reached a late, committed stage of fusion. The fusion wall partitioning the cells was thinner than the outer, zygote wall. These forms also predominated in mating mixtures observed by Nomarski optics microscopy (above) or fluorescence microscopy using membrane stains (not shown) providing assurance that the forms observed by EM were typical of a larger population.

The [ste6(cef1-1) × wild-type] prezygotes had a symmetric appearance even though only one partner was mutant, suggesting that communication or coordination occurred during cell fusion such that a defect in one cell affected processes of the other (Fig. 3, C, E, and F). Nuclei, when visible, were located near the fusion zone. Though we did not stain specifically to enhance membranes, in some cases apparent secretory vesicles were visible and appeared to be localized to the cell surface of the fusion wall region (Fig. 3 F). Uncommonly, one cell of a [ste6(cef1-1) × wild-type] prezygote appeared to have formed a bubble or lysed into its partner (Fig. 3 D). We interpret these features to be consistent either with a failure to degrade some elastic component of the partitioning fusion wall or with a defect in membrane fusion. Rare [wild-type × wild-type] prezygotes appeared similar in appearance to [ste6(cef) × wild-type] prezygotes but were too infrequent (<1% of mating pairs) to characterize in detail (not shown). Overall, the [ste6(cef1-1) × wild-type] prezygotes gave the appearance expected of normal intermediates in mating. The morphological forms observed in ste6(cef) prezygotes resemble in many regards the normal intermediates of mating that have been reported for the budding yeast *H. wingei* (6). Only fusion wall degradation remained incomplete, preventing cytoplasmic mixing. The arrest phenotype of the [ste6(cef1-1) × wild-type] prezygotes might reflect either failure of signaling required to initiate the final, irreversible, steps of cell fusion, or the lack of a component required to carry out these steps.

**Ability of ste6(cef1) to Induce a-factor Responses in MAT α Cells**

Null mutations in STE6 are defective in the secretion of a-factor. The obvious question was whether the reduced a-factor export of the ste6(cef) strain was sufficient to activate mating responses. We measured the ability of a ste6(cef) strain to promote responses in its partner under mating conditions. First, we determined the ability of culture supernatants from the parent strain LM23-3az to arrest MAT α cells in the G1 phase of the cell cycle. Using a bud-arrest assay we showed that supernatants from the two strains had a similar ability to arrest growth. After 3 h the control cells incubated with MAT α supernatant had 24% unbudded cells whereas those incubated with wild-type MAT α supernatant had 62% unbudded cells and those incubated with the MAT α ste6(cef1-1) supernatant had 67% unbudded cells. Thus, the levels of a-factor produced by ste6(cef) strain seemed capable of inducing initial cell cycle arrest in its partner.

The ability of the MAT α ste6(cef1) strain to induce transcription of the fusion-gene (FUS1) in a MAT α partner was determined. Mating conditions were imposed by fil-
tering a MATa strain containing the FUSI-lacZ reporter with MATa strains in a 1:1 ratio and incubating the filter at 30°C for 3 h on a YPD plate. Under these conditions induction of FUSI-lacZ should reflect induction by the partner attempting to mate. The ste6(cefl) strain (LE4-89u) induced FUSI-lacZ in the partner MATa strain LM23-316az to a level 70% of that induced by a wild-type MATa strain (LM104) (5.3 vs 7.5 Miller β-galactosidase units, standard error 10-20%) whereas a Δste6::URA3 strain, LM110, induced FUSI-lacZ to only 14% of wild-type (1.1 units). Presumably the induction by the Δste6::URA3 strain reflects the background induction by a-factor release from lysed cells or nonspecific export through other transporters. These experiments were consistent with the

Figure 3. Electron microscopy of yeast mating pairs. Electron micrographs of cell pairs after 3.5 h of mating are shown. (A and B) [LM23-3az(Cef⁺) × FC139(Cef⁺)], zygotes; (C-F) [LE4-89(cefl) × FC139(Cef⁺)], prezygotes; the six panels contain representative, mating pairs. SV, secretory vesicles; FW, fusion wall. Bars: (A-C) 1 μm; (D) 0.2 μm.
Comparison of α-factor Expression by Strains with Various STE6 Alleles

Preliminary studies indicated that the ste6(cef) allele conferred a modest α-factor export defect. To further study the role of reduced α-factor export in the cell fusion defect we compared a strain expressing ste6(cef) to a strain expressing a leaky ste6 mutation. For these experiments we used ste6(cef) expressed from a plasmid in Δste6 strains to eliminate the effect of any secondary mutations in the original strain. Leaky ste6 mutants have been described by Berkower and Michaelis that confer reduced α-factor expression due to mutations in ATP-binding domains (1). Several of these were screened to find one expressing α-factor at levels similar to those of ste6(cef). Biological activity was assayed in semi-purified preparations that, in our hands, most reproducibly reflected activity of strains. An important aid to reproducibility of α-factor assays was the inclusion of non-ionic detergent, 0.05% Triton X-100, in medium for growth arrest assays. In the absence of detergent, growth inhibition zones were frequently asymmetric and unreliable. In the presence of detergent, zones were larger and reproducible from day to day. We speculated that the extremely hydrophobic α-factor may migrate preferentially at the air-water interface in the absence of detergent, rendering assays sensitive to plate medium dryness and subtle differences in lawn spreading or top agar. Nonetheless, quantitation of α-factor remains approximate.

The ste6(K1093A) strain, LM110(pSM401), expressed ∼twofold to fourfold more α-factor than the ste6(cef) strain, LM110(pLE131). Both expressed slightly less α-factor than a STE6+ strain (Fig. 4). The original ste6(cef1-1) strain, in which ste6(cef) was not overexpressed, expressed substantially less α-factor than the LM110(pLE131) strain. A Δste6:URA3 strain, as reported by others, still expressed significant levels of α-factor, though substantially less than strains expressing the other STE6 alleles (Fig. 4) (44).

Examination of growth inhibition induced by the dilutions of α-factor preparations (Fig. 4) revealed an unexpected pattern in which growth arrest turbidity and diameter appeared somewhat independent. Most strikingly, the preparation from the undiluted LM110(pLE131) strain seemed to produce more complete growth arrest far from the site of factor application than near (Fig. 4). This observation has been repeated several times. Growth arrest by the Δste6 preparation, though very transient, also occurred over a relatively wide area and in a pattern distinct from that observed using low concentrations of synthetic α-factor (not shown). Since the solvent for these preparations was DMSO, it seemed unlikely that free and cell-bound pools of α-factor that have been described remained distinct (2, 32). It is possible though that MAT a cells export by a Ste6-independent pathway a biologically active molecule in addition to α-factor.

Quantitation of Prezygote Accumulation during Mating by Various Strains

Prezygote accumulation was determined microscopically by determining the ratio of prezygotes that clearly had not undergone fusion to the total number of cells attempting fusion (prezygotes + zygotes). The accumulation of prezygotes was similar in the genomic ste6(cef) strain LE4-89 (71%) and the 2μ-based ste6(cef) strain LM110(pLE131) (63%) (Table III) though the strains differed greatly in α-factor production (Fig. 4). The parental and ste6(K1093A) strains on the other hand did not accumulate prezygotes in mating mixtures (<1%) (Table III). The total number of mating cell pairs observed microscopically were comparable with ste6(cef) and ste6(K1093A) strains (Table III). However, this number is difficult to measure accurately because of clumping in mating mixtures. The completed mating frequencies of the ste6(K1093A) and 2μ-based ste6(cef) strains were comparable, suggesting fusion might be delayed, but not entirely blocked in the ste6(cef) strain (Table IV). Other known leaky alleles of STE6 (1) were tested. None led to accumulation of prezygotes during mating (data not shown). Thus the prezygote phenotype may reflect a very narrow threshold of α-factor expression that efficiently promotes early mating steps but is insufficient for some late fusion step. Somewhat incompatible with this model is the observation that though strains expressing ste6(cef) from a single genomic copy or overexpressing ste6(cef) from a multicopy plasmid differed greatly in their levels of expression of α-factor (Fig. 4), they accumulated prezygotes to a similar extent during mating (Table IV).

Effect of Mating to a Partner with Increased Sensitivity to α-factor

The experiments described above suggested that the cell fusion defect of ste6(cef1) strains might be caused by reduction of α-factor expression below a specific threshold. It has been shown that a mating defect induced by a failure to produce sufficient pheromone can be suppressed by mating to a partner with increased pheromone sensitivity (4). If the cell fusion defect were due to reduced ability to activate pheromone responses in a partner cell then mutations in the partner that restored a high level of signaling should suppress the accumulation of prezygotes. We quantitated the effect on prezygote formation of mating a ste6(cef1) strain to an sst2 partner. Strains defective in SST2 have an ∼100-fold increased sensitivity to pheromones (5, 7). We examined the cell fusion phenotype of ste6(cef1-1) mated to an sst2 partner. Prezygotes accumulated to a similar extent when a ste6(cef) strain was mated to an SST2+ strain or to an sst2− strain (71% vs 61% prezygotes) (Table III, lines 2 and 8). Similar results were ob-

![Figure 4](https://example.com/figure4.png)

Quantitation of a-factor exported by various strains. Dilutions of semi-purified a-factor preparations (see Materials and Methods) were spotted on lawns of the a-factor-sensitive strain RC884. Strains assayed: LM23-3az, [STE6(CEF)]; LE4-89, [ste6(cef)]; LM112(pLE131), [2μ-ste6(cef)]; LM112(pSM401), [ste6(K1093A)]; LM110, [ste6::URA3]; a-factor solvent, [DMSO].
Table IV. Mating Ability of Strains Expressing Various Alleles of STE6

| Strain*                | STE6 allele | Mating Frequency1 (%) |
|------------------------|-------------|-----------------------|
| LM23-3az               | Wild-type   | 60                    |
| LE4-89                 | ste6(cef)   | 0.1                   |
| LM112 (pRS425)         | Δste6 (vector) | <0.0001               |
| LM112 (pLE131)         | ste6(cef)   | 1.2                   |
| LM112 (pSM401)         | ste6(K1093A)| 1.4                   |

* All strains isogenic to LM23-3az parent.
1 Mean of two determinations. Mating partner strain IH1701.

Discussion

We have identified S. cerevisiae mutants specifically defective in cell fusion steps during conjugation. The cell fusion defects were observed when only one partner was defective. Three of these mutants carried independent alleles of STE6, ste6(cef), which reduced a-factor expression. The MAT a-specific ste6(cef) mutations led to accumulation of mating pairs at a unique stage of conjugation in which the fusion wall remained intact. Cells of ste6(cef) strains mated with a wild-type partner arrested with a zygote-like cell wall surrounding both cells and with nuclei and secretory vesicles near the fusion wall partition, suggesting that many early mating functions remained intact. The ste6(cef)-induced defect in fusion wall degradation may not be mediated by a simple failure to induce pheromone-mediated responses in a partner cell since it was not suppressed by a sst2 mutation in the partner.

The previously characterized fis mutants seem to accumulate a variety of prezygote forms arrested at many stages of cell fusion (9, 10, 29, 42, 43). The ste6(cef) prezygotes resembled a subpopulation of fis prezygote forms that had advanced to a stage of fusion in which the cell wall at the margins of the region of cell contact had fused and the region of cell wall in the fusion zone had thinned. The symmetry of [ste6(cef) X wild-type] prezygotes suggested that early cell wall remodeling steps of mating that are dependent on pheromones proceeded normally (Fig. 3) (17, 37). The ste6(cef) alleles, unlike null ste6 alleles, did not interfere with early steps in mating required for mating pair formation.

The Ste6 transporter is a member of the ABC transporter family and is required for the export of a-factor, the pheromone produced by MATa cells (18, 30, 31, 44). Our ste6(cef) alleles of STE6, unlike deletions, were only partially defective in the transport of a-factor. The ste6(cel1-1) allele that we characterized contained an amber mutation which probably leads to a reduced level of expression of full-length Ste6 protein. Efficient read-through of nonsense codons in other regions of STE6 has been previously reported (12). It is unclear whether the fusion defect phenotype is a consequence of expression of a truncated product or to reduced expression of the full-length transporter protein.

A reasonable model for the behavior of cef alleles of STE6 is that they act by reducing the amount of a-factor exported. A strain expressing slightly more a-factor than the ste6(cef) strain did not accumulate prezygotes, suggesting that a sharp threshold of a-factor production might exist, below which cell fusion could be initiated, but not efficiently completed. Such a threshold for cell fusion might reflect distinct control of expression of some gene product required for later stages of mating. If such a pheromone-threshold had its basis only in the requirement to induce responses via the pheromone pathway then a mutation in the partner cell that intensified responses would be expected to suppress the fusion defect. However, an sst2 mutation that greatly increased pheromone sensitivity failed to suppress the ste6(cef) fusion defect. A plasmid overexpressing the structural gene for a-factor led to increased a-factor export by a ste6(cef) strain, but also failed to suppress the mating fusion defect. The role of a-factor in the late stages of mating may be complex, or Ste6 itself may play a role in cell fusion distinct from its role as a transporter.

We thank Susan Michaelis, Ira Herskowitz, Fred Chang, George Sprague, Jr., Gerald Fink, Elaine Elion, and Joshua Trueheart for strains and plasmids; Frank Macaluso for technical assistance with electron microscopy; and Jon Warner, Margaret Kielian, Greg Prellich, Anjani Shah, Malini Sen, and Tamar Michaeli for helpful comments on the manuscript.

This work was supported by Public Health Services grant GM43365 to L. Marsh and Cancer Core support grant P30 CA13330 from the National Cancer Institute.

Received for publication 26 September 1995 and in revised form 22 July 1996.

References

1. Berkower, C., and S. Michaelis. 1991. Mutational analysis of the yeast a-factor transporter STE6, a member of the ATP binding cassette (ABC) protein superfamily. EMBO J. (Eurol. Mol. Biol. Organ.) 10:3777–3785.
2. Betz, R., and W. Duntze. 1979. Purification and partial characterization of a-factor, a mating hormone produced by mating-type-a cells from Saccharomyces cerevisiae. Eur. J. Biochem. 95:469–475.
3. Byers, B., and L. Goetsch. 1975. Behavior of spindles and spindle plaques in the cell cycle and conjugation of Saccharomyces cerevisiae. J. Bacteriol. 124:511–523.
4. Chan, R.K., L.M. Melnick, L.C. Blair, and J. Thorner. 1983. Extracellular suppression allows mating by pheromone-deficient sterile mutants of Saccharomyces cerevisiae. J. Bacteriol. 155:903–906.
5. Chan, R.K., and C.A. Otte. 1982. Isolation and genetic analysis of Saccharomyces cerevisiae mutants supersensitive to G1 arrest by a factor and a factor pheromones. Mol. Cell. Biol. 2:11–20.
6. Conti, S.F., and T.D. Brock. 1965. Electron microscopy of cell fusion in conjugating Hansenula wingesi. J. Bacteriol. 90:524–533.
7. Dietzel, C., and J. Kurjan. 1987. Pheromonal regulation and sequence of the Saccharomyces cerevisiae SST2 gene: a model for desensitization to pheromone. Mol. Cell. Biol. 7:4169–4177.
8. Einarsson, L., E. Renstrom, C. Ammala, P.-O. Berggren, A.M. Bertorelli, B. Bokvist, A. Chilblain, J.T. Deeney, P.R. Platt, J. Gabel et al. 1996.
PKC-dependent stimulation of exocytosis by sunfonylureas in pancreatic beta-cells. Science (Wash. DC). 271:813-815.

9. Elion, E. P.L. Grisafi, and G.R. Fink. 1990. FUS3 encodes a cdc2/CDC28-related kinase required for the transition from mitosis into conjugation. Cell 63:465-466.

10. Elion, E.A., J. Trueheart, and G.R. Fink. 1995. Fus2 localizes near the site of cell corpses during programmed cell death in Caenorhabditis elegans. Genetics. 129:79-94.

11. Fearon, K., V. McClendon, B. Bouetti, and D.M. Bedwell. 1994. Premature translation termination mutations are efficiently suppressed in a highly conserved region of yeast Ste7p, a member of the ATP-binding cassette (ABC) transporter family. J. Biol. Chem. 269:17802-17808.

12. Fenn, J.C., A. Shaikh, G. Spatafora, and P. Fives-Taylor. 1995. The fimA locus of Streptococcus paraurethralis encodes an ATP-binding membrane transport system. Molec. Microbiol. 15:849-853.

13. Higgins, C.F. 1995. The ABC of Channel regulation. Cell. 82:693-696.

14. Hoffman, C.S., and F. Winston. 1987. Ten min DNA preparation from yeast efficiently releases autonomously plasmids for transformation of E. coli. Gene (Amst.). 57:267-272.

15. Hyde, S.C., P. Esmy, M.J. Hartshorn, M.M. Mimmack, U. Gileadi, S.R. Pearce, M.F. Gallagher, D.R. Gill, R.E. Hubbard, and C.F. Higgins. 1990. Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. Nature (Lond.). 346:362-365.

16. Jackson, C.L., and L.H. Hartwell. 1990. Courtship in S. cerevisiae: both cell types choose mating partners by responding to the strongest pheromone signal. Cell 63:1039-1051.

17. Kuchler, K., R.E. Sterne, and J. Thorner. 1989. Saccharomyces cerevisiae STE6 gene product: a novel pathway for protein export in eukaryotic cells. EMBO (Eur. Mol. Biol. Organ.) J. 15:3973-3984.

18. Kunihara, L.J., C.T. Beh, M. Latterich, R. Schekman, and M.D. Rose. 1994. Structural homology among membrane-associated domains of CFTR and certain transporter proteins. J. Protein Chem. 12:279-290.

19. Marsh, L. 1992. Substitutions in the hydrophobic core of the alpha-factor receptor of Saccharomyces cerevisiae permit response to Saccharomyces kluveri alpha-factor and to antagonist. Mol. Cell. Biol. 12:3959-3966.

20. Marsh, L., and I. Herskowitz. 1988. STE2 protein of Saccharomyces cerevisiae is a member of the rhodopsin/beta-adrenergic receptor family and is responsible for recognition of the peptide ligand alpha-factor. Proc. Natl. Acad. Sci. USA. 85:3855-3859.

21. McCaffrey, G. F. Clay, K. Kelsey, and G.F. Sprague. Jr. 1987. Identification and regulation of a gene required for cell fusion during mating of the yeast Saccharomyces cerevisiae. Mol. Cell. Biol. 7:2680-2690.

22. McGrath, J.P., and A. Varshavsky. 1989. The yeast STE5 gene encodes a homologue of the mammalian multidrug resistance P-glycoprotein. Nature (Lond.). 340:400-404.

23. McNew, J.S. 1993. STE6, the yeast alpha-factor transporter. Semin. Cell Biol. 4: 17-27.

24. McCaffrey, G., F. Clay, K. Kelsey, and G.F. Sprague, Jr. 1987. Identification and regulation of a gene required for cell fusion during mating of the yeast Saccharomyces cerevisiae. Mol. Cell. Biol. 7:2680-2690.

25. McNew, J.S. 1993. STE6, the yeast alpha-factor transporter. Semin. Cell Biol. 4:17-27.

26. Michaelis, S. 1991. Genetic analysis of G protein-coupled receptors and signal transduction during yeast karyogamy pathway. J. Cell Biol. 90:8332-8336.

27. Michaelis, S., and I. Herskowitz. 1988. The alpha-factor pheromone of Saccharomyces cerevisiae is essential for mating. Mol. Cell. Biol. 8:1309-1318.

28. Sen, M., and L. Marsh. 1994. Noncontiguous domains of the alpha-factor receptor of yeasts confer ligand specificity. J. Biol. Chem. 269:968-973.

29. Segall, J.E. 1993. Polarization of yeast cells in spatial gradients of alpha mating factor. Proc. Natl. Acad. Sci. USA. 90:8332-8336.

30. Strazdis, J.R., and V.L. MacKay. 1982. Reproducible and rapid methods for the isolation and assay of a-factor, a yeast mating hormone. J. Bacteriol. 151:1153-1161.