

# Constitutive mutants of the protein kinase STE11 activate the yeast pheromone response pathway in the absence of the G protein

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**STE4** encodes the  $\beta$ -subunit of a heterotrimeric guanine nucleotide-binding protein (G protein) that is an early and essential component of the pheromone signal transduction pathway. From a *ste4* deletion strain we have isolated both dominant and recessive suppressors that show increased transcription of pheromone responsive genes and have regained the ability to mate, albeit at a low level. Each of these suppressor mutations suppresses *ste4* and *ste5* deletions but not deletions in *STE7*, *STE11*, or *STE12*. Among the dominant mutations, we have identified two alleles of *STE11*, a gene that encodes a protein kinase activity essential for mating. One allele contains an alteration in the putative regulatory domain of the protein kinase; the second allele has an alteration in the catalytic site. In strains carrying these mutations, a second protein kinase required for mating, *STE7*, becomes hyperphosphorylated, just as it does in wild-type cells treated with pheromone. Thus, a protein kinase cascade appears to be an essential feature of the response pathway and probably connects the receptor/G protein to an identified transcription factor, *STE12*.

[Key Words: STE11 protein kinase; yeast pheromone response; signal transduction]

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The response to extracellular signals is an essential element in the control of the growth and differentiation of all living cells. In haploid cells of the yeast *Saccharomyces cerevisiae*, the differentiation pathway from vegetative growth to the sexual cycle is controlled by the mutual exchange of extracellular, diffusible peptide pheromones: Cells of mating type  $\alpha$  produce  $\alpha$ -factor and respond to *a*-factor, and cells of mating type *a* produce *a*-factor and respond to  $\alpha$ -factor. Exposure of cells to the opposite mating pheromone generates an intracellular signal that leads to a variety of physiological changes that prepare the cell for mating. This suite of changes includes transcription induction of genes required for cell and nuclear fusion, arrest of the mitotic cell cycle in the  $G_1$  phase, and emergence of a projection from the cell body (shmoo formation). These last two changes also result, at least in part, from altered transcription of appropriate genes. Thus, to a first approximation, the pheromone response pathway is a pathway in which the pattern of transcription is altered (for review, see Cross et al. 1988; Marsh et al. 1991).

Components or regulators of the signal transduction pathway have been identified by mutations that abolish

pheromone response or, in a few cases, by mutations that lead to constitutive signaling even in the absence of pheromone. Genetic and physiological experiments with these mutant strains have led to the following picture of the response pathway. Detection of pheromone is mediated by specific cell-surface receptors, encoded by *STE3* in  $\alpha$  cells and by *STE2* in *a* cells, that belong to the seven-transmembrane receptor family (Jenness et al. 1983; Burkholder and Hartwell 1985; Nakayama et al. 1985; Hagen et al. 1986). These receptors couple to a heterotrimeric G protein (Dietzel and Kurjan 1987; Miyajima et al. 1987; Nakafuku et al. 1987; Jahng et al. 1988; Whiteway et al. 1989; Blumer and Thorner 1990) and thereby activate an intracellular pathway that is common to *a* and  $\alpha$  cells (Bender and Sprague 1986; Nakayama et al. 1987). Binding of pheromone to receptor is believed to lead to the exchange of GTP for GDP on the  $G_\alpha$  subunit and to the attendant release of  $G_{\beta\gamma}$ . In contrast to most mammalian cells and to fission yeast (Obara et al. 1991), in *S. cerevisiae*, free  $G_{\beta\gamma}$  propagates the signal to downstream targets. This conclusion follows from the observation that deletion of  $G_\alpha$  [encoded by *GPA1* (*SCG1*)] or overexpression of  $G_\beta$  (encoded by *STE4*) activates the pathway in the absence of pheromone (Dietzel and Kurjan 1987; Miyajima et al. 1987; Jahng et al. 1988; Cole et al. 1990; Nomoto et al. 1990;

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Whiteway et al. 1990). Although the immediate target of  $G_{\beta\gamma}$  is not known, a terminal target is STE12, a DNA-binding protein that binds to the promoter regions of inducible genes (Dolan et al. 1989; Errede and Ammerer 1989). These genes include *FUS1* and *KAR3*, required for cell and nuclear fusion (McCaffrey et al. 1987; Trueheart et al. 1987; Meluh and Rose 1990), and *FUS3* and *FAR1*, required for  $G_1$  arrest (Chang and Herskowitz 1990; Elion et al. 1990). STE12, therefore, plays a pivotal role in the execution of the pheromone-induced changes in cellular physiology.

Although there is a fairly concrete view of the events that occur at the cell surface and in the nucleus, how the signal is transmitted between these two sites is not known. Five genes that may be involved in transmitting the signal have been identified by the isolation of non-responsive mutants. Four of these genes—*STE7*, *STE11*, *FUS3*, and *KSS1*—are predicted to encode protein kinases (Teague et al. 1986; Courchesne et al. 1989; Elion et al. 1990; Rhodes et al. 1990). In vitro kinase activity has been demonstrated for the STE11 protein (Rhodes et al. 1990). The role of the fifth gene, *STE5*, is elusive. Because loss-of-function mutations in these genes block the phenotype associated with the absence of  $G_{\alpha}$  or over-expression of  $G_{\beta}$ , it has been inferred that these genes control steps subsequent to the G protein. Other interpretations are possible, however. For example, the *STE* gene products might be required to make the G protein competent to transmit a signal. The relationship between gene products thought to participate in the same process is rigorously established only by examining the phenotype of reciprocal double mutants involving gain-of-function and loss-of-function alleles for both genes. Nevertheless, in keeping with the possibility that at least one protein kinase functions at a post-G protein step in signal transmission, STE12 has been shown to be phosphorylated rapidly after pheromone treatment, and the degree of its phosphorylation correlates with its transcriptional activity (Song et al. 1991).

To gain insight into how the pheromone-generated signal is propagated from  $G_{\beta\gamma}$  to STE12, we have isolated suppressors that restore signal propagation in a strain lacking  $G_{\beta}$  as a result of a deletion of the structural gene (*STE4*). We reasoned that mutations that activate the pathway downstream of the G protein would include dominant mutations in known *STE* genes or would identify new components in the signaling cascade. Here, we report the isolation of dominant *STE11* alleles that appear to encode hyperactive or unregulated forms of the protein kinase. Strains harboring these mutations exhibit a high level of expression of pheromone-inducible genes, exhibit morphological alterations characteristic of pheromone response, and have regained some capacity to mate. Strikingly, these mutants also exhibit hyperphosphorylation of the STE7 protein kinase, a biochemical alteration seen in wild-type cells after pheromone treatment (Z.-Q. Zhou and B. Errede, unpubl.). Thus, these genetic and biochemical data suggest that STE11 phosphorylates a protein kinase and that a kinase cascade is an integral part of the pheromone response pathway.

## Results

### *Isolation of ste4Δ suppressors that restore mating*

The *FUS1* gene is transcriptionally regulated by pheromone and by the products of the *STE4*, *STE5*, *STE7*, *STE11*, and *STE12* genes. In a wild-type *STE+* strain, *FUS1* exhibits a low basal level of transcription that is increased 10-fold or more by treatment of the cell with pheromone. In addition, *FUS1* transcription is undetectable in strains harboring null mutations in any of these five *STE* genes, implying that the basal transcription results from endogenous activity of the pathway in the absence of pheromone stimulation (McCaffrey et al. 1987; Hagen et al. 1991). We exploited these properties of the *FUS1* promoter to select suppressors of a deletion in the *STE4* gene (*ste4Δ::LEU2*). A fragment that includes the *FUS1* upstream activation sequence was joined to the *HIS3* open reading frame to create a hybrid gene (*FUS1::HIS3*), which was integrated at the *FUS1* locus by one-step gene replacement (see Materials and methods). Transcription of *FUS1::HIS3* was inducible by pheromone and dependent on the *STE* genes (data not shown). Thus, *his3Δ STE+* cells containing *FUS1::HIS3* grew without the addition of exogenous histidine, whereas *his3Δ ste4Δ::LEU2* cells containing *FUS1::HIS3* did not (Table 1).

We used the *his3Δ ste4Δ::LEU2 FUS1::HIS3* strain (SY1436) and selected mutants that could grow in the absence of histidine (see Materials and methods). To identify suppressor mutations that led to activation of the pathway, rather than *cis*-acting mutations that affected only the expression of the *FUS1::HIS3* construct, the initial collection of His<sup>+</sup> mutants was screened for suppression of two other *ste4Δ* phenotypes. First, *ste4Δ* mutants secrete much less pheromone than wild-type strains do because an intact pathway is required for efficient transcription of the pheromone structural genes (Fields and Herskowitz 1985; Fields et al. 1988). Second, *ste4Δ* mutants cannot mate. Nine suppressors that secreted substantial amounts of  $\alpha$ -factor (Fig. 1) and had regained some degree of mating ability were isolated by selection and subsequent screening of  $\sim 10^8$  cells.

To obtain a quantitative measure of the degree of suppression conferred by these mutations, three assays were performed. First, the level of expression of *FUS1::HIS3* was assessed by determining the level of resistance that the mutants exhibited to aminotriazole (AT), a competitive inhibitor of the HIS3 enzyme (Klopotoski and Wiater 1965; Struhl and Davis 1977). All suppressors were at least as resistant to AT as the isogenic *STE+* strain, and four were able to grow in the presence of 40 mM AT, the highest concentration tested (Table 1). A second measure of transcriptional activity, expression of a *FUS1-lacZ* reporter plasmid, revealed that all mutants expressed substantial levels of  $\beta$ -galactosidase, comparable to or greater than an isogenic *STE+* strain (Table 1). Four of the mutants expressed higher levels of  $\beta$ -galactosidase than the wild-type strain did after treatment with pheromone. Finally, by a quantitative assay, the suppressor mutants mated one to four orders of magni-

**Table 1.** Transcription and mating phenotypes of the *ste4Δ* suppressors

Strain <sup>a</sup>	Dominance <sup>b</sup>	AT resistance (mm) <sup>c</sup>	<i>FUS1-lacZ</i> expression <sup>d</sup>	Mating efficiency <sup>e</sup>
<i>ste4Δ</i>		His <sup>-</sup>	0.3	$<5 \times 10^{-8}$
1	D	40	240	$9.3 \times 10^{-5}$
4	D	40	204	$2.7 \times 10^{-4}$
12	D	10	6.9	$3.1 \times 10^{-6}$
13	D	10	22.3	$5.3 \times 10^{-6}$
14	D	1	6.3	$8.6 \times 10^{-6}$
25	R	2.5	58.3	$1.3 \times 10^{-6}$
26	D	40	247	$5.1 \times 10^{-4}$
27	R	10	147	$4.0 \times 10^{-5}$
29	D	40	11.3	$6.7 \times 10^{-5}$
<i>STE</i> <sup>+</sup>		1	13.9	0.81
<i>STE</i> <sup>+</sup> + <i>a</i> -factor		ND <sup>f</sup>	104	ND <sup>f</sup>

<sup>a</sup>All strains are isogenic to SY1390 (*MATα STE<sup>+</sup> FUS1::HIS3*) except as indicated. Strains containing suppressor mutations were derived from SY1436 or SY1491 (*MATα ste4Δ::LEU2 FUS1::HIS3*).

<sup>b</sup>Suppressor mutations were determined to be dominant (D) or recessive (R) with respect to wild type, as described in Materials and methods.

<sup>c</sup>Highest concentration of AT tested that allowed growth on SD–His plates.

<sup>d</sup>β-Galactosidase activity was determined as described in Materials and methods. The values reported are the average of assays of at least three independent transformants.

<sup>e</sup>Absolute mating efficiencies are presented, determined as described in Materials and methods.

<sup>f</sup>(ND) Not determined.

tude better than the parental *ste4Δ::LEU2* strain (Table 1).

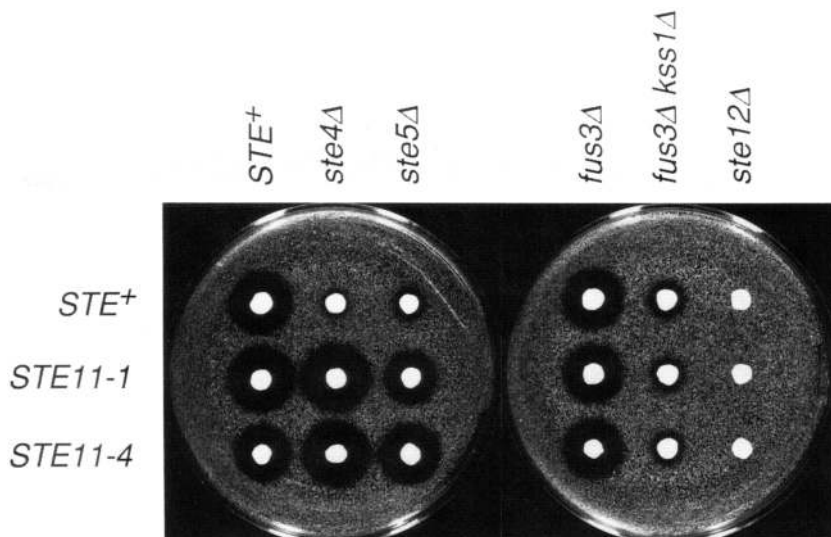
To test whether the suppressor mutations were dominant or recessive, *MATα/MATα* diploids that were heterozygous for the suppressor mutation were created by protoplast fusion. Seven of the suppressor mutations conferred growth in the absence of histidine and were therefore dominant. Two suppressors (25 and 27) were His<sup>-</sup> and therefore recessive.

#### Suppressors include dominant alleles of STE11

The mutants were isolated as suppressors of the transcription and mating defects caused by a deletion in the *STE4* gene. To discover whether the suppressors required

the activity of other response pathway genes, the *STE5*, *STE7*, *STE11*, or *STE12* gene was deleted in each suppressor mutant and the resultant strain was tested for its ability to grow without exogenous histidine. By this criterion, all nine mutants could suppress a deletion in *STE5* but not deletion of *STE7*, *STE11*, or *STE12* (Table 2).

To test the possibility that the suppressor mutations were alterations of *STE7*, *STE11*, or *STE12*, we used gene disruption cassettes to knock out each of the candidate *STE* genes, reasoning that a dominant allele would be destroyed by a gene disruption. In particular, we used the *ste7*, *ste11*, and *ste12* deletion strains constructed above and assessed the His phenotype following introduction of a plasmid-borne version of the relevant wild-type *STE*



**Figure 1.** α-factor production in suppressor strains. *STE*<sup>+</sup> and suppressor strains (designated *STE11-1* and *STE11-4*, as explained later in the text) were spotted onto a lawn of supersensitive XMB4-12b (*MATa sst1*) cells. Where indicated, the strains carried *ste4*, *ste5*, *fus3*, *fus3* and *kss1*, or *ste12* mutations. The plates were incubated at room temperature for 24 hr. The diameter of the zone of growth inhibition (halo) surrounding the spotted strains reflects the amount of α-factor secreted.

**Table 2.** Genetic requirements for suppressor activity

Suppressor strain	<i>ste7Δ</i>							
	<i>ste5Δ</i>	<i>ste7Δ</i>		<i>ste11Δ</i>		<i>ste12Δ</i>		
		-pSTE7	+pSTE7	-pSTE11	+pSTE11	-pSTE12	+pSTE12	
1	+	-	+	-	-	-	+	
4	+	-	+	-	-	-	+	
12	+	-	+	-	-	-	+	
13	+	-	+	-	-	-	+	
14	+	-	+	-	-	-	+	
25	+	-	+	-	+	-	+	
26	+	-	+	-	-	-	+	
27	+	-	+	-	+	-	+	
29	+	-	+	-	-	-	+	

All strains are isogenic to SY1491 (*MATα ste4Δ::LEU2 FUS1::HIS3*) except as indicated. (+) His<sup>+</sup> phenotype; (-) His<sup>-</sup> phenotype.

gene (see Materials and methods). If the suppressor mutation was ablated by a *ste* gene disruption, then the plasmid-borne *STE* gene would not restore the His<sup>+</sup> phenotype to the *ste4Δ::LEU2* suppressor strain. Conversely, if the suppressor mutation was not affected by the disruption, then the plasmid-borne *STE* gene would restore the original phenotype. By this criterion, the two recessive mutations (25 and 27) were not alleles of *STE7*, *STE11*, or *STE12* (Table 2). On the other hand, all seven of the dominant mutations appeared to be *STE11* alleles. This possibility was confirmed by rescuing the *STE11* allele from each of the mutants by gap repair (Rothstein 1991; see Materials and methods). Upon introduction into a naive *ste4::LEU2* strain, the repaired plasmids isolated from these seven mutants (1, 4, 12, 13, 14, 26, and 29) conferred the original suppressor phenotype: The cells were His<sup>+</sup> and produced high levels of pheromone.

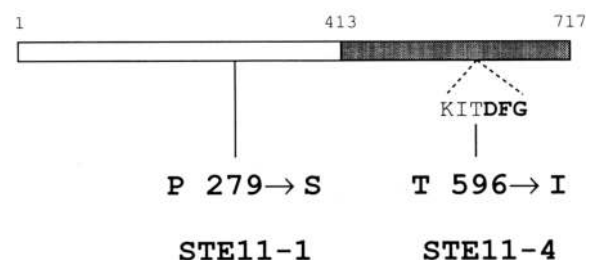
Because the segment of DNA cloned by gap repair corresponded closely to the *STE11* locus and because overexpression of wild-type *STE11* does not lead to activation of *FUS1::HIS3* (B. Stevenson and G. Sprague, unpubl.), we reasoned that the mutations were likely within the *STE11*-coding sequence. In fact, DNA sequence analysis of suppressor 1 revealed a C → T transition at nucleotide 950 [following the DNA sequence numbering of Rhodes et al. (1990)], which substitutes serine for proline at amino acid residue 279 (Fig. 2). This allele was designated *STE11-1*. The altered residue is within the large amino-terminal domain which, by analogy with other protein kinases, may have a regulatory function (Taylor et al. 1990). A transition mutation (C → T) at nucleotide position 1902 was present in the six remaining mutants, leading to the replacement of threonine 596 by isoleucine. This allele was designated *STE11-4*. The altered residue is adjacent to the invariant Asp-Phe-Gly (DFG) triplet in subdomain VII according to the classification of Hanks et al. (1988) and lies at the heart of the proposed kinase catalytic site.

*STE11-1 and STE11-4 exhibit similar, but distinct, phenotypes*

The variability of phenotypes exhibited by the original

isolates carrying the *STE11-4* mutation, and the possibility that secondary mutations within the strains could alter the suppressor phenotype, prompted us to re-examine both *STE11* alleles in defined isogenic backgrounds. In this isogenic setting, we investigated the effect of the *STE11* alleles in strains that could respond to pheromone (*STE4*<sup>+</sup>) or in strains that carried deletions in known components of the response pathway.

The wild-type *STE11* gene in strain SY1390 (relevant genotype: *MATα STE<sup>+</sup> FUS1::HIS3*) was replaced by either the *STE11-1* or the *STE11-4* allele by two-step gene replacement (see Materials and methods). As expected, both alleles conferred a His<sup>+</sup> phenotype and high basal expression of *FUS1-lacZ* in an otherwise wild-type genetic background (Table 3). However, by several criteria, the *STE11-4* allele conferred a stronger phenotype than the *STE11-1* allele. First, the expression of *FUS1-lacZ* was higher in *STE11-4* strains than in *STE11-1* strains. Second, although both alleles suppressed the mating defect caused by loss of the  $\alpha$ -factor receptor (*ste3Δ*) or of  $G_{\beta}$  (*ste4Δ*), strains carrying the *STE11-4* allele exhibited a mating efficiency about two orders of magnitude greater than *STE11-1* strains. Third, cells carrying the *STE11-4* allele in an otherwise wild-type background grew noticeably slower than either isogenic *STE11-1* or



**Figure 2.** Location of the amino acid substitutions caused by the *STE11-1* and *STE11-4* mutations. The long rectangle represents the *STE11* protein kinase; the region of protein kinase homology is shaded. Numbering of amino acids is from Rhodes et al. (1990). Conserved catalytic subdomain VII is shown, with invariant amino acid residues in boldface type (Hanks et al. 1988).

**Table 3.** Transcription and mating activities of STE11-1 and STE11-4 in various genetic backgrounds

Strain <sup>a</sup>	AT resistance (mM) <sup>b</sup>	<i>FUS1-lacZ</i> expression <sup>c</sup>	Mating efficiency <sup>d</sup>
<i>STE11-1</i>	40	233.8	0.90
<i>STE11-1 ste3Δ</i>	40	207.5	$1.3 \times 10^{-5}$
<i>STE11-1 ste4Δ</i>	40	160.4	$2.9 \times 10^{-5}$
<i>STE11-1 ste5Δ</i>	1	4.8	$<4 \times 10^{-8}$
<i>STE11-1 ste7Δ</i>	His <sup>-</sup>	0.1	$<7 \times 10^{-8}$
<i>STE11-1 ste12Δ</i>	His <sup>-</sup>	0.1	$<4 \times 10^{-8}$
<i>STE11-1 fus3Δ</i>	40	147.7	$1.7 \times 10^{-2}$
<i>STE11-1 fus3Δ kss1Δ</i>	His <sup>-</sup>	ND <sup>e</sup>	$<4 \times 10^{-8}$
<i>STE11-4</i>	40	341.9	0.93
<i>STE11-4 ste3Δ</i>	40	258.4	$7.1 \times 10^{-4}$
<i>STE11-4 ste4Δ</i>	40	300.6	$2.0 \times 10^{-3}$
<i>STE11-4 ste5Δ</i>	30	49.4	$<4 \times 10^{-8}$
<i>STE11-4 ste7Δ</i>	His <sup>-</sup>	0.1	$<9 \times 10^{-8}$
<i>STE11-4 ste12Δ</i>	His <sup>-</sup>	0.1	$<6 \times 10^{-8}$
<i>STE11-4 fus3Δ</i>	40	321.5	$2.2 \times 10^{-2}$
<i>STE11-4 fus3Δ kss1Δ</i>	His <sup>-</sup>	ND <sup>e</sup>	$<3 \times 10^{-8}$
<i>STE</i> <sup>+</sup>	1	17.4	0.81
<i>ste3Δ</i>	1	16.8	$<4 \times 10^{-8}$
<i>ste4Δ</i>	His <sup>-</sup>	0.3	$<5 \times 10^{-8}$
<i>ste5Δ</i>	His <sup>-</sup>	0.3	$<2 \times 10^{-8}$
<i>ste12Δ</i>	His <sup>-</sup>	0.1	$<5 \times 10^{-8}$
<i>STE</i> <sup>+</sup> <i>fus3Δ</i>	5	29.3	$1.0 \times 10^{-2}$
<i>STE</i> <sup>+</sup> <i>fus3Δ kss1Δ</i>	His <sup>-</sup>	ND <sup>e</sup>	$<3 \times 10^{-8}$

<sup>a</sup>All strains are isogenic to SY1390 (*MATα STE<sup>+</sup> FUS1::HIS3*) except as indicated.

<sup>b</sup>Highest concentration of AT tested that allowed growth on SD-His plates.

<sup>c</sup>β-Galactosidase activity was determined as described in Materials and methods. The values reported are the average of assays of at least three independent transformants.

<sup>d</sup>Absolute mating efficiencies are presented, determined as described in Materials and methods.

<sup>e</sup>(ND) Not determined.

*STE11* strains. Microscopic examination of exponential phase *STE11-4* cells revealed a significant proportion of large cells with projections or other aberrant morphologies, which were not present in the isogenic *STE11* culture (Fig. 3). *STE11-4 ste4Δ* strains also exhibited projection formation (Fig. 3), as did *STE11-4 ste3Δ* (data not shown), but *STE11-4 ste12Δ* strains exhibited wild-type morphology (Fig. 3). These findings are again consistent with the idea that the pathway is activated at a step downstream of *G<sub>β</sub>* but before *STE12*.

To complete the epistasis analysis in these isogenic strains, we constructed *ste5*, *ste7*, and *ste12* derivatives of the *STE11-1* and *STE11-4* mutants. Both alleles conferred a His<sup>+</sup> phenotype in *ste5Δ* strains but not in *ste7Δ* or *ste12Δ* strains (Table 3), confirming the results obtained with the original suppressor mutants. However, quantitative mating analysis and transcription assays revealed that *ste5Δ* was suppressed only partially (Table 3). Moreover, the growth and morphological defects of the *STE11-4* allele were not seen in the *ste5Δ* strain (data not shown). These results imply that the phenotype of the

*STE11* mutant strains is a composite of the signal generated by the mutant kinases and of a *STE5*-dependent signal.

Recently, two additional genes required for the activity of the response pathway have been identified. These genes, *FUS3* (Elion et al. 1990) and *KSS1* (Courchesne et al. 1989), are partially redundant in function and appear to encode related protein kinases. Deletion of *FUS3* alone has little effect on transcription induction but blocks cell cycle arrest in response to pheromone. Deletion of both genes blocks transcription induction and cell cycle arrest (Elion et al. 1991; G. Ammerer, A. Amon, and K. Nasmyth, pers. comm.). As shown in Table 3, deletion of both genes abolished both the transcription and mating phenotypes of the *STE11-1* and *STE11-4* strains, suggesting that *FUS3/KSS1* functions at a step after *STE11* in the pathway. Although these genetic data do not exclude more complicated relationships, biochemical data from Gartner et al. (this issue) support this simple relationship (see Discussion).

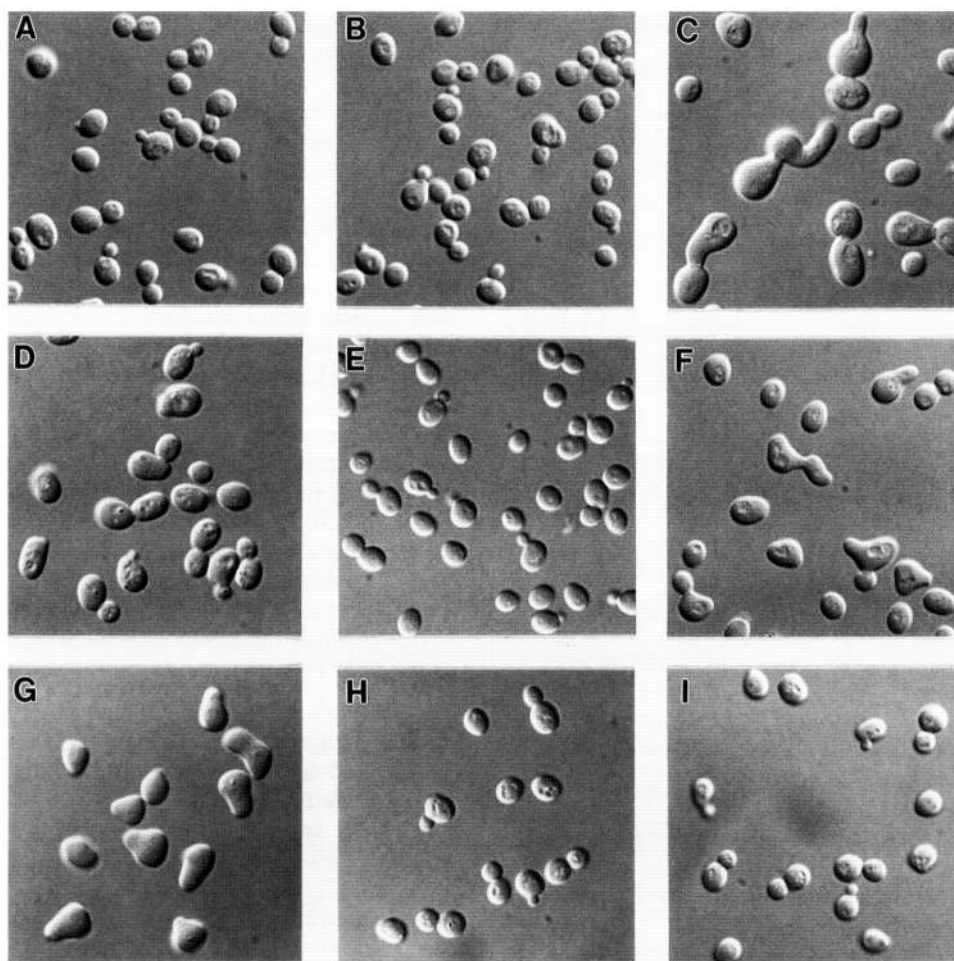
#### *STE11 controls the phosphorylation state of the STE7 protein kinase*

The *STE7* protein kinase is hyperphosphorylated in cells treated with pheromone (Z.-Q. Zhou and B. Errede, unpubl.). To determine whether *STE11* activity affects the phosphorylation state of *STE7*, we examined the *STE7* protein in strains expressing either the *STE11-1* or *STE11-4* allele. It is not possible to detect wild-type levels of *STE7*; hence, *STE7* was overproduced to permit immunodetection. Because overproduction of *STE7* does not influence pheromone response in otherwise wild-type cells, these experiments are likely to give an accurate representation of the post-translational changes in *STE7* during response. Treatment of wild-type cells with pheromone increased the intensity of the slower migrating forms of *STE7*, which are characteristic of hyperphosphorylation (Fig. 4, lane 3; Z.-Q. Zhou and B. Errede, unpubl.). In the absence of pheromone, expression of the *STE11-1* or *STE11-4* allele led to an increase in the proportion of slower migrating forms of *STE7* (Fig. 4, cf. lanes 7 and 8 with lane 4). This result suggested that *STE11* affects the phosphorylation state of *STE7* and reinforced the idea that *STE11* acts before *STE7* in the pathway.

The phenotypes conferred by the dominant *STE11* alleles require a functional *FUS3/KSS1* activity (Table 3). In concert with this physiological relationship between *STE11* and *FUS3/KSS1*, the hyperphosphorylated forms of *STE7* were depleted in *STE11-1* (or *STE11-4*) *fus3Δ kss1Δ* strains. The extent of *STE7* hyperphosphorylation was indistinguishable from that seen in an untreated *STE<sup>+</sup> fus3Δ kss1Δ* strain (Fig. 4, cf. lanes 10 and 11 with lane 9). These findings indicate that *FUS3/KSS1* activity is required for *STE7* modification by the constitutive *STE11* kinases.

#### Discussion

In the yeast pheromone response pathway, a signal is



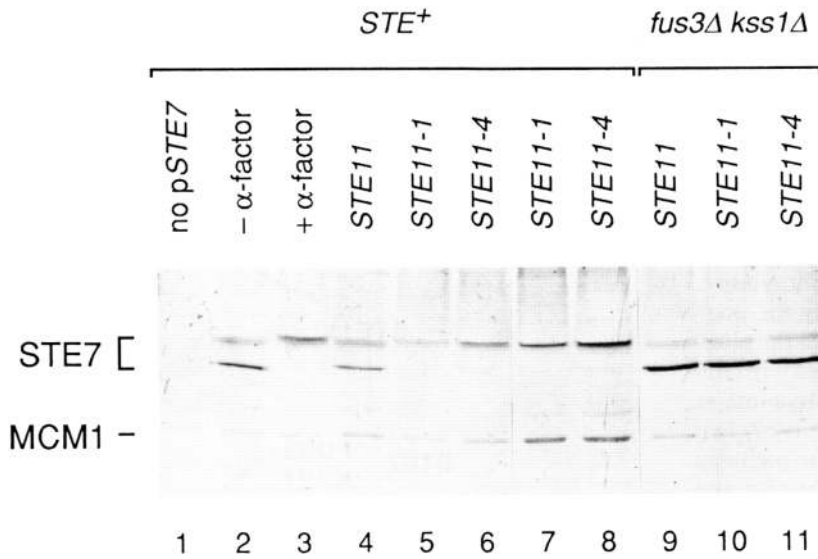
**Figure 3.** Cellular morphology of *STE11-1* and *STE11-4* strains. Isogenic strains were grown in rich medium to exponential phase. **A**, *STE*<sup>+</sup>; **D**, *ste4*Δ; **G**, *STE*<sup>+</sup> after treatment with *a*-factor; **B**, *STE11-1*; **E**, *STE11-1 ste4*Δ; **H**, *STE11-1 ste12*Δ; **C**, *STE11-4*; **F**, *STE11-4 ste4*Δ; **I**, *STE11-4 ste12*Δ. Cells were fixed and photographed as described in Materials and methods.

transmitted from the cell surface to the nucleus, where a change in transcription of select genes occurs, leading ultimately to the characteristic physiological response. The events that take place at the cell surface and in the nucleus have been well studied, but the nature and order of events that constitute the signal that connects these two locales are poorly understood. To gain insight into this central portion of the pathway, we identified mutants with a constitutively active pathway. A number of these mutants harbor dominant mutations in *STE11*, which encodes a protein kinase required for activity of the pathway. Below, we discuss the implications of these findings for the organization of the pathway and for the regulation of kinase activity.

#### *Organization of the pheromone response pathway*

The majority of components that participate in the pheromone response pathway have been identified by the isolation of mutants with a nonresponsive phenotype (MacKay and Manney 1974; Hartwell 1980). However,

because the mutants exhibit an identical phenotype, the functional relationships among the gene products are largely unknown. Prior to this work, the relative positions of two components had been established by genetic analyses. First, the G protein acts at an early, but post-receptor, step in the pathway. This conclusion came from the analysis of strains in which perturbations in the activity or quantity of *STE4* ( $G_{\beta}$ ) led to constitutive activation of the pathway (Blinder et al. 1989; Cole et al. 1990; Whiteway et al. 1990; Elion et al. 1991). The constitutive phenotype allowed strains lacking receptors to mate, but the phenotype was blocked by loss-of-function mutations in *STE5*, *STE7*, *STE11*, *STE12*, and *FUS3/KSS1*. These results place the G protein after the receptors, but its relationship to the *STE* and *FUS3/KSS1* products is uncertain. These products may function after the G protein, or they may be required for its activity. Second, a transcription factor, *STE12*, required for the transcription of genes whose products execute response, was placed at the end of the pathway. This placement follows from the finding that overexpression of *STE12*



**Figure 4.** Post-translational modification of the STE7 protein kinase. STE7 protein was detected in protein extracts by western analysis as described in Materials and methods. STE7 exists in two forms; the slower migrating species is hyperphosphorylated (Z.-Q. Zhou and B. Errede, unpubl.). The level of MCM1 protein served as a control for the amount of protein extract loaded in each lane. (Lane 1) *STE*<sup>+</sup> no pN279; (lane 2) *MATa STE*<sup>+</sup>; (lane 3) *MATa STE*<sup>+</sup> treated with 5 mM  $\alpha$ -factor for 3 hr (lane 4) *STE*<sup>+</sup>; (lanes 5,7) *STE11-1*; (lanes 6,8) *STE11-4*; (lane 9) *STE*<sup>+</sup> *fus3-6::LEU2 kss1Δ::URA3*; (lane 10) *STE11-1 fus3-6::LEU2 kss1Δ::URA3*; (lane 11) *STE11-4 fus3-6::LEU2 kss1Δ::URA3*. All strains are *MAT $\alpha$*  and contained pN279 (*CYC1-STE7*), unless otherwise stated. Over-expression of STE7 in *STE11-1* and *STE11-4* strains that are otherwise wild type for the signal transduction pathway leads to a growth defect. Because plasmid loss provides a means

to overcome the detrimental effect of STE7, only ~30% of the cells in cultures of these strains contain the STE7 plasmid, compared with ~80% of the cells in cultures of the other strains (data not shown). Consequently, there is less STE7 protein in the extracts loaded in lanes 5 and 6 than in other lanes. To facilitate comparison with other lanes, three times more protein from these extracts was loaded in lanes 7 and 8.

led to a constitutive phenotype even in strains lacking the *STE* and *FUS3/KSS1* products (Dolan and Fields 1990; Elion et al. 1991).

The isolation of *STE11* alleles that confer a constitutive phenotype has allowed us to infer the relationships among additional pathway components. First, we conclude that STE11 functions after STE4. The dominant *STE11* alleles suppress all defects associated with loss of STE4 activity. Second, we conclude that STE11 functions after at least some STE5 activities because the dominant *STE11* mutations suppress the lack of transcription of *FUS1* and other genes seen in *ste5Δ* strains. However, the *STE11* alleles do not suppress the mating defect of *ste5Δ* strains. Two models can be proposed to explain the quantitative difference in suppression of *ste4Δ* and *ste5Δ*. The simpler model supposes that STE4, STE5, and STE11 exhibit a linear relationship and supposes further that STE5 is required for optimal activity of the STE11 kinase. The second model supposes that the pheromone response pathway branches at the STE4 or STE5 step, creating two independent signals, one of which operates through STE11. Together, these signals influence the activity of STE12 and perhaps other proteins involved in mating.

Finally, we conclude that STE11 acts before STE7 and *FUS3/KSS1*. The genetic data establish that STE7 and *FUS3/KSS1* are required for the *STE11* mutant phenotype but do not establish their relationship in the pathway. However, biochemical analysis of wild-type and mutant strains reveals that STE11 influences the phosphorylation state and presumably the activity of STE7 and *FUS3/KSS1*. When wild-type cells are treated with pheromone, STE7 protein is phosphorylated rapidly in a STE11-dependent manner. The increased phosphoryla-

tion of STE7 apparently increases its kinase activity (Z.-Q. Zhou and B. Errede, unpubl.), although the possibility that the phosphorylation represents an adaptive response to pheromone has not yet been excluded. Hyperphosphorylation of STE7 is also observed in the *STE11-1* and *STE11-4* constitutive mutants, even in the absence of added pheromone. In a parallel effort, Gartner et al. (this issue) have shown that *FUS3* rapidly becomes phosphorylated after pheromone treatment. As is thought to be the case for STE7, the phosphorylated form of *FUS3* appears to be the active kinase. Phosphorylation of *FUS3* requires STE11 function and occurs in *STE11-1* and *STE11-4* strains even in the absence of pheromone. These data therefore place STE7 and *FUS3* after STE11 in the pheromone response pathway.

What is the relationship between STE7 and *FUS3*? In this case, evidence suggests an interdependent rather than linear relationship. Phosphorylation of STE7, either on pheromone stimulation or in the *STE11* mutant strains, requires *FUS3/KSS1* (this work; Zhou and Errede 1992), and likewise, phosphorylation of *FUS3* under either condition requires STE7 (Gartner et al., this issue). Thus, we suggest that STE7 and *FUS3/KSS1* require each other to become active. Perhaps they carry out mutual phosphorylation or form a complex. This model rationalizes the properties of a presumptive gain-of-function mutation in *FUS3*. The phenotype conferred by this allele is abolished by deletion of *STE7* or *STE11* (J. Brill, E. Elion, and G. Fink, pers. comm.), not because STE7 and STE11 function after *FUS3* but because STE11 and STE7 must operate to generate phosphorylated *FUS3*, which appears to be the active form of the protein.

The genetic requirements for phosphorylation of STE7 and *FUS3* are the same whether mediated by pheromone

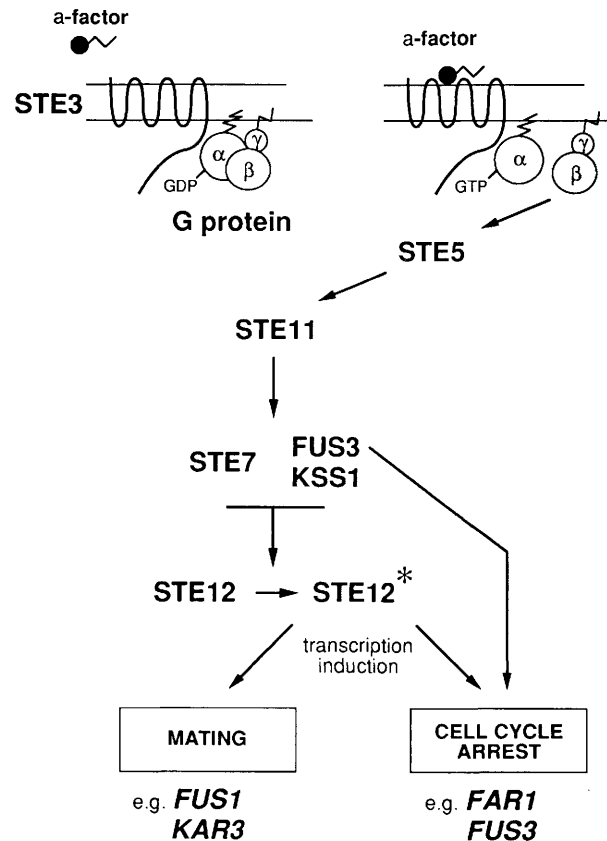
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or by *STE11* mutation, implying that the two conditions are equivalent mechanistically. Thus, we suggest that in the normal course of pheromone response the activity of *STE11* increases, which leads to increased phosphorylation of *STE7* and *FUS3* and an attendant increase in the activity of those kinases. Whether *STE11* acts directly on *STE7* and *FUS3* or acts through an intermediary protein or proteins is not known. Targets of *STE7* and *FUS3* may include *STE12*, which is believed to exhibit increased ability to activate transcription on phosphorylation (Song et al. 1991). Our results therefore imply the presence of a protein kinase cascade at the center of the pheromone response pathway. This insight, coupled with the results of others (Dietzel and Kurjan 1987; Miyajima et al. 1987; Nakayama et al. 1988; Blinder et al. 1989; Dolan and Fields 1990), suggests that most of the pathway components function in a linear sequence. Thus far, the only exception is the relationship between *STE7* and *FUS3*. As discussed above, there may be a branchpoint at the *STE5* step, but in the absence of direct evidence for this possibility, we will assume the simpler, linear relationship (Fig. 5).

#### Regulation of the kinase activity of *STE11*

Protein kinases typically consist of a catalytic domain and a regulatory domain, which is either part of the same polypeptide or is present on a second subunit (Taylor et al. 1990). The protein kinase encoded by *STE11* is a large protein comprised of 717 residues. The kinase catalytic domain occupies the carboxy-terminal half of the protein (Fig. 3; Rhodes et al. 1990). The function of the large amino-terminal portion of the protein is unknown, but by analogy with other protein kinases this domain may have a role in regulating the activity of the catalytic domain. The position and nature of the amino acid substitutions in the constitutive *STE11* mutants suggest how the activity of *STE11* may be regulated.

The *STE11-1* mutant protein has serine substituted for proline in the middle of the amino-terminal putative regulatory domain. Although in principle this domain could have either a positive or negative regulatory role, we favor the latter possibility for several reasons. In this view, the negative regulatory function has been inactivated by the amino acid substitution, which leads to increased activity of the catalytic domain as revealed by phosphorylation of *STE7*. Consistent with this proposed negative role is the finding that overproduction of the amino-terminal domain of *STE11* makes cells resistant to  $\alpha$ -factor-induced cell-cycle arrest (N. Rhodes and B. Errede, unpubl.). A similar result has been obtained for *byr2*, a putative protein kinase from *Schizosaccharomyces pombe* (Wang et al. 1991), which is 42% identical to *STE11* (B. Stevenson, unpubl.) and is required for mating and sporulation by that organism. Overexpression of the *byr2* amino terminus reduces sporulation, indicative of a reduction in signal strength in that pathway (Wang et al. 1991). Finally, the amino-terminal segments of other serine/threonine kinases, for example, Raf-1 (Stanton 1989), are known to have negative regulatory roles.



**Figure 5.** A model for the pheromone response pathway. This schematic incorporates results from this work and elsewhere. In  $\alpha$  cells, the binding of  $\alpha$ -factor to the receptor encoded by *STE3* (or  $\alpha$ -factor binding to *STE2* in  $\alpha$  cells) results in the exchange of GTP for GDP on the  $\alpha$ -subunit of the G protein [encoded by *GPA1* (*SCG1*)], and its dissociation from the  $\beta$  and  $\gamma$  subunits (encoded by *STE4* and *STE18*).  $G_{\beta\gamma}$  transmits the signal through *STE5* and the protein kinases, causing a change in the modification state of the transcription factor *STE12*. *STE12\** represents the phosphorylated form that appears to have transcriptional activity. As discussed in the text, *STE7* and *FUS3/KSS1* may have an interdependent relationship. Examples of inducible genes involved in mating and cell cycle arrest are given. *FUS1* is required for cell fusion and *KAR3* for nuclear fusion. *FAR1* and *FUS3* cause cell cycle arrest by affecting  $G_1$  cyclin accumulation. Pheromone-mediated post-translational modification of *FAR1* and *FUS3*, in addition to their increased abundance as a result of increased transcription, is likely essential to achieve cell cycle arrest.

The *STE11-4* mutant protein has isoleucine substituted for threonine in the catalytic domain, adjacent to the invariant Asp-Phe-Gly triplet, which is thought to be involved in ATP binding at the catalytic site (Hanks et al. 1988; Knighton et al. 1991). An intriguing possibility is that the threonine residue is a site of negative regulation by phosphorylation, which would interfere with ATP binding. Substitution by isoleucine would remove this regulatory site, resulting in a constitutively active kinase. It is noteworthy that the analogous position in *byr2* is a serine residue (Wang et al. 1991). Another pos-



sibility is that the substitution enables STE11 to interact more efficiently with its substrate. In this view, the properties of the mutant kinase do not result from loss of a negative regulatory mechanism but from a gain in intrinsic activity.

#### Implications for signal transduction in other organisms

Recent evidence indicates that the pheromone response pathway as deduced for the budding yeast *S. cerevisiae* is highly conserved in the fission yeast *S. pombe*. Pheromone receptors homologous to those of budding yeast have been identified for fission yeast (Kitamura and Shimoda 1991; M. Yamamoto, pers. comm.), and those receptors are coupled to G proteins (Obara et al. 1991). Equally noteworthy, two putative protein kinases, *byr1* and *byr2*, which are homologous to STE7 and STE11, are essential components of the pathway (Nadin-Davis and Nasim 1988; Wang et al. 1991; A. Neiman and I. Herskowitz, pers. comm.; B. Stevenson, unpubl.). Moreover, genetic analysis reveals that *byr2* and *byr1* function in the same order as do their budding yeast counterparts. Recently, DNA sequences that can encode a protein with homology to STE7 have been isolated from *Drosophila melanogaster* (A. Neiman, pers. comm.). As yet, no close relatives to STE11, other than *byr2*, have been identified in other species. However, given that *S. cerevisiae* and *S. pombe* have very similar signal transduction pathways despite the great evolutionary distance that separates them, it will be interesting to learn whether STE11 and STE7 homologs are key components in signal transduction processes in multicellular organisms.

## Materials and methods

### Strains, media, and microbiological techniques

The majority of yeast strains used in this work are isogenic with SY1390 (*MAT $\alpha$  STE<sup>+</sup> FUS1::HIS3 leu2 ura3 trp1 his3 $\Delta$ 200::ura3 pep4 $\Delta$ ::ura3 can1*), which was derived from 246-1-1 (*MAT $\alpha$  leu2 ura3 trp1 his4-519 can1*, provided by K. Tatchell) by one-step gene replacement at the relevant loci (Rothstein 1991). This strain contains the *FUS1::HIS3* gene fusion at the *FUS1* locus and is phenotypically *Fus1<sup>-</sup>*. A *ste4 $\Delta$ ::LEU2* version of this strain (SY1491) and its *lys2* derivative (SY1436) were used for mutant isolation. In the genetic analysis of the mutants, *ste* deletion derivatives were produced by one-step gene replacement using *ste5 $\Delta$ ::URA3*, *ste7 $\Delta$ ::URA3*, *ste11 $\Delta$ ::URA3*, and *ste12 $\Delta$ ::URA3* DNA constructs. Strains containing the *STE11-1* (SY1865) or *STE11-4* (SY1866) alleles, in an otherwise wild-type background, were produced by integration of pSL1654 and pSL1655, respectively, at the *STE11* locus in strain SY1390. Resolution of the tandem *STE11* duplication was selected by resistance to 5-fluoro-orotic acid (Boeke et al. 1984), and *Ura<sup>-</sup>* colonies were screened phenotypically for the presence of the mutant allele. Deletion derivatives of SY1390 (*STE<sup>+</sup>*), SY1865 (*STE11-1*), and SY1866 (*STE11-4*) were produced by one-step gene replacement using *ste3 $\Delta$ ::LEU2*, *ste4 $\Delta$ ::LEU2*, *ste5 $\Delta$ ::LEU2*, *ste12 $\Delta$ ::LEU2*, *fus3-6::LEU2*, and *kss1 $\Delta$ ::URA3* DNA constructs. Three additional strains were used: DC5 (*MAT $\alpha$  leu2-3, -112 his3 gal2 can1*;

provided by J. Strathern); 227 (*MAT $\alpha$  lys1 cry1*; provided by I. Herskowitz); XMB4-12b (*MAT $\alpha$  sst1-1 arg9 ilv3 ura1 his4*; provided by L. Blair).

Yeast and bacterial strains were propagated using standard methods. YEPD and SD media have been described (Rose et al. 1990). Yeast transformations were performed as described by Beggs (1978) or by electroporation (Becker and Guarente 1991), except that sonicated, denatured salmon sperm DNA was included as carrier. Bacterial transformations, bacterial DNA preparations, and plasmid constructions were performed by standard methods (Sambrook et al. 1989).

### Plasmids

*FUS1::HIS3* (pSL1497) contains the *HIS3* open reading frame driven by the *FUS1* promoter, flanked by sequences to direct integration of the gene fusion to the *FUS1* locus. Appropriate sites in *FUS1* and *HIS3* for the fusion point were created by *Bal* 31 digestion and *Sall* linker addition. The exact end points of the deletions used for the gene fusion were determined by DNA sequencing: for the *FUS1* promoter, 3 bp upstream of the ATG; for the *HIS3* open reading frame, 22 bp upstream of the ATG. The *FUS1::HIS3* fusion was created by ligating the *FUS1* and *HIS3* fragments in the pSPT18 vector (Pharmacia); subsequently, a 1.2-kb *HincII* fragment containing downstream *FUS1* sequences was ligated into the *SmaI* site of the polylinker. Digestion with *EcoRI* releases a *FUS1::HIS3* fragment that can recombine with and replace the native *FUS1* locus. The *FUS1-lacZ* plasmid used to assay *FUS1* expression has been described (pSL307; McCaffrey et al. 1987).

A collection of plasmids was used to introduce deletions at the chromosomal *STE* loci: *ste4 $\Delta$ ::LEU2* (p121; Whiteway et al. 1989); *ste5 $\Delta$ ::URA3* (pSURE1, provided by J. Thorner); *ste5 $\Delta$ ::LEU2* (pSL1180; this work); *ste7 $\Delta$ ::URA3* (pSL1077; this work); *ste7 $\Delta$ 3::LEU2* (pNC113; Company and Errede 1988); *ste11 $\Delta$ ::URA3* (pSL1094; this work); *ste12 $\Delta$ ::URA3* (pSL1311; this work); *ste12 $\Delta$ ::LEU2* (pSUL-16; Fields and Herskowitz 1987); *fus3-6::LEU2* (pYEE98; Elion et al. 1990); and *kss1 $\Delta$ ::URA3* (pGA1850, provided by G. Ammerer).

CEN-ARS plasmids with a selectable *TRP1* marker were used to introduce *STE* genes into *ste* deletion strains: pSL1363, *STE11* on a 5.6-kb *Sall-BamHI* fragment in pRS314 (Sikorski and Hieter 1989); pSL1364, *STE12* on a 5.4-kb *Clal* fragment in pRS314; pSTE7<sup>+</sup>, *STE7* on a 5-kb *SacI* fragment in pNC160 (Rhodes et al. 1990). pSL1506, used to clone the *STE11* alleles, was constructed by replacing the *BamHI-Sall* fragment of YCp50 (Rose et al. 1987) with a 5.3-kb *BamHI-Sall* fragment from pSTE11.1 (Chaleff and Tatchell 1985) and deleting the 3.6-kb *XbaI* fragment that contains the entire *STE11* gene. pSL1654 and pSL1655 contain the *STE11-1* and *STE11-4* alleles, respectively, as 3.6-kb *XbaI* fragments in a modified pRS306 vector (Sikorski and Hieter 1989) lacking the *KpnI* site in the polylinker. pNC279, a *TRP1* CEN-ARS plasmid containing *STE7* driven by the *CYC1* promoter (provided by Z. Zhou), was used to facilitate detection of *STE7* in Western analysis.

### Isolation and analysis of *ste4 $\Delta$* suppressor mutants

Mutations that restore expression of *FUS1::HIS3* to a *ste4 $\Delta$ ::LEU2* strain arise spontaneously at a frequency of  $\sim 10^{-5}$  of cells plated on SD-His. In one experiment, aliquots of eight different cultures of strain SY1436 (relevant genotype *MAT $\alpha$  ste4 $\Delta$ ::LEU2 FUS1::HIS3 ura3 lys2*) containing pSL307, a *URA3* 2- $\mu$ m *FUS1-lacZ* reporter plasmid, were plated on SD-His-Ura. Only 1 colony of the 96 tested restored mating ability to the *ste4 $\Delta$*  strain. This isolate (mutant 1) also produced more

$\alpha$ -factor than the isogenic *STE*<sup>+</sup> parent (SY1390). To isolate more mutants with these phenotypes a culture of SY1491 (*MAT $\alpha$  ste4 $\Delta$ ::LEU2 FUS1::HIS3*) was mutagenized with ethylmethanesulfonate (70% survival; Moir et al. 1982), and cells that expressed *FUS1::HIS3* were selected on SD–His plates. The frequency of colony formation was  $1.5 \times 10^{-4}$ . Eight mutants (4, 12, 13, 14, 25, 26, 27, and 29) were selected for further study on the basis of secondary screens for pheromone production and mating competence.

The dominant or recessive nature of the suppressor mutations was determined by testing the expression of *FUS1::HIS3* in *MAT $\alpha$ /MAT $\alpha$*  diploids, formed by protoplast fusion (Schnell et al. 1989) of the *ste4 $\Delta$*  suppressor mutants with the parental *ste4 $\Delta$*  strain. A His<sup>−</sup> phenotype indicated that the mutation was recessive, whereas a His<sup>+</sup> phenotype indicated that the mutation was dominant. *MAT $\alpha$ /MAT $\alpha$*  diploids heterozygous for the mutation in suppressor mutant 1 grew poorly on SD–His and produced a level of  $\alpha$ -factor intermediate between the original mutant and the parental *ste4 $\Delta$*  strain, suggesting that the mutation was incompletely dominant (codominant).

The ability of each *ste4 $\Delta$*  suppressor mutant to suppress deletions in other *STE* genes was determined by replacing the chromosomal copy of the relevant *STE* gene with a deletion allele marked by a *URA3* gene (Rothstein 1991). Following confirmation of the genotype by Southern analysis, the His and pheromone production phenotypes of the deletion mutants were evaluated. In cases where the mutation did not suppress a particular *ste* deletion, the possibility that the suppressor mutation was allelic to the *STE* gene was determined by introducing a plasmid-borne copy of the *STE* gene. Failure to express *FUS1::HIS3* or to produce more  $\alpha$ -factor in the presence of the *STE* gene suggested that the suppressor was allelic to that gene. This result was confirmed by cloning the suppressor allele and testing its effect on the expression of *FUS1::HIS3* in a naive *ste4 $\Delta$ ::LEU2* strain.

#### Cloning and sequencing of the *STE11* alleles

The *STE11* alleles were cloned by the gap repair method (Rothstein 1991). Strains suspected of carrying a mutant *STE11* allele were transformed to uracil prototrophy with pSL1506 linearized at the unique *Xba*I site. The 3.6-kb *Xba*I fragment rescued from each suppressor mutant was subcloned into M13mp18 for sequencing (Sanger et al. 1977), using synthetic primers complementary to the *STE11* sequence. The DNA sequence of the entire *STE11*-coding region was determined for the *STE11* alleles rescued from mutants 1 and 4, and from the wild-type isogenic strain (SY1491). A single nucleotide difference was detected between wild type and each of the mutants. Only the sequence spanning the change in *STE11-4* was determined for alleles rescued from mutants 12, 14, 26, and 29. These alleles contain the same mutation present in *STE11-4*.

#### $\beta$ -Galactosidase, mating, and halo assays

For  $\beta$ -galactosidase assays, strains carrying pSL307 were grown to mid-log phase at 30°C in selective medium. Cells were prepared and assayed as described previously (Jarvis et al. 1988). Quantitative mating assays were performed by a filter mating assay as described (Clark and Sprague 1989). Qualitative mating and pheromone production assays (halo assays) were performed according to Sprague (1991).

#### Preparation of yeast protein extracts and detection of *STE7*

*STE11-1*, *STE11-4*, and *STE*<sup>+</sup> strains harboring pNC279 were grown to saturation in SD–Trp and used to inoculate 250 ml of

S broth–Trp medium containing 1% sucrose as the carbon source (to induce *CYC1–STE7* expression). Cultures were harvested at a density of  $\sim 2 \times 10^7$  cells/ml, and protein extracts were prepared as described (Company et al. 1988). Aliquots of protein (25  $\mu$ g/lane, from 40% ammonium sulfate fractions) were fractionated on an 8% SDS–polyacrylamide gel (Laemmli 1970) and transferred to nitrocellulose. Rabbit anti-*STE7* polyclonal antibodies were used to detect *STE7* protein. Mouse anti-MCM1 polyclonal antibodies (a gift of G. Ammerer) were used to detect MCM1 protein. The Promega Protoblot system with goat anti-rabbit or goat anti-mouse IgG conjugated to alkaline phosphatase was used to detect the primary antibody by colorimetric methods.

#### Microscopy

Cells were fixed in 3.7% formaldehyde/0.15 M NaCl and sonicated briefly before mounting. Micrographs were taken on a Zeiss Axioplan photomicroscope with Nomarski optics, using a 100 $\times$  objective.

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## Constitutive mutants of the protein kinase STE11 activate the yeast pheromone response pathway in the absence of the G protein.

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