Role of the ABC Transporter Ste6 in Cell Fusion during Yeast Conjugation

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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 **a**-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 **a**-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 **a**-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 **a**-factor than *ste6(cef)* did not accumulate prezygotes during mating which could indicate a tight threshold of **a**-factor signaling required for mating. However, mating to an *sst2* partner which has a greatly increased sensitivity to **a**-factor did not suppress the fusion defect of a *ste6(cef)* strain. Overexpression of the structural gene for **a**-factor also did not suppress the fusion defect. It is possible that **a**-factor or *STE6* play more complex roles in cell fusion.

YEAST cells of **a** 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).

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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 **a**-factor is exported directly from the cell by the Ste6 transporter and is required for mating (18, 30, 32). The modified **a**-factor peptide activates the G protein-coupled **a**-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 **a**-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 ~68,700 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 *ADE6* 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 **a**-factor was assayed by patching mutant strains onto a lawn of strain RC884, α *sst2*, which is sensitive to **a**-factor. Strains 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* **a** 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* **a** 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* **a**/*MAT* α *CEF1*/*cef1* strain. This was converted to a *MAT* **a**/*MAT* **a** strain by introducing a

Table I. Yeast Strains and Plasmids Used in This Study

Strain	Genotype	Source
LM23-3az (parental strain)	*MAT***a** *STE6 (CEF1+) bar1 [FUS1-lacZ::URA3] leu2 ura3 his4 lys5 met1*	26
LE4-89	*MAT***a** *ste6 (cef1)-1 bar1 [FUS1-lacZ::URA3] leu2 ura3 his4 lys5 met1*	This work
LE4-75	*MAT***a** *ste6 (cef1)-2 bar1 [FUS1-lacZ::URA3] leu2 ura3 his4 lys5 met1*	This work
LE4-65	*MAT***a** *ste6 (cef1)-3 bar1 [FUS1-lacZ::URA3] leu2 ura3 his4 lys5 met1*	This work
LE4-89u	Isogenic to LE4-89 but cured of *[FUS1-lacZ::URA3]*	This work
LE4-75u	Isogenic to LE4-75 but cured of *[FUS1-lacZ::URA3]*	This work
LE4-65u	Isogenic to LE4-65 but cured of *[FUS1-lacZ::URA3]*	This work
LE1-5	*MAT***a** *fus3 bar1 [FUS1-lacZ::URA3 leu2 ura3 his4 lys5 met1*	This work
IH1701	*MAT*α *his2 ade6*	I. Herskowitz
FC139	*MAT*α *HMR***a** *HML***a** *ura3-52 met1 lys5*	26
RC884	*MAT*α *sst2-3 his6 leu1 met1 trp5 ura1 can1 cyh2 rme*	5
LM104	Isogenic to LM23-3az but cured of *[FUS1-lacZ::URA3]*	This work
LM110	Isogenic to LM104 but Δ*ste6::URA3*	This work
LM112	Isogenic to LM104 but Δ*ste6*	This work
LM23-116az	*MAT*α *bar1 STE2::mTn3 [TRP1]-4 leu2 ura3-52 lys5 met1 FUS1-lacZ*	26
SM1229	*MAT***a** *mfa1-D1::LEU2 mfa2-D1::URA3 trp1 leu2 ura3 his4 can1*	24
Plasmids		
pC2-1	YCp50 genomic library clone containing *STE6*	This work
pLE89G1	*ste6 (cef1)* allele on a *CEN*-based plasmid isolated from strain LE4-89	This work
pLE131	*ste6 (cef1-1)* in YEp13	This work
pSM192	*STE6* in pRS316	1
pSM322	*STE6* in pRS315	1
pSM401	ste6 (K1093A) in pRS315	1
pSL324	*FUS1* in YEp13	29
pSB257	*FUS2* in YEp24	43
pLE426	*MFA2* in pRS426	This work

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 *MAT* **a**/*MAT* **a** *CEF1*/*cef1* strain mated efficiently as a *MAT* **a** strain indicating that *cef1* was recessive. The *cef1* gene was cloned by complementation of the mating defect of the *cef1* 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.** A *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 **a**-factor export. Presence of the deletion/replacement in LM110 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 pYB4.0. Integration was targeted to the *STE6* gene of FC139 by digesting the plasmid DNA with endonuclease SpeI 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(cef1-1) 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 StuI 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 (15) into *Escherichia coli* and analyzed to ensure that they contained DNA corresponding to a complete *STE6* gene. Plasmids were transformed into a Δ*ste6*::*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(cef1-1)* coding region (contained in a BamHI–NcoI restriction fragment).

Construction of a Plasmid for Overexpression of a-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 a-factor Preparations

Partially purified **a**-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 dH_2O until washes were clear, followed by a wash with 30 ml 40% methanol. The **a**-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 *sst2*) 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 **a**-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 MAT α 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

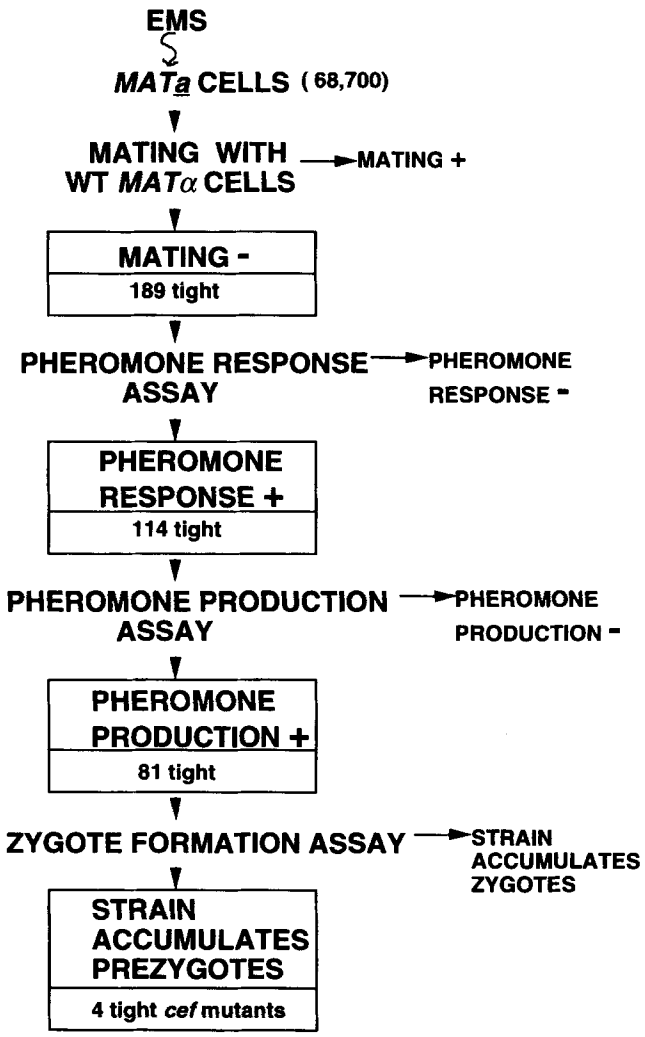


Figure 1. Schematic diagram of the multi-tiered screen for mutants blocked in the cell fusion step of mating.

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* **a** 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 **a**-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 *S. cerevisiae* genomic library (33). The *cef1-1* mutation alone reduced mating 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, (1) 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)*.

Table II. Complementation of cef1 Strains by STE6

Strain	Mating Efficiency*	Plasmid: pC2–1 (genomic clone, *STE6*)	Plasmid: pSM192‡ (*STE6*)
LM23–3az *STE6*$^+$	23%	ND	ND
LE4–89 *ste6(cef1–1)*	0.05%	18%	40%
LE4–75 *ste6(cef1–2)*	0.07%	31%	17%
LE4–65 *ste6(cef1–3)*	0.06%	15%	55%
LM110 Δ*ste6::URA3*	<0.0001%	ND	17%

*Mating efficiency was measured as the percent of the *MAT* **a** cells able to form prototrophic diploid colonies when incubated with an excess of the *MAT* α tester strain, IH1701 (39). Mean of duplicate determinations. See Materials and Methods.
‡For LM110 complementation the *STE6* plasmid pSM322 was used.

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 *3*). 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 hydrophobic 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* α partner§	Mating pairs observed*			
		Prezygotes	Zygotes	Prezygotes	Pairs/10^4 CFU‖
		no.	*no.*	%	
1. LM23–3az *STE6*$^+$	*SST2*$^+$	1	334	<1	120
2. LE4–89 *ste6 (cef1–1)*	"	80	32	71	75
3. LM110 (pLE131) Δ*ste6* (pSte6 (cef1–1))	"	107	99	52	412
4. LM112 (pSM401) Δ*ste6* (pSte6 (K1093A))	"	1	330	<1	166
5. LM112 (pLE131, pLE426) pSte6 (cef1–1), pMFA2		25	16	61	n.d.
6. LM110 Δ*ste6::URA3*	"	0	0	-	<1
7. LM23–3az *STE6*$^+$	*sst2*	1	645	<1	231
8. LE4–89 *ste6 (cef1–1)*	"	251	158	61	108
9. LM110 (pLE131) *ste6* (pSte6 (cef1–1))	"	346	266	57	204
10. LM110 Δ*ste6*	"	0	0	-	<1

*See Materials and Methods for mating conditions.
‡*MAT* **a** partner and relevant genotype. All are isogenic with LM23–3az.
§*MAT* α partners: *SST2*$^+$, FC139; *sst2*$^-$, RC884.
‖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(cef1-1) in a Δste6 Strain

Though the *ste6(cef1-1)* mutation segregated in crosses as a single, *MAT* **a**-specific mutation, we further examined the phenotype conferred by a *ste6(cef1-1)* 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(cef1-1)* plasmid, pLE131, exhibited a cell fusion defect (Table III, line *3*). Thus the *ste6(cef1-1)* mutation is both necessary and sufficient to produce the cell fusion defect. The *ste6(cef1-1)* 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

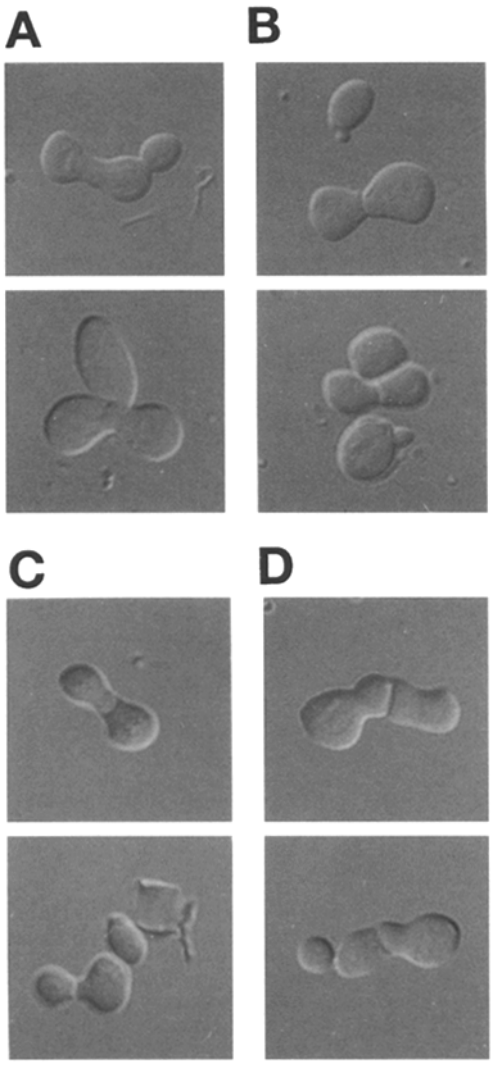


Figure 2. Light microscopy of yeast mating pairs. Photomicrographs of typical mating cell pairs using Nomarski optics. (*A*) LM23-3az × IH1701, wild-type zygotes. (*B*) LE4-89 (*ste6(cef)* × IH1701, prezygotes. (*C*) LM110(pLE131) (*ste6(cef)*) × IH1701, prezygotes. (*D*) LE1-5 (*fus3*) × IH1701, prezygotes.

progressed to a late stage of fusion. The significance of this difference is unclear but might indicate that the *fus* 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(cef1)* strain to promote responses in its partner under mating conditions. First, we determined the ability of culture supernatants from the parent strain LM23-3az or the *ste6(cef1)* strain LE4-89 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 cells incubated with wild-type *MAT* **a** supernatant had 62% unbudded cells and those incubated with the *MAT* **a** *ste6(cef-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* **a** *ste6(cef1)* strain to induce transcription of the fusion-gene (*FUS1*) in a *MAT* α partner was determined. Mating conditions were imposed by fil-

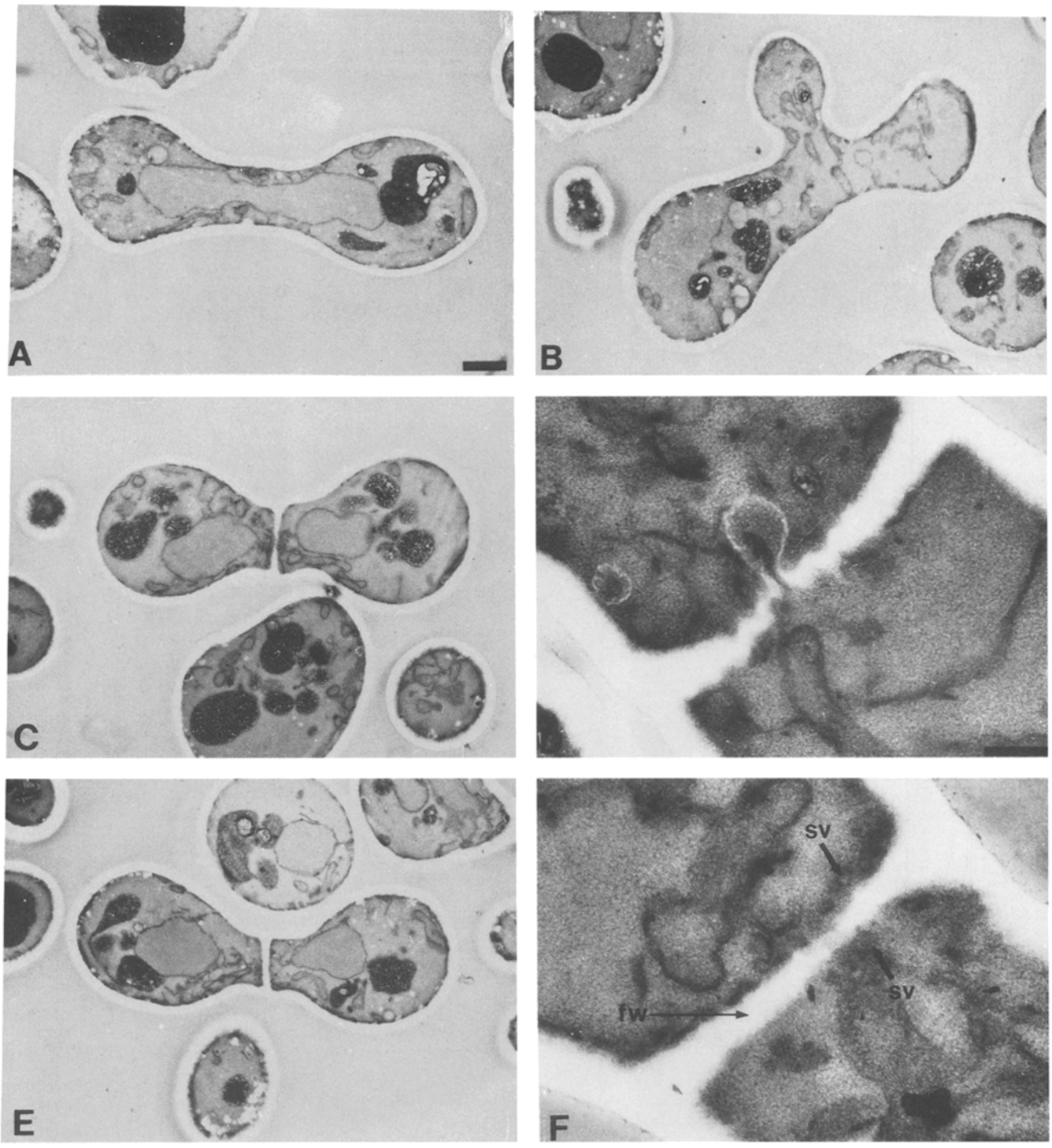
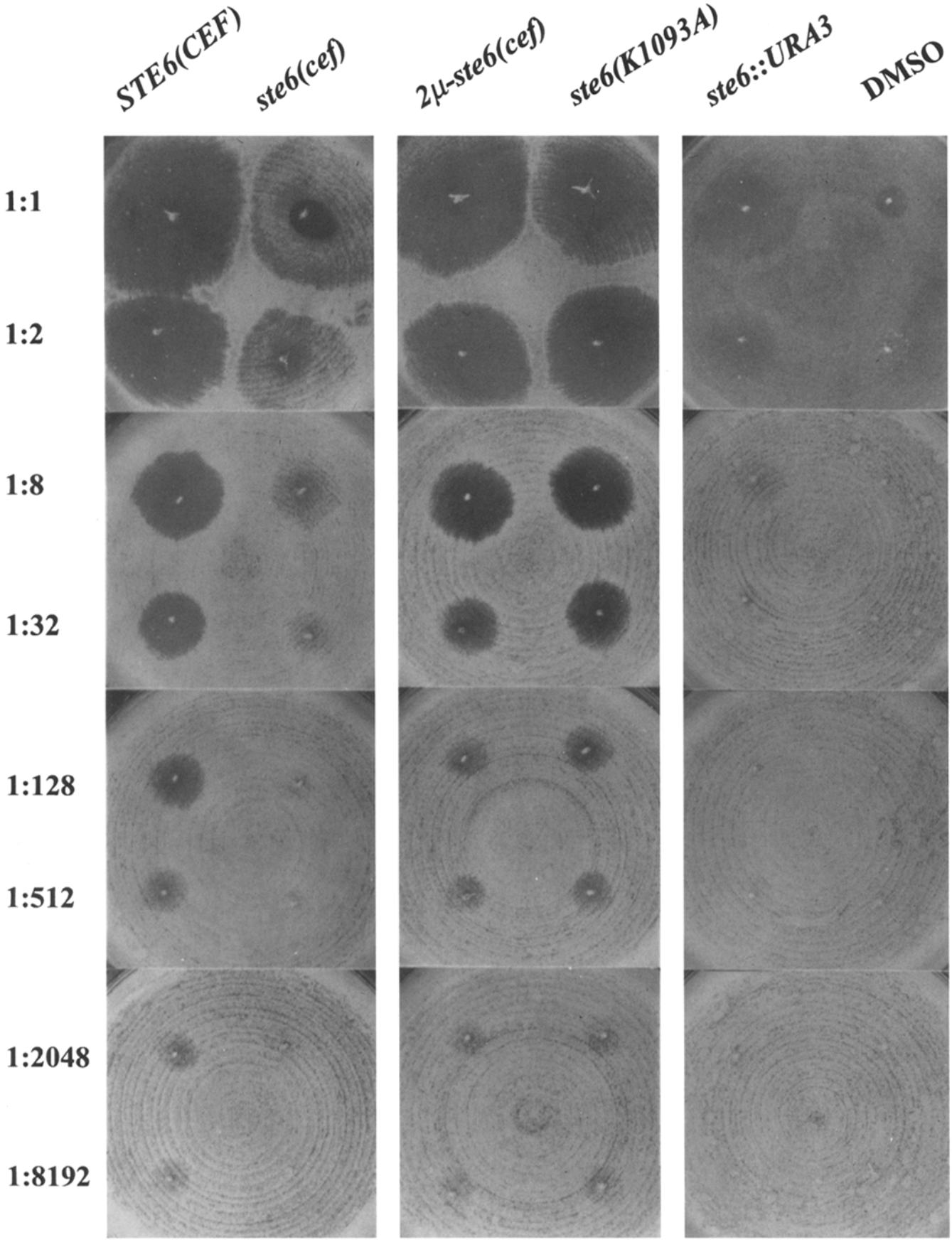


Figure 3. Electron microscopy of yeast mating pairs. Electron micrographs of cell pairs after 3.5 h of mating are shown. (*A* and *B*) [LM23-3az(Cef$^+$) × FC139(Cef$^+$)], zygotes; (*C–F*) [LE4-89(*cef1*) × FC139(Cef$^+$)], prezygotes; the six panels contain representative, mating pairs. SV, secretory vesicles; FW, fusion wall. Bars: (*A–C*) 1 μm; (*D*) 0.2 μm.

tering a *MAT* α strain containing the *FUS1-lacZ* reporter with *MAT* **a** 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 *FUS1-lacZ* should reflect induction by the partner attempting to mate. The *ste6(cef1)* strain (LE4-89u) induced *FUS1-lacZ* in the partner *MAT* α strain LM23-116az to a level 70% of that induced by a wild-type *MAT* **a** strain (LM104) (5.3 vs 7.5 Miller β-galactosidase units, standard error 10–20%) whereas a Δ*ste6*::*URA3* strain, LM110, induced *FUS1-lacZ* to only 14% of wild-type (1.1 units). Presumably the induction by the Δ*ste6*::*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


STE6(CEF)
ste6(cef)
2μ-ste6(cef)
ste6(K1093A)
ste6::URA3
DMSO
1:1
1:2
1:8
1:32
1:128
1:512
1:2048
1:8192

electron microscopy data that suggested that the *ste6(cef)* strain was capable of inducing many, though perhaps not all, mating responses in its partner.

Comparison of a-factor Expression by Strains with Various STE6 Alleles

Preliminary studies indicated that the *ste6(cef)* allele conferred a modest **a**-factor export defect. To further study the role of reduced **a**-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 Δ*ste6* 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 **a**-factor expression due to mutations in ATP-binding domains (1). Several of these were screened to find one expressing **a**-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 **a**-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 speculate that the extremely hydrophobic **a**-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 **a**-factor remains approximate.

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

Examination of growth inhibition induced by the dilutions of **a**-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 Δ*ste6* preparation, though very transient, also occurred over a relatively wide area and in a pattern distinct from that observed using low concentrations of synthetic **a**-factor (not shown). Since the solvent for these preparations was DMSO, it seemed unlikely that free and cell-bound pools of **a**-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 **a**-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 **a**-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 **a**-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 **a**-factor (Fig. 4), they accumulated prezygotes to a similar extent during mating (Table IV).

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

The experiments described above suggested that the cell fusion defect of *ste6(cef1)* strains might be caused by reduction of **a**-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. 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), [2l-*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 Frequency‡
		%
LM23–3az	Wild-type	60
LE4–89	*ste6(cef)*	0.1
LM112 (pRS425)	Δ*ste6* (vector)	<0.0001
LM112 (pLE131)	*ste6(cef)*	1.2
LM112 (pSM401)	*ste6*(K1093A)	1.4

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

served for *ste6(cef)* expressed from a plasmid mated to an *SST2*$^+$ or *sst2* partner (52 vs 57% prezygotes) (Table III lines *3* and *9*). In contrast, less than 1% prezygotes were observed in mating of the parental strain or the *ste6*(K1093A) strain to either an *SST2*$^+$ or an *sst2* partner. If reduced **a**-factor expression underlies the *ste6(cef)* fusion defect then we expected that increasing **a**-factor expression would suppress the fusion defect. Introduction of a plasmid (pLE426) overexpressing a structural gene for **a**-factor (*MFA2*) led to increased expression of **a**-factor (not shown) from a *ste6(cef)* strain (LM112(pLE131)). However, the **a**-factor plasmid failed to suppress the mating fusion defect (Table III, line *5*). Thus the fusion defect appears to not lie only in the inability of *ste6(cef)* strains to activate pheromone responses in their partners.

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 *fus* 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 *fus* 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)* × 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 *MAT* **a** 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(cef1-1)* 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 supress 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.

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