Genetic fine-structural analysis of the *Saccharomyces cerevisiae* α -pheromone receptor

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The α -pheromone receptor encoded by the *STE2* gene contains seven potential transmembrane domains. Its ability to transduce the pheromone signal is thought to require the action of a G protein. As an initial step toward defining the structural features of the receptor required for its activity, we examined the phenotypic consequences of linker insertion mutations (12 bp) at 10 different sites in the *STE2* gene. Three mutant classes, which correspond to three different regions of the receptor protein, were observed. 1) The two mutants affecting the C-terminal region (C-terminal mutants) were essentially wild type for mating efficiency, pheromone binding, and pheromone sensitivity. 2) The three mutants in the N-terminus mated with reduced efficiency, showed reduced pheromone binding capacity, and were partially defective in pheromone induction of agglutinin production and cell division arrest. Increased gene dosage of these N-terminal alleles suppressed their mutant phenotypes, whereas the *sst2-1* mutation, which blocks adaptation to pheromone, did not result in suppression. Thus, the N-terminal mutants were apparently limited by receptor production, but not by the adaptation function *SS72*. 3) The five mutants in the central region containing the seven transmembrane segments (central mutants) were completely defective for mating and did not respond to pheromone, but could be distinguished by their ability to bind pheromone. Inserts in or near transmembrane domains 2 and 4 blocked pheromone binding, whereas inserts into transmembrane domains 1, 5, and 6 retained partial pheromone bind-

ing activity even though they failed to transduce a signal. The central mutants were not suppressed by increased gene dosage, and one mutant (*ste2-1101*) was partially suppressed by *sst2-1*. Furthermore, the central core mutants were also distinguished from one another in that three of the five mutants were able to partially complement the temperature sensitivity of *ste2-3*.

Introduction

Conjugation in *Saccharomyces cerevisiae* occurs between haploid cells of opposite mating type and results in the creation of a diploid cell (Cross *et al.*, 1988). Mating type, *a* or α , is determined by information encoded at the *MAT* locus (Nasmyth and Shore, 1987; Herskowitz, 1989). Conjugation requires the action of pheromones produced by cells of the two mating types. *MATa* cells secrete *a*-pheromone, a dodecapeptide covalently attached to farnesyl by a thioether bond to a cysteine residue (Anderegg *et al.*, 1988), and *MAT α* cells secrete α -pheromone, a tridecapeptide (Stotzler *et al.*, 1976). Cells stimulated by the appropriate pheromone increase in agglutinability (Suzuki and Yanagishima, 1985), arrest mitotic cell division in G1 (Bucking-Throm *et al.*, 1973), and undergo cellular morphogenesis to form a conjugation tube (Byers, 1981). Other functions are induced that direct cell and nuclear fusion (Rose *et al.*, 1986; McCaffrey *et al.*, 1987; Trueheart *et al.*, 1987). After prolonged exposure to pheromone, the cells that do not mate can adapt to the stimulus and resume cell division (Moore, 1984).

The α -pheromone receptors present on the surface of *MATa* cells play a complex role in mating. α -pheromone receptors, encoded by the *STE2* gene (Jenness *et al.*, 1983; Blumer *et al.*, 1988), are structurally related to the *a*-pheromone receptors, encoded by *STE3* (Nakayama *et al.*, 1985; Hagen *et al.*, 1986), and to the β -adrenergic/rhodopsin family of receptors in that they all contain seven putative transmembrane domains (Stryer and Bourne, 1986). Additional members of this family include receptors for cyclic AMP (cAMP; Klein *et al.*, 1988), serotonin (Julius *et al.*, 1988), and the peptide neurotransmitter substance K (Masu *et al.*, 1987). The α -pheromone

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receptor may also be functionally related to the β -adrenergic/rhodopsin family; receptors in this group transduce their signal by activation of heterotrimeric G proteins (Stryer and Bourne, 1986). Genetic evidence (Dietzel and Kurjan, 1987a; Miyajima *et al.*, 1987; Jahng *et al.*, 1988; Blinder *et al.*, 1989; Fujimara, 1989; Whiteway *et al.*, 1989) suggests that the α -pheromone receptor activates a G protein with α , β , and γ subunits encoded by *GPA1* (*SCG1*) (Dietzel and Kurjan, 1987a; Miyajima *et al.*, 1987), *STE4* (Whiteway *et al.*, 1989), and *STE18* (Whiteway *et al.*, 1989), respectively. The ability of cells carrying a mutation in *GPA1* or overexpressing *STE4* to mate in the absence of pheromone receptor suggests that G protein activation is the only essential signal induced by the pheromone receptors (Miyajima *et al.*, 1987; Jahng *et al.*, 1988; Blinder *et al.*, 1989; Cole *et al.*, 1990; Nomoto *et al.*, 1990; Whiteway *et al.*, 1990). However, other aspects of receptor function may be required for high-efficiency mating (Cross *et al.*, 1988). The α -pheromone receptor is also similar to the β -adrenergic receptor and to rhodopsin in that C-terminal sequences of each receptor are thought to be involved in receptor desensitization (Konopka *et al.*, 1988; Reneke *et al.*, 1988). α -pheromone bound receptors are also down-regulated by internalization, and the receptor ligand complexes are probably degraded in the vacuole (Chvatchko *et al.*, 1986; Jenness and Spatrick, 1986).

A variety of mutagenesis procedures have been employed to study the relationship between the structure and function of the receptors belonging to the β -adrenergic/rhodopsin family. For example, extensive study of the adrenergic receptors has identified regions that are involved in ligand binding (Dixon *et al.*, 1987a,b; Strader *et al.*, 1987; Fraser *et al.*, 1988), G protein activation (Frielle *et al.*, 1988; Kobilka *et al.*, 1988), and receptor desensitization (Bouvier *et al.*, 1988). Analysis of frameshift mutations and partial deletions of the *STE2* gene indicate that the C-terminal sequences of the α -pheromone receptor are nonessential for signaling but mediate an adaptive response to pheromone (Konopka *et al.*, 1988; Reneke *et al.*, 1988). The present study examines the phenotypic consequences of small insertion mutations that do not shift the reading frame of the *STE2* gene.

Results

Effects of linker insertions on Ste2 protein production

Ten mutant alleles of *STE2* were created by insertion of 16 bp *EcoRI* linkers at the sites indi-

cated in Figure 1. The construction and effects of these frameshift mutations were described previously (Konopka *et al.*, 1988). To examine the effects of insertions that do not shift the reading frame, we reduced the 16-bp insertion to 12 bp by using Mung Bean nuclease to remove the 4-bp overhang left after *EcoRI* digestion. Re-ligation of these plasmids resulted in the predicted loss of the *EcoRI* site and acquisition of a *BalI* site at the corresponding position. Mutants are designated *ste2-l* followed by the number of the *STE2* codon disrupted by the insertion.

The creation of in-frame insertion alleles of *STE2* was confirmed by the use of Western immunoblot analysis to detect the production of full-length Ste2 protein. *STE2* alleles were carried on a single-copy YCp plasmid vector (pJBK-007). The host strain (DJ213-6-3) contained a chromosomal deletion of the *STE2* gene (*ste2-10::LEU2*). As shown in Figure 2A, control cells transformed with the vector alone (*ste2Δ*) did not produce proteins that cross-reacted with the Ste2 antibodies. Analysis of cells carrying a *STE2*⁺ plasmid (YCp-*STE2*) resulted in detection of a 43-kDa band and two carbohydrate-modified species of the Ste2 protein at 48 and 53 kDa, as previously described (Blumer *et al.*, 1988; Konopka *et al.*, 1988). An additional band of lower gel mobility was also detected at a position expected for a dimer of Ste2 protein (Konopka *et al.*, 1988). The mutant cell extracts are shown in the subsequent lanes; the numbers refer to the codon disrupted by the insertion. Strains containing *STE2* plasmids with linker inserts that disrupt the C-terminal half of the protein (*ste2-1169*, *1229*, *1261*, *1326*, or *1416*) produced readily detectable full-length Ste2 protein. However, strains containing linker inserts that disrupted codons corresponding to the N-terminal half of the protein (*ste2-145*, *147*, *152*, *162*, and *1101*) produced reduced levels of Ste2 protein that were difficult to detect. To gain increased sensitivity for this analysis, the *STE2* genes were subcloned onto the multicopy plasmid vector YEp24. Expression of *STE2*⁺ on this plasmid resulted in 10-fold overproduction of protein (Figure 2B; Konopka *et al.*, 1988). Increased protein was detected for all 10 mutant strains, confirming the presence of full-length protein product. Ste2 proteins disrupted by insertions into the N-terminal half of the molecule (*ste2-145*, *147*, *152*, *162*, and *1101*) migrated predominantly as heterogeneous species of lower gel mobility; however, significant levels of Ste2 protein were detected at the 43-, 48-, and 53-kDa positions. Two of the mutants with inserts

Figure 1. *ste2* insertion mutants. The corresponding positions of the linker insertions are indicated on the Ste2 protein model by arrows labeled with the number of the *STE2* codon disrupted. The topology of the Ste2 protein in the membrane shown in the diagram is predicted on the basis of Ste2 hydropathy plots (Burkholder and Hartwell, 1985; Nakayama *et al.*, 1985) and similarity to rhodopsin (Engelman *et al.*, 1982). Potential N-linked glycosylation sites are indicated as CHO.

in the C-terminal half of Ste2 produced proteins that differed from wild type. *ste2-1229* produced more of the 43-kDa form, and *ste2-1416* yielded faster migrating proteins that may be proteolytic products.

Functional analysis of *ste2* insertion mutants

The efficiency with which cells mate is a sensitive assay for detecting defects in receptor function. The mating efficiency of the *ste2* insertion mutant strains was determined by the use of a quantitative mating assay (Hartwell, 1980). When a *ste2-10::LEU2* strain carrying the wild-type *STE2* gene on a YCp plasmid (DJ213-6-3 + YCp-*STE2*) was mixed with *MAT α* cells, ~30% of input cells formed diploids in 4 h at 23°C; this level of mating efficiency was assigned a relative value of 100% (Table 1). In contrast, an *ste2-10::LEU2* strain (DJ213-6-3 + YCp vector) did not mate detectably (mating efficiency $\leq 0.001\%$). The *ste2* insertion mutants differed widely in their mating efficiency but could be grouped into three classes according to the position of the insert in the Ste2

protein (Table 1). The first class of mutants disrupted the C-terminal domain (*ste2-1326* and *1416*) and mated with wild-type efficiency. This result agrees with previous findings indicating the C-terminal domain is not essential for mating (Konopka *et al.*, 1988; Reneke *et al.*, 1988). The second class of mutants disrupted the N-terminal domain (*ste2-145*, *147*, and *152*) and showed reduced mating efficiencies (30.5, 30.9, and 2.5%, respectively). Although the N-terminal mutants produced Ste2 proteins with altered electrophoretic properties (Figure 2), they produced enough functional cell-surface receptors to support mating. The third class of mutants disrupted the central region of Ste2 containing the seven hydrophobic segments (*ste2-162*, *1101*, *1169*, *1229*, and *1261*). These mutants lacked detectable mating ($\leq 0.001\%$).

All mutants were assayed for specific changes in pheromone responsiveness. α -pheromone causes wild-type *a* cells to arrest cell division in the G1 phase and stimulates the appearance of agglutinin molecules on the cell surface (Cross *et al.*, 1988). The *STE2*⁺ strain

Figure 2. Western immunoblot analysis of Ste2 protein. (A) Ste2 antiserum was used to probe a Western immunoblot containing extracts of *ste2-10::LEU2* strain DJ213-6-3 transformed with the single-copy CEN plasmid vector lacking an insert (*ste2Δ*) or containing a *STE2* gene (*STE2+*). Subsequent lanes contain extracts of cells carrying insertion mutant plasmids and are labeled according to the *STE2* codon disrupted by the linker insert. (B) Extracts from *ste2-10::LEU2* strain DJ213-6-3 containing multicopy plasmid YEp24 lacking an insert (*ste2Δ*) or containing the *STE2* gene (*STE2+*). Subsequent lanes contain extracts of cells carrying *STE2* insertion mutant genes on YEp24 and the lanes are labeled according to the *STE2* codon disrupted.

(DJ213-6-3 + YCp-*STE2*) produced a low level of agglutinin in the absence of α -pheromone (agglutinin index, 0.1) and a high level in 10^{-7} M α -pheromone (agglutinin index, 0.8). This level of agglutinin induction was assigned a relative value of 100% in Table 1. The *ste2Δ* control strain (DJ213-6-3 + YCp-vector) was unresponsive because it produced a low level of agglutinin that did not increase in the presence of pheromone. All of the insertion mutants produced a low level of agglutinin in the absence of pheromone, indicating that they are not locked into constitutive signaling, as has been observed for *ste2-9* (Burkholder, 1986). The relative level of agglutinin induction for each strain is indicated in Table 1. The mutants in the central region (DJ213-6-3 + YCp-*ste2-162*; -1101; -1169; -1229; or -1261) lacked significant induction of agglutinin (<16%). N-terminal insertion mutant strain *ste2-152* induced a low level of agglutinin (25%). The other two N-terminal insertion strains, *ste2-145* and *ste2-147*, were able to induce higher levels of agglutinin (70 and 91%, respectively). The C-terminal mutants induced essentially wild-type levels of agglutinin (106 and 98%).

Two assays were used to monitor cell division arrest in response to α -pheromone. First, because yeast cells normally bud at the onset of

DNA synthesis, the proportion of unbudded cells reflects the number of cells in the G1 phase of the cell division cycle. The α -pheromone responsiveness of the various strains is indicated in Table 1 as the relative increase in the number of unbudded cells after a 4-h incubation in α -pheromone. In the second assay, filter disks containing α -pheromone are placed on solid medium that has been seeded with the cells to be tested. After incubation (48 h), the responsive strains produce halos surrounding the filter disk because of inhibition of the division cycle by α -pheromone (Figure 3). Table 1 indicates the relative amount of α -pheromone required to produce a standard response. The central region mutants (*ste2-162*, 1101, 1169, 1229, and 1261) show no α -pheromone response in either of the cell division arrest assays. In contrast, the mutants of the C-terminal domain (*ste2-1326* and -1416) show wild-type responses in both assays. Two of the N-terminal domain mutants (*ste2-145* and -147) were responsive in the shorter (4 h) budding assay (45 and 63% of wild type, respectively), but they were unresponsive in the longer (48 h) halo assay. The other N-terminal domain mutant (*ste2-152*) was unresponsive in both assays. It appears that the N-terminal mutants can initiate the responses, as

Table 1. Analysis of *STE2* insertion mutants

<i>STE2</i> allele ^a	Mating efficiency (%) ^b	Agglutinin induction (%) ^c	Cell division arrest	
			% unbudded ^d	% halo ^e
<i>ste2Δ</i>	<0.001	2	7	0
<i>STE2</i> ⁺	100	100	100	100
<i>ste2-I45</i>	30.5	70	44	0
<i>ste2-I47</i>	30.9	91	63	0
<i>ste2-I52</i>	2.5	25	0	0
<i>ste2-I62</i>	<0.001	0	0	0
<i>ste2-I101</i>	<0.001	16	0	0
<i>ste2-I169</i>	<0.001	8	0	0
<i>ste2-I229</i>	<0.001	6	0	0
<i>ste2-I261</i>	<0.001	4	0	0
<i>ste2-I326</i>	88.5	106	84	100
<i>ste2-I416</i>	111	98	91	200

^a *STE2* genes carried on YCp plasmids in *ste2-10::LEU2* strain DJ213-6-3. *ste2Δ* indicates the YCp-vector control lacking a *STE2* gene.

^b Mating efficiency with MAT α strain PT2 was determined by a filter mating assay.

^c Agglutinin was induced with 10⁻⁷ M α -pheromone and quantitated by agglutination with MAT α strain EMS63. Relative values are reported with the *STE2*⁺ strain normalized to 100%.

^d Cells were incubated in presence or absence of 10⁻⁷ M α -pheromone for 4 h and then the increase in the number of unbudded G1 phase cells was determined by microscopic observation. Relative values are reported with the *STE2*⁺ strain normalized to 100%.

^e Halo assay for cell division arrest as demonstrated in Figure 3. Relative values are reported for the concentration of pheromone required to produce a halo with a 5-mm radius. The *STE2*⁺ strain was normalized to 100%.

judged by the 1-h agglutination assay (*ste2-I45*, *-I47*, *-I52*) and by the budding assay (*ste2-I45* and *-I47*), but they are unable to sustain a longer response, as judged by the halo test. On close inspection it appears that *ste2-I45* and *ste2-I47* may produce turbid halos that probably result from their inability to maintain the pheromone-arrested state.

α -pheromone binding

The insertion mutants were tested for α -pheromone binding activity to determine whether the mutant receptors could still bind their ligand (Figure 4 and summarized in Table 2). To achieve optimal sensitivity, the *STE2* genes were expressed on multicopy plasmid YEp24 in strain DJ213-7-3. This strain lacks the α -pheromone degrading protease encoded by the *BAR* gene (MacKay *et al.*, 1988). Overexpression of wild-type *STE2* (DJ213-7-3 + YEp-*STE2*) resulted in ~4.3-fold more α -pheromone bound to the cells than the same strain containing a single copy of *STE2* (YCp-*STE2*). The control strain lacking a *STE2* gene (DJ213-7-3 + YEp24) lacked detectable binding. Strains that overexpressed the N-terminal mutants *ste2-I45*, *ste2-I47*, and *ste2-*

I52 bound α -pheromone at levels comparable to a wild-type strain containing a single copy of *STE2* (4202-15-3). Strains that overexpressed central core mutants *ste2-I101* and *ste2-I169* lacked significant binding activity, suggesting that the mutational lesions either disrupt the ligand binding site or prevent accumulation of receptors on the cell surface. Strains overexpressing the *ste2-I62*, *ste2-I229*, and *ste2-I261* alleles bound 15, 18, and 27%, respectively, compared with the single copy *STE2* control strain. Because these mutants show no response to pheromone (Table 1), the occupied receptors must be unable to produce the proper intracellular signal(s). This behavior could result from specific defects in a signaling domain of the receptor. Alternatively, the occupied mutant receptors could be hypersensitive to a negative regulatory function (e.g., receptor downregulation; Jenness and Spatrick, 1986).

Suppressibility of the *STE2* mutants

Patterns of suppression were used as a criterion to distinguish the properties of the various insertion mutants. The mutants were tested for their ability to be suppressed by increased gene

Figure 3. Halo assays demonstrating effects of *Ste2* overproduction and the *sst2-1* mutation on pheromone sensitivity. Each row contains a set of halo assays for the *STE2* gene indicated on the left. Strain DJ213-6-3 (*ste2-10::LEU2*) carried *STE2* genes on single-copy YCp plasmids (column 1) or multicopy YEp plasmids (column 2) and strain 7416-12-3 (*ste2-10::LEU2 sst2-1*) carried YCp plasmids. Sterile filter disks containing 2×10^{-6} , 10^{-5} , or 5×10^{-5} M α -pheromone (left to right) were placed on an agar plate spread with the indicated strain. Plates were photographed after a 48-h incubation at 23°C. The halo is the zone of growth inhibition detected surrounding the filter disk.

dosage of the mutant *ste2* allele (Table 2); they were also tested for suppression by mutation *sst2-1* (Table 3). Expression of the wild-type *STE2* allele on a high-copy plasmid, YEp24, resulted in ~ 10 -fold overproduction of the Ste2 protein (Figure 2B) and had little effect on mating efficiency (Table 2). Overexpression of the five central mutants (YEp-*ste2-I62*, *ste2-I101*, *ste2-I169*, *ste2-I229*, and *ste2-I261*) did not result in detectable mating. In contrast, the three N-terminal insertion mutants (*ste2-I45*, *ste2-I47*, and *ste2-I52*) increased in mating efficiency as a result of overexpression. In particular, the YEp-*ste2-I52* strain increased 15-fold to a mating efficiency of 37.5%. Overexpression of the N-terminal mutant alleles also resulted in a

stronger cell division arrest response in the halo assay (Figure 3).

The *sst2-1* mutation results in hypersensitivity to pheromone and failure to adapt when cells are exposed to pheromone for prolonged periods (Chan and Otte, 1982; Dietzel and Kurjan, 1987b). An *sst2-1 ste2-10::LEU2* strain (7416-12-3 + YCp vector) did not mate detectably, demonstrating that mating is still dependent on receptor activity (Table 3). The same strain containing a YCp-*STE2* plasmid mated at a reduced efficiency (13%, Table 2) compared with an *SST2* strain (Table 1). Of the five *sst2-1* strains containing the central mutants, the *ste2-I62*, *ste2-I169*, and *ste2-I229* strains still did not exhibit detectable mating. The *ste2-I261* strain

Figure 4. Pheromone binding. [³H]-α-pheromone binding to strain DJ213-6-3 (*ste2-10::LEU2 bar1-1*) carrying multicopy plasmid vector YEp24 without an insert (*ste2Δ*), with the *STE2* gene (multicopy *STE2*), or with one of the linker insertion mutant genes (indicated by the number of the *STE2* codon disrupted). A strain (4202-15-3) carrying a single chromosomal copy of *STE2* is shown for comparison (single-copy *STE2*). Cells were harvested in log phase and then incubated with [³H]-α-pheromone (2×10^{-8} M). The α-pheromone binding is reported as cpm bound per 10^7 cells.

showed barely detectable levels of mating (20 000-fold lower than the *STE2*⁺ strain). Only the *ste2-1101* allele showed a significant level of suppression; the *sst2-1 ste2-1101* strain mated 1200-fold more efficiently than the *SST2 ste2-1101* strain. This was surprising because the *SST2 ste2-1101* strain did not detectably bind pheromone (Figure 4). Possibly a small number of functional receptors are formed in this strain that facilitate mating.

The effects of the *sst2-1* mutation on the responsiveness of the N-terminal insertion mutants to α-pheromone was determined directly by examining the levels of agglutinin production and cell division arrest at varying doses of pheromone. *SST2* (DJ213-6-3) or *sst2-1* (7416-12-3) strains lacking the *STE2* gene (*ste2-10::LEU2*) did not detectably induce agglutinin. The constitutive level of agglutinin production increased

in the *sst2-1 ste2-10::LEU2* strain (Figure 5A), which is consistent with the proposal that *SST2* acts to antagonize the postreceptor signal (Blinder and Jenness, 1989; Dietzel and Kurjan, 1987b). Analysis of strains carrying YCp-*STE2* demonstrated that the *sst2-1* mutation resulted in ≥30-fold increased sensitivity for agglutinin induction (Figure 5B) and halo formation (Figure 3). In contrast, agglutinin induction (Figure 5C) and halo formation (Figure 3) were enhanced <3-fold by *sst2-1* in strains carrying YCp-*ste2-145*. However, the halos formed by the YCp-*ste2-145 sst2-1* strain were clearer than in the *ste2-145 SST2* strain, suggesting that the *sst2-1* mutation enabled the cells to maintain cell division arrest longer. Similar results were observed for the other N-terminal mutants (*ste2-147* and *ste2-152*) (Figure 3 and data not shown). Thus, the N-terminal mutants are suppressed

Table 2. High-copy YEp plasmid expression of *STE2* mutants

<i>STE2</i> allele ^a	Mating efficiency (%) ^a	YEp/YCp mating efficiency ^b	α -pheromone binding (%) ^c
<i>ste2</i> Δ	<0.001	ND	NDB
<i>STE2</i> ⁺	78	0.78	100
<i>ste2-145</i>	41	1.34	24
<i>ste2-147</i>	49.3	1.6	27
<i>ste2-152</i>	37.5	15	22
<i>ste2-162</i>	<0.001	ND	3.6
<i>ste2-1101</i>	<0.001	ND	NDB
<i>ste2-1169</i>	<0.001	ND	0.7
<i>ste2-1229</i>	<0.001	ND	4.2
<i>ste2-1261</i>	<0.001	ND	6.1

^a Mating efficiency of *STE2* genes on YEp24 in *ste2-10::LEU2* strain DJ213-6-3.

^b Ratio of mating efficiency for strains carrying *STE2* genes on YEp plasmid to their mating efficiency when carrying *STE2* genes on YCp plasmids. ND indicates ratio was not determined because of low mating efficiency.

^c Relative α -pheromone binding capacity as determined in Figure 4 with YEp-*STE2* normalized to 100%. NDB indicates no detectable binding above background.

by increased gene dosage, whereas they are not significantly affected by mutation *sst2-1*. In contrast, the mating ability of the *ste2-1101* mutant is suppressed by *sst2-1*, but increased gene dosage failed to restore detectable levels of mating.

Intragenic complementation

Intragenic complementation was used as a second method for distinguishing the properties of the central mutants (*ste2-162*, *-1101*, *-1169*, *-1229*, and *-1261*). Strains were constructed that contained a plasmid-born *ste2* insertion mutant and a chromosomal *ste2-3^{ts}* allele. These heterogenotes were then tested for correction of the *ste2-3* temperature-sensitive mating defect. The control strain that contained a chromosomal copy of *ste2-3* and the YCp-vector mated with low efficiency (0.005%, Table 4) at the restrictive temperature (34°C), as reported previously (Hartwell, 1980). Increased gene dosage of *ste2-3* corrected the temperature sensitive-mating defect; i.e., introduction of an additional *ste2-3^{ts}* gene on the YCp-plasmid resulted in 820-fold increased mating and the YEp24-*ste2-3* plasmid resulted in wild-type mating efficiency (9000-fold increase; Table 4). The central mutants on YCp or YEp plasmids showed no detectable mating when carried by a *ste2* Δ strain (DJ213-6-3) (Tables 1 and 2). However, three of

the central mutants on the YCp plasmid partially restored mating in the *ste2-3^{ts}* strain (Table 4). Mating efficiency of the *ste2-3^{ts}* strain increased 14-fold with YCp-*ste2-162*, 198-fold with YCp-*ste2-1229*, and 940-fold with YCp-*ste2-1101*. YCp-*ste2-1169* and YCp-*ste2-1261* failed to restore the mating defect of *ste2-3*. Thus intragenic complementation was allele specific.

Expression of the *ste2* insertion alleles on high-copy YEp-plasmids was used to determine whether the level of complementation would depend on the relative quantities of the mutant gene products. YEp-*ste2-162* complemented chromosomal *ste2-3* 61-fold more efficiently than did the YCp-*ste2-162* plasmid (Table 4). Thus, complementation was limited by the amount of *ste2-162* gene product. In contrast, the ability of *ste2-1101* and *ste2-1229* to complement *ste2-3* was unaffected by increasing the plasmid copy number (Table 4) even though the level of their protein products increased (Figure 2). This suggests that the *ste2-1101* and *ste2-1229* gene products had saturated their ability to participate in complementation. Perhaps the *ste2-3* product can form a stoichiometric complex with the *ste2-1101* or *ste2-1229* products to give active pheromone receptors.

Discussion

The fine structural analysis of the *STE2* gene reported here indicates that the N-terminus and the central region containing the seven transmembrane segments are very sensitive to the effects of the structural perturbations caused by the 12-bp insertions. All eight mutants within these regions showed decreased or no re-

Table 3. Suppression of *ste2* mutants by *sst2-1*

<i>STE2</i> allele	Mating efficiency (%) ^a	<i>sst2⁻/SST2⁺</i> mating efficiency ^b
<i>ste2</i> Δ	<0.001	ND
<i>STE2</i>	12.6	0.13
<i>ste2-145</i>	34.4	1.1
<i>ste2-147</i>	52	1.7
<i>ste2-152</i>	2.8	1.1
<i>ste2-162</i>	<0.001	ND
<i>ste2-1101</i>	1.2	≥ 1200
<i>ste2-1169</i>	<0.001	ND
<i>ste2-1229</i>	<0.001	ND
<i>ste2-1261</i>	<0.005	≥ 5

^a Mating efficiency of *sst2-1 ste2-10::LEU2* strain 7416-12-3 carrying *STE2* genes on YCp plasmids.

^b Ratio of mating efficiency in *sst2-1* strain 7416-12-3 to mating efficiency in *SST2⁺* strain DJ213-6-3 (Table 1).

Figure 5. Dose-response for agglutinin induction. Agglutinin induction for *MATa* strains DJ213-6-3 (*SST2 ste2-10::LEU2*; open figures) and 7416-12-3 (*sst2-1 ste2-10::LEU2*; closed figures) carrying the CEN plasmid vector lacking an insert (YCp-vector; A), containing the *STE2* gene (YCp-*STE2*; B), or the *ste2-145* gene (YCp-*ste2-145*; C). *MATa* cells were incubated with the indicated α -pheromone concentration (x-axis) and then the extent of agglutination with *MATa* strain EMS63 was quantitated.

sponse to pheromone. The genetic techniques employed in this study made it possible to distinguish among the mutants and infer the nature of their defects. Biochemical analysis of these mutant receptors will also be informative when appropriate in vitro assays for receptor function are developed. Overall, the properties of these mutants define three phenotypic classes, which are associated with each of the three domains of the proposed receptor structure: 1) the N-terminal domain that is probably extracellular, 2) the central domain containing the seven putative transmembrane-spanning segments, and 3) the C-terminal domain that is probably intracellular. Small differences among the central mutants suggest that portions of this domain may be functionally distinct as well. Previous analysis of frameshift and deletion mutations of the *STE2* gene (Konopka *et al.*, 1988; Reneke *et al.*, 1988) indicate that the C-terminal domain is involved in regulation of the pheromone response but is otherwise unnecessary for pheromone binding and signal transduction. The mutants described in this study that contain insertions in the C-terminus did not have detectable effects on mating efficiency, pheromone binding, or pheromone sensitivity.

N-terminal domain

The *ste2-145*, *ste2-147*, and *ste2-152* alleles appear to interrupt the N-terminal portion of the receptor at or near the beginning of the first transmembrane segment (Figure 1); the *ste2-152* allele may affect residues within the first transmembrane segment. Strains containing these mutations exhibited reduced mating ability and decreased responsiveness to α -pheromone. This phenotype was at least partially a consequence of a reduction in the number of receptors, because overexpression of the mutant alleles on high-copy plasmids resulted in suppression. The reduced number of surface receptor sites could reflect either a faster rate of turnover or a slower rate of synthesis and delivery to the cell surface. The temperature-sensitive allele, *ste2-3*, which also affects this region (ala 52 to thr), blocks the delivery and accelerates turnover of the surface receptor sites at the nonpermissive temperature (Jennness, Kane, and Spatrick, unpublished result). Sequences adjacent to the hydrophobic domains of the β -adrenergic receptor have also been found to be important for stable receptor production (Dixon *et al.*, 1987a).

The *sst2-1* mutation caused a 30- to 100-fold

Table 4. Intragenic complementation of *STE2* alleles

<i>STE2</i> allele on plasmid	Mating efficiency in <i>ste2-3^{ts}</i> strain ^a				YE _p /YC _p ^c
	YC _p plasmid	Fold increase ^b	YE _p plasmid	Fold increase ^b	
<i>ste2Δ</i>	0.005		0.011		
<i>STE2</i>	100	20 000	100	9 091	0.46
<i>ste2-162</i>	.07	14	9.4	855	61.1
<i>ste2-1101</i>	4.7	940	10.5	955	1.0
<i>ste2-1169</i>	.007	1.4	.015	1.4	1.0
<i>ste2-1229</i>	.99	198	.97	88.2	0.45
<i>ste2-1261</i>	.005	1	.085	7.7	7.7
<i>ste2-3^{ts}</i>	4.1	820	101	9 182	11.2

^a Mating efficiency of *ste2-3^{ts}* strain 7419-2-3 carrying *STE2* genes on single-copy YC_p or multicopy YE_p plasmids at 34°C.

^b Fold increase in mating efficiency relative to the *ste2-3^{ts}* strain carrying the *ste2D* plasmid.

^c Ratio of the fold increase in mating as the result of multicopy YE_p or single-copy YC_p plasmid expression of *STE2* genes.

increase in the pheromone sensitivity of *STE2*⁺ strains but had a relatively minor influence on the sensitivity of the N-terminal mutants (<3-fold). In contrast, the sensitivity of *STE2*⁺ strains was unaffected by increased copy number of *STE2* (Konopka *et al.*, 1988), whereas additional copies of the N-terminal mutant alleles resulted in nearly full restoration of pheromone sensitivity. Thus, the pheromone sensitivity of the N-terminal mutants appears to be limited by their ability to produce receptors, whereas the sensitivity of the wild-type strains is limited by the activity of the *SST2* gene product. Strains that overproduced the N-terminal mutants displayed greater enhancement of pheromone sensitivity in the *sst2-1* strain (unpublished observation), indicating that the differential enhancement of pheromone sensitivity may be an effect of receptor number and not an intrinsic property of the N-terminal mutant receptors.

Central domain

The *ste2-162*, *ste-1101*, *ste2-1169*, *ste2-1229*, and *ste2-1261* alleles comprise a class of insertion mutants that exhibit no detectable mating or pheromone responsiveness. Each of the mutants affects a different transmembrane segment in the central domain. The severe phenotype indicates that the central domain is essential either for pheromone binding or for signal transduction. Our results suggest that the binding and signaling functions are affected differentially by these mutants.

Three of the central mutants retain some α -pheromone binding activity. Strains overpro-

ducing *ste2-162*, *ste2-1229*, and *ste2-1261* bound 15, 18, and 27%, respectively, of the amount of α -pheromone bound by a single copy *STE2* control strain, indicating that part of their defects may be attributed to reduced pheromone binding (Figure 4). However, the amount of pheromone bound is significant, because the half-maximal induction of agglutinin by a *STE2* strain occurred at a pheromone concentration corresponding to $\sim 10\%$ receptor occupancy (Figure 5). The lack of detectable pheromone response or mating for the *ste2-162*, *ste2-1229*, and *ste2-1261* strains indicates that they are defective in signal transduction. The behavior of these mutants also implicates their insertion sites (in or near hydrophobic segments 1, 5, and 6) as being important for signal transduction. Hydrophobic segments 5 and 6 and intracellular loop 3 are thought to be sites of G protein interaction for the β -adrenergic receptor (Kobilka *et al.*, 1988) and rhodopsin (Franke *et al.*, 1988). Consistent with the view that the central domain is important for signal transduction is the finding that this region is most highly homologous between the *STE2* genes of *S. cerevisiae* and *S. kluyveri* (Burkholder and Hartwell, 1985; Nakayama *et al.*, 1985; Marsh and Herskowitz, 1988). In particular, the subregion containing hydrophobic segments 5 and 6 and intracellular loop 3 contains the highest stretch of identity. However, sequence homology is apparently not necessary for functional homology, because the *STE3* gene (α -pheromone receptor) lacks extended stretches of identity with *STE2* even though they are thought to activate the same G protein signal transduction pathway (Bender and Sprague, 1986; Nakayama *et al.*, 1987).

Of the five central mutants, only *ste2-1101* and *ste2-1169* lacked detectable pheromone binding (Figure 4). These strains contain inserts near or at hydrophobic segments 2 and 4 and thereby implicate these segments in pheromone binding or in assembly of properly folded receptors on the cell surface. Both of the mutant receptors appear to enter the secretory pathway, because the carbohydrate-modified forms, p53 and p48, are produced (Figure 2). The *ste2-1169* mutant produces nearly wild-type levels of p53 and p48. The lack of pheromone binding indicates either that the binding site is defective or that the mature receptors are sequestered in a cellular compartment inaccessible to pheromone. The *ste2-1101* mutant, however, contains only minor amounts of p53 and p48; thus, it is possible that insufficient quantities of this mutant receptor may be present on the cell surface.

Suppression of mating defects by mutation *sst2-1* was detected only for the *ste2-1101* mutant and, to a much lesser degree, for the *ste2-1261* mutant. This result was surprising because no detectable receptor sites were present on the *ste2-1101* cells when compared with the other mutants. Even though the N-terminal mutants (*ste2-145*, *-147* and *-152*) showed a relatively high number of surface receptor sites, they were unable to sustain a pheromone response and they were not significantly suppressed by mutation *sst2-1*. Perhaps the *ste2-1101* mutant receptor can sustain a weak signal that is sufficient to promote mating in an *sst2-1* genetic background. A more sustained signal would conceivably result if the mutant receptor were no longer subject to one or more of the negative control mechanisms that operate on wild-type receptors. Such control mechanisms could include receptor downregulation (Jenness and Spatrick, 1986) or the adaptive response mediated by the C-terminal domain of the receptor (Konopka *et al.*, 1988; Reneke *et al.*, 1988).

The ability to complement *ste2-3* divides the central mutants into groups that are apparently unrelated to the severity of their mutant phenotypes. Three of the five central mutants (*ste2-162*, *-1101*, and *1229*) were able to complement partially the temperature-sensitive mating defect of a *ste2-3* mutant. The *ste2-3* mutation affects residue 52, which is in or near the first transmembrane-spanning segment (Jenness, Spatrick, and Kane, unpublished result); the mating efficiency of *ste2-3* mutants increases disproportionally with gene dosage, implying that the mutant receptors may compete with each other for degradation or that they may titrate a negative regulatory factor. The *ste2-162*

mutation also affects the first transmembrane segment. On a high-copy plasmid, this allele enhanced the mating of the *ste2-3* mutants, whereas on a single-copy plasmid no complementation was observed. This dosage-dependent effect suggests that the *ste2-162* product may also compete for destruction or inactivation of the *ste2-3* receptor. In contrast, the *ste2-1101* and *ste2-1229* alleles (affecting transmembrane segments 2 and 5, respectively) complemented the *ste2-3* strain by a mechanism that was independent of plasmid copy number. This observation implies a different mechanism for complementation; for example, the *ste2-1101* and *ste2-1229* mutant products may interact directly with the *ste2-3* product. Intramolecular interaction among transmembrane segments has been proposed for the β -adrenergic receptor (Frielle *et al.*, 1988; Kobilka *et al.*, 1988); perhaps intermolecular interaction can occur in the case of the α -pheromone receptor.

Methods

Strains and media

Yeast strains used in this study include PT2 (*MATa hom3 ilv1 can1*) (Shuster, 1982), X2180-1 (*MATa gal2*) (Hartwell, 1980), and EMS63 (*MATa his2*) (Hartwell, 1980). Congenic strains derived from strain 381G (Hartwell, 1980) were DJ213-6-3 (*ste2-10::LEU2 leu2 ura3 ade2-1 his4-580 lys2 trp1 tyr1 cry1 SUP4-3^{ts}*), DJ213-7-3 (*ste2-10::LEU2 bar1-1 leu2 ura3 ade2-1 his4-580 lys2 trp1 tyr1 cry1 SUP4-3^{ts}*), 7416-12-3 (*ste2-10::LEU2 sst2-1 leu2 ura3 ade2-1 his4-580 lys2 trp1 cry1 SUP4-3ts*), and 7419-2-3 (*ste2-3^{ts} leu2 ura3 ade2-1 his4-580 lys2 trp1 cry1 SUP4-3^{ts}*). Cells were grown in synthetic medium containing adenine, amino acids, and 0.1% casamino acids but lacking uracil to select for plasmid maintenance (Sherman *et al.*, 1979). Inhibitor medium was described previously (Jenness *et al.*, 1983).

Construction of STE2 mutants

Construction of *STE2* expression plasmids was previously described (Konopka *et al.*, 1988). A single-copy YCp plasmid derived from pDK263 (*CEN3 ARS1 URA3*) (Koshland *et al.*, 1985) carried the *STE2* gene (YCp-*STE2*; previously designated pJBK-008). The *STE2* gene was also cloned into multicopy plasmid YEp24 to create YEp-*STE2* (previously designated pJBK-036). Sixteen-base-pair *EcoRI* linker inserts into the *STE2* gene that created frameshift mutations were constructed by the sup-linker method of Lobel and Goff (1984). The linker sequence (CTG GCT GGA ATT CCA G) was inserted into blunt-end restriction sites within the coding sequence of the *STE2* gene that disrupted the indicated amino acid codons: *Hpa* I (codon 45), *Ssp* I (101 and 261), *EcoRV* (169), and *Alu* I (229, 326, and 416), as previously described (Konopka *et al.*, 1988). To convert the 16-bp linkers to 12 bp linkers (CTG GCT GGC CAG), we used *EcoRI* to digest the linker sequence in the plasmid and then mung bean nuclease to digest the 4-bp single-stranded overhang remaining after *EcoRI* digestion. Re-ligation of the plasmid

creates a *Bal* I restriction site in the 12-bp linker sequence; this site was used to assess whether the clones isolated contained the predicted linker sequence at the expected position in the plasmid. Standard recombinant DNA procedures were utilized and restriction enzymes (New England Biolabs, Beverly, MA) and mung bean nuclease (PL Biochemicals, Piscataway, NJ) were used according to manufacturers' instructions.

Pheromone-induced responses

Wild-type or mutant *STE2* plasmids were introduced into the appropriate *MATa* strain by the lithium acetate transformation method (Ito *et al.*, 1983). Agglutination index was determined spectrophotometrically, as described previously (Hartwell, 1980). *MATa* cells were induced with synthetic α -pheromone (Sigma Chemical, St. Louis, MO) and agglutinated with *MATa* strain EMS63, which was induced with α -pheromone supernatant from X2180-1A. α -pheromone-induced cell division arrest was quantitated by determining the fraction of unbudded cells in a liquid culture, as previously described (Moore, 1983), or by a halo assay on agar plates. Halo assays were performed by adding 25 μ l α -pheromone to a sterile filter disk (Difco, Detroit, MI) which was then placed on a synthetic medium agar plate that had been spread with a lawn of 10^5 cells. After incubation for 48 h at 23°C, halo sizes were determined by measuring the distance from the edge of the filter disk to the edge of the zone of growth inhibition. Values reported represent the average of two independent experiments. Projection formation was determined microscopically after incubating *MATa* strains in the presence of α -pheromone as previously described (Moore, 1983). The mating efficiency of *MATa* strains with *MATa* strain PT2 was determined by a filter mating assay as previously described (Hartwell, 1980).

α -pheromone binding and receptor internalization assays

α -pheromone binding capacity was determined as previously described (Jenness and Spatrick, 1986), except that the cells were cultured in minimal medium plus 0.1% casamino acids instead of YM-1. Cells were grown overnight (22°C) to a final density between 10^6 and 10^7 cells/ml, diluted to 5×10^6 cells/ml, and then incubated with 2×10^{-8} M [3 H]- α -factor (13.2 Ci/mMole) in inhibitor medium. The control reaction contained 2×10^{-6} M cold α -pheromone. After 30 min (25°C), 90 μ l were diluted with 2 ml inhibitor medium, cells were collected on a premoistened 2.5-cm Whatman (Hillsboro, OR) GF/C filter, and the filter was rinsed twice with 2 ml inhibitor medium. The rinsing time was 0.4 min. Filters were dried and counted in a liquid scintillation counter. The specific binding was calculated as the CPM bound to the experimental filter minus the CPM bound to the control filter. The [3 H]- α -pheromone was described elsewhere (Jenness *et al.*, 1986). The [3 H]- α -pheromone concentration refers to the 33% of radioactive peptides that were able to bind to *MATa* cells.

Immunoblotting

Antiserum to the 60 C-terminal amino acids of *STE2* was raised in rabbits immunized with bacterially expressed *trpE-STE2* fusion protein as previously described (Konopka *et al.*, 1988). In preparation for immunoblotting, $\sim 10^9$ log phase cells were harvested, washed once in water, and then resuspended in 0.5 ml 10 mM tris(hydroxymethyl)aminomethane (Tris) 1 mM EDTA, pH 7.5. Cells were disrupted by vortexing in the presence of glass beads for 5 min at 4°C and then extracts were centrifuged ($10\,000 \times g$) for 30 min.

The pellet was extracted with 0.1 ml of 1% deoxycholate, 10 mM Tris, pH 7.5, for 30 min on ice. After centrifugation the solubilized material was mixed with an equal volume of sodium dodecyl sulfate (SDS)-gel sample buffer containing 8 M urea and resolved by electrophoresis on a 9.5% SDS-polyacrylamide gel (Laemmli, 1970). Samples were then electrophoretically transferred to nitrocellulose and probed with *STE2* antisera diluted into a solution of 1% nonfat powdered milk (Carnation, Los Angeles, CA) in 10 mM Tris, 250 mM NaCl, pH 7.5. The *STE2* bands were then detected by the use of horseradish peroxidase-conjugated goat anti-rabbit IgG according to the manufacturer's directions (BioRad, Richmond, CA).

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