Order of action of components in the yeast pheromone response pathway revealed with a dominant allele of the STE11 kinase and the multiple phosphorylation of the STE7 kinase

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The signal transduction pathway that mediates the response of haploid yeast cells to peptide mating pheromones involves several components including the protein kinases STE7 and STE11. We have isolated and characterized a dominant allele of the *STE11* gene and have demonstrated that expression of an amino-terminally truncated form of STE11 protein causes constitutive activation of the mating pathway. Expression of this dominant *STE11* allele also restored mating ability to certain sterile strains. In conjunction with the results of others, our epistasis results establish the following order of action of pathway components: STE2, GPA1(SCG1), STE4, STE5, STE11, STE7, STE12. Transduction of the signal from STE11 to STE7 may involve phosphorylation because STE7 displays several phosphorylation forms, and STE7 is multiply phosphorylated in response to either pheromone or coexpression of dominant STE11 protein. Further signal propagation appears to require STE7 protein kinase activity, because a catalytically impaired STE7 mutant is defective in the mating response.

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Signal transduction from the cell surface to targets inside the nucleus plays an important role in many cellular and developmental processes. Current studies of signaling pathways focus on defining the components involved and elucidating the mechanisms underlying signal propagation. Of particular interest are the diverse pathways mediated by heterotrimeric G proteins (for review, see Stryer and Bourne 1986; Gilman 1987). Several of these systems in higher eukaryotes appear to employ protein phosphorylation to transduce the signal downstream of the G protein, but defining the kinases involved and identifying their substrates has proved difficult. The mating pheromone signal transduction pathway present in the budding yeast *Saccharomyces cerevisiae* is a genetically tractable model system for studying the role of phosphorylation in signaling systems mediated by G proteins. Mating between haploid *a* and α yeast cells is achieved by the exchange of peptide mating pheromones, with conjugation resulting in the formation of diploid *a*/ α cells (Duntze et al. 1970; Hartwell 1973; Kurjan 1985; Bender and Sprague 1986). The response of haploid *a* cells to the mating pheromone α -factor involves the

interaction of a specific transmembrane pheromone receptor with a signal transduction pathway that coordinates all cellular events necessary for conjugation: arrest of the cell cycle in G₁ phase, the rapid transcriptional induction of several genes required for mating, and the attendant physiological changes necessary for cell and nuclear fusion (for review, see Cross et al. 1988; Herskowitz 1989). An analogous pathway is present in haploid α cells (Bender and Sprague 1986; Nakayama et al. 1987). Genetic experiments have identified several components in the pheromone response pathway in a haploid cell, including the products of the *STE2*, *STE4*, *STE5*, *STE7*, *STE11*, *STE12*, and *STE18* genes. Deletions or conditional mutations in any of these genes cause sterility (MacKay and Manney 1974a,b; Hartwell 1980). The *STE2* gene encodes the transmembrane receptor for α -factor and displays a transmembrane topology similar to the rhodopsin/ β -adrenergic family of receptors (Jenness 1983; Nakayama et al. 1985; Dixon et al. 1986). The nucleotide sequences of the yeast genes *GPA1*(SCG1), *STE4*, and *STE18* implicate them as the respective α , β , and γ subunits of the G protein coupled to the STE2 receptor in the yeast mating pathway (Dietzel and Kurjan 1987; Miyajima et al. 1987; Whiteway et al. 1989).

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The $\beta\gamma$ -complex STE4/STE18, and not the α -subunit GPA1(SCG1), is believed to be responsible for transducing the signal in the mating pathway (Blinder et al. 1989; Cole et al. 1990; Nomoto et al. 1990; Whiteway et al. 1990). In mammalian cells, signal propagation by $\beta\gamma$ subunits has been demonstrated in the activation of K^+ channels by phospholipase A_2 (Kim et al. 1989) and in the stimulation of adenylyl cyclase II (Federman et al. 1992).

Although the pheromone receptor and the heterotrimeric G protein subunits have been studied intensively, propagation of the signal downstream of these components is poorly understood. Only the putative target of the mating pathway, the DNA-binding transcriptional activator STE12, has been characterized in detail (Dolan et al. 1989; Errede and Ammerer 1989). In response to pheromone, STE12 has been shown to be phosphorylated rapidly (Song et al. 1991). In addition to the uncharacterized STE5 gene product, the pathway is believed to include four putative protein kinases; STE7, STE11, FUS3, and KSS1 (Chaleff and Tatchell 1985; Courchesne et al. 1989; Elion et al. 1990). Although both the STE7 and STE11 gene products are absolutely required for mating, deletion of either the FUS3 gene or the KSS1 gene has only a mild effect on mating efficiency (Courchesne et al. 1989; Elion et al. 1991). The combination of deletions of FUS3 and KSS1, however, confers sterility (Elion et al. 1991). The nucleotide sequences of the STE7 and STE11 genes show significant homology to serine/threonine protein kinases (Teague et al. 1986; Hanks et al. 1988; Rhodes et al. 1990). Although STE11 protein kinase activity is essential for the mating response, and a potential novel substrate of STE11 has been reported, direct involvement of STE11 in propagation of the signal has not been demonstrated; neither the phosphorylation nor the catalytic activity of STE11 protein is affected by the presence of pheromone (Rhodes et al. 1990).

Our characterization of a dominant STE11 allele provides further evidence that STE11 protein is directly involved in signal propagation. Our results suggest the presence of a catalytic and a regulatory domain in STE11 protein, and our analysis of double mutants establishes the functional order of several of the STE gene products in the mating pathway. In addition, we demonstrate that the phosphorylation of STE7 protein is correlated with mating pathway activation and that STE7 kinase activity is important for an efficient mating response.

Results

Isolation of a dominant allele of STE11

An inducible yeast expression library was prepared by fusing sheared, size-selected yeast genomic DNA to the GAL1 promoter in pYES-R (Elledge et al. 1991). Although fusion of DNA fragments to the GAL1 promoter allows their transcription to be regulated by carbon sources (Johnston and Davis 1984), this version of the GAL1 promoter does not provide an initiator for their translation. Rather, an initiation codon must be present in the cloned DNA, with the initiation of translation

occurring predominantly at the initiator closest to the 5' end of the resulting mRNA. In addition to yielding full-length clones, this library may contain truncation mutants. A clone derived from a shearing event within the coding region of a gene can only be expressed if initiation of translation occurs at an internal AUG codon, leading to the synthesis of an amino-terminally truncated protein.

The library was screened for clones that cause galactose-dependent transcriptional activation of FUS1, a gene whose mRNA level increases 40-fold after the addition of mating pheromone (McCaffrey et al. 1987). The yeast strain NNY19, containing a chromosomally integrated copy of a FUS1-lacZ construct (Nomoto et al. 1990), was transformed with library DNA, and transformants were selected on uracil-deficient media containing glucose. This selection permitted growth of the transformants, but the presence of glucose repressed transcription of the cloned DNA. Colonies transferred to nitrocellulose filters were incubated on uracil-deficient media containing galactose to induce transcription from the GAL1 promoter. Clones whose expression caused activation of the mating pathway were revealed by β -galactosidase expression (Schena et al. 1989). A plasmid containing an allele of the STE11 gene under control of the GAL1 promoter was isolated and identified by Southern blot analysis with a STE11 probe (data not shown). Details of the preparation and characterization of the genomic DNA expression library, including a complete description of the genetic screen and other activators of the mating response pathway, will be presented elsewhere (S. Ramer and R. Davis, in prep.).

Characterization of a STE11 amino-terminal truncation mutant

We sequenced this STE11 allele and discovered that a large part of the STE11-coding region was absent; only the carboxy-terminal coding region of the STE11 gene remained fused to the GAL1 promoter (pYGU-11 Δ N; Fig. 1A). Sequencing revealed that the first nucleotide of STE11 present in pYGU-11 Δ N is an adenosine at position 1051 (with numbering according to Rhodes et al. 1990), whereas the initiator for the wild-type STE11 gene is located at nucleotide position 116–118, and the stop codon is located at position 2267–2269. This deletion resulted in the synthesis of a mutant form STE11 protein (STE11 Δ N), which lacks its amino terminus. Protein sequence comparison shows that STE11 Δ N protein contains all of the conserved catalytic kinase subdomains (Hanks et al. 1988; Rhodes et al. 1990). Use of the GAL1 promoter confers catabolite regulation of transcription to the STE11 Δ N allele (Johnston and Davis 1984). In the absence of repressing carbon sources such as glucose or sucrose, the addition of galactose induces the transcription of STE11 Δ N at least 20-fold (data not shown).

Three lines of reasoning indicate that translational initiation of STE11 Δ N begins at the first in-frame AUG codon, located at nucleotide position 1139–1141: (1) The first AUG codon corresponds to the translation initia-

Figure 1. Plasmids employed in the expression and analysis of STE11 and STE7 protein. (A) Schematic representation of the plasmids directing STE11 expression. The shaded area represents the coding region of *STE11*. pYGU-STE11 contains the wild-type *STE11* gene under control of the *GAL1* promoter. pYGU-STE11ΔN contains ~4 kb of uncharacterized DNA 3' to the *STE11*-coding region, which is removed in pYGL-STE11ΔN without any loss of function. (B) Schematic representation of the plasmids directing STE7 expression. pYGU-STE7 contains the wild-type *STE7* gene under control of the *GAL1* promoter. pYGU7-A220 encodes a mutant form of *STE7* protein in which the lysine at amino acid position 220 has been replaced with an arginine. (C) Schematic representation of the β-galactosidase reporter plasmid used to monitor STE12-dependent activation of transcription (pUZ4). The upstream activating sequence (UAS) contains three consensus-binding sites (ATGAAACA) for the DNA-binding protein STE12 (Kronstad et al. 1987; Van Arsdell and Thorner 1987; Dolan et al. 1989). These binding sites are tandemly repeated and are represented by arrows. Abbreviations of restriction sites: (Bn) *Ban*II; (C) *Cla*I; (H) *Hind*III; (K) *Kpn*HI; (N) *Nco*I; (R) *Eco*RI; (Xb) *Xba*I.

tion site in 95% of yeast mRNA (Cigan and Donahue 1987); (2) initiation at the next in-frame AUG codon (nucleotides 1412–1414) would result in deletion of the entire first catalytic subdomain of the kinase; and (3) the presence of two additional out-of-frame AUG codons (nucleotides 1233–1235 and 1236–1238, respectively) between the first two in-frame AUG codons precludes the use of the initiator at positions 1412–1414. In addition, immunoblot analysis of STE11ΔN protein (with a polyclonal antibody raised against recombinant STE11ΔN protein) revealed a protein with an approximate mass of 48 kD (Fig. 2A). On the basis of SDS-PAGE of native STE11 protein, which comprises 717 amino acids and migrates at ~93 kD, translational initiation at position 1139 should result in a protein of 366 amino acids that migrates at ~47 kD, in agreement with our findings.

STE11ΔN is a dominant STE11 allele that causes pheromone-independent activation of the mating pathway

STE11ΔN protein function was initially assessed by complementation of the mating defect conferred by a deletion of the *STE11* gene (with YSC-AΔste11). Patches of YSC-AΔste11 that express STE11ΔN protein were able to mate efficiently with tester lawns. No diploids were formed with control patches of YSC-AΔste11 not expressing STE11ΔN protein (data not shown). The effect of the *STE11ΔN* allele on STE12-dependent transcription was examined in three wild-type strains (KMY211-A, YPH499, and W303-1A). All genes activated by the mat-

ing pathway (in MATa cells) contain two or more binding sites for the STE12 protein. Transcriptional activation was monitored with the β-galactosidase reporter plasmid pUZ4 (Fig. 1C), which contains three consensus STE12-binding sites (Kronstad et al. 1987; Van Arsdell and Thorner 1987; Dolan et al. 1989) upstream of the β-galactosidase gene. In all three strains, STE11ΔN expression activated STE12-dependent transcription to levels equal to or greater than levels observed by the addition of pheromone. Furthermore, high levels of STE12-dependent transcriptional activation (179 units) were observed when both STE11ΔN protein (with pYGL-STE11ΔN; Fig. 1A) and STE11 wild-type protein (with pYGU-STE11; Fig. 1A) were overexpressed, indicating that the *STE11ΔN* allele is dominant.

Expression of STE11ΔN induces phenotypes similar to those observed following treatment with mating pheromone

Activation of the mating pathway by mating pheromone causes the arrest of the cell cycle in G₁ phase, and several phenotypes including the accumulation of large unbudded cells and the formation of pear-shaped cells (shmoos) (Buckling-Throm et al. 1973; Wilkinson and Pringle 1974; Lipke et al. 1976). After 18 hr of STE11ΔN expression, >70% of cells accumulated as large unbudded cells, and ~15% of these cells displayed projections typically observed upon treatment with a high concentration of mating pheromone (Fig. 3). These phenotypes were not a result of growth in galactose, nor did they occur when

Figure 2. Immunoblot analysis of wild-type STE11 and STE11ΔN protein. (A) Whole-cell extracts and immunoblots were prepared as described in Materials and methods. All lanes contain ~5 μg of whole-cell extract derived from YPH499 transformants. Each was grown for 8 hr in the presence of galactose to induce expression from the following plasmids. (Lane 1) pYGU-11ΔN; (lane 2) pYGU-STE11; (lane 3) no plasmid. The positions of wild-type STE11 protein and STE11ΔN protein are indicated by labeled arrows. Positions of molecular mass markers (kD) run in another lane of the same gel are indicated at right. (B) Schematic representation of the STE11-coding region, including the amino acid coordinates of the initiators of wild-type and truncated STE11 protein, the first amino acid of the first conserved catalytic subdomain, and the last amino acid.

wild-type STE11 was expressed for 18 hr in YPH499 with pYGU-STE11 (data not shown). Further experiments are required to determine whether STE11ΔN expression affects the phenotype and the progression of the cell cycle by the same mechanisms as does pheromone treatment.

Ordering the components of the mating pathway with STE11ΔN

The epistatic relationships of the STE11ΔN allele with other pathway components were assessed by determining the effect of STE11ΔN expression in strains lacking individual components of the mating pathway. In all cases, their ability to mate with an α-tester strain and their ability to activate STE12-dependent transcription were determined (Table 1). STE11ΔN protein was ex-

pressed in strains isogenic to KMY211-A, each lacking the STE4, STE5, STE7, STE11, or STE12 gene. In addition, the relationship of the STE11ΔN effect to the FUS3 and KSS1 kinases was tested by the expression of STE11ΔN in strains isogenic to YPH499 lacking either the FUS3 or KSS1 gene. Strains that lack either FUS3 alone or KSS1 alone are able to mate efficiently; only the double mutant Δfus3Δkss1 is sterile (Elion et al. 1991). Therefore, STE11ΔN protein was also expressed in a YPH499 derivative lacking both the FUS3 and KSS1 genes.

Expression of STE11ΔN protein in a strain lacking either the STE4 or STE5 gene restored a significant level of mating ability. In both of these strains, expression of STE11ΔN protein also resulted in high levels of STE12-dependent transcription. In contrast, expression of STE11ΔN protein in a strain lacking either the STE7 or STE12 gene did not restore mating ability, nor did it

Figure 3. Phenotypes of yeast cells expressing STE11ΔN protein. (A) The wild-type strain YPH499 was transformed with pYGU-11ΔN and grown to mid-log phase in medium containing 2% sucrose to prevent expression of STE11ΔN. (B) The transformants described in A were centrifuged and resuspended in medium containing 2% galactose and grown for an additional 18 hr. Images were obtained with Normarski optics.

Table 1. Effect of the dominant STE11 allele on mating and STE12-dependent transcription

Strain	Deletion	Expression Plasmid pYGU–	Mating ^a	β-Galactosidase ^b	
				sucrose	galactose
KMY211-A	none (wild type)	STE11	+++	<1	<1
KMY211-A	none (wild type)	11ΔN	+++	2	281
YSC-AΔste4 ^c	Δste4	11ΔN	+	2	267
YSC-AΔste5 ^c	Δste5	11ΔN	++	<1	208
YSC-AΔste7 ^c	Δste7	11ΔN	–	<1	<1
YSC-AΔste11 ^c	Δste11	11ΔN	+++	<1	169
YSC-AΔste12 ^c	Δste12	11ΔN	–	<1	<1
YPH499	none (wild type)	STE11	+++	2	2
YPH499	none (wild type)	11ΔN	+++	2	444
YPHAΔfus3 ^d	Δfus3	11ΔN	+++	<1	325
YPHAΔkss1 ^d	Δkss1	11ΔN	+++	<1	464
YPHAΔfus3Δkss1 ^d	Δfus3, Δkss1	11ΔN	–	<1	<1

^aMating tests were performed by the patch method as described in Materials and methods. (+) Patches comprising 10–30 individual colonies form within 48 hr. (++) Patches of diploids form within 48 hr of replica plating. (+++) Indistinguishable from wild-type. Dense patches of diploids form within 36 hr of replica plating. (–) No diploids form within 96 hr of replica plating.

^bβ-Galactosidase assays, with the reporter plasmid pUZ4 (Fig. 1C), are in Miller units as described in Materials and methods.

^cKMY211-A strain background.

^dYPH499 strain background.

cause any enhancement of STE12-dependent transcription. In addition, overproduction of wild-type STE11 protein with pYGU–STE11 did not restore mating ability to any of the deletion strains tested except YSC-AΔste11. Assuming a linear relationship of mating pathway components, these results suggest that the STE11ΔN protein functions downstream of the STE4 and the STE5 gene products and upstream of the STE7 and STE12 gene products.

Expression of STE11ΔN protein in strains lacking either the FUS3 or KSS1 genes also caused high levels of STE12-dependent transcription. In contrast, expression of STE11ΔN protein in the Δfus3Δkss1 strain neither activated STE12-dependent transcription nor restored mating ability. These results localize the function of the FUS3 and KSS1 gene products downstream of the STE11 gene product and provide further evidence that the FUS3 and KSS1 kinases play redundant roles in activating gene expression (Elion et al. 1991).

The STE7 kinase is a phosphoprotein

As our data are consistent with the STE11 gene product functioning upstream of the STE7 gene product, we sought to identify the effects of the dominant STE11 mutation on the biochemical properties of the STE7 kinase. Polyclonal antisera were raised against recombinant STE7 protein and verified by immunoblot analysis of both the native recombinant protein (Fig. 4, lane 4) and a recombinant λCII fusion protein (data not shown).

As a result of the scarcity of STE7 protein in wild-type cells, we enhanced the levels of STE7 protein by directing STE7 synthesis from the galactose-inducible GAL1 promoter (Johnston and Davis 1984) with the plasmid pYGU–STE7 (Fig. 1B). Patches of the yeast strain YSC-AΔste7, which lacks the STE7 gene, expressing STE7 protein with pYGU–STE7, mated efficiently with the

α-mating tester strain DC17 (data not shown), demonstrating functional complementation of the mating defect under these conditions. For immunoblot analysis, whole-cell extracts were prepared from a KMY211-A derivative lacking the STE7 gene (YSC-AΔste7), the wild-type strain KMY211-A, and a KMY211-A transformant that expressed STE7 protein with pYGU–STE7 (Fig. 4, lanes 1–3). Under these conditions, immunoblots did not reveal any STE7-dependent protein in extracts derived from wild-type cells (cf. lanes 1 and 2). When STE7 protein was expressed with pYGU–STE7, however, several galactose-dependent species appeared (lane 3), migrating with apparent masses between 64 and 76 kD. The most immunoreactive species was the fastest migrating and

Figure 4. Immunoblot analysis of STE7 protein. Whole-cell extracts and immunoblots were prepared as described in Materials and methods from the following strains. (Lane 1) YSC-AΔste7, a KMY211-A derivative lacking the STE7 gene; (lane 2) KMY211-A (wild-type); (lane 3) KMY211-A transformed with pYGU–STE7 expressing STE7 from the GAL1 promoter (lanes 1–3 contain 5 μg of extract per lane); (lane 4) ~2.0 ng of recombinant STE7 protein purified from *E. coli*. The arrow indicates the position of unphosphorylated STE7 protein; the asterisk (*) indicates the position of the multiply phosphorylated form of the STE7 protein. Positions of molecular mass markers (kD) run in another lane of the same gel are indicated at left.

was shown to comigrate with STE7 protein produced in *Escherichia coli* (lane 4). In addition, a doublet representing the slowest-migrating species also exhibited a high level of immunoreactivity.

To determine whether these immunoreactive species represented different phosphorylation forms of STE7 protein, the lysates were treated with calf intestinal phosphatase (Fig. 5). This treatment converted all slow-migrating forms to an apparent single species migrating at ~64 kD (lane 2). This conversion did not occur in mock-treated lysates (lane 3) and was blocked by phosphatase inhibitors, indicating that the conversion was caused by dephosphorylation and not by proteolysis of STE7 protein.

Dephosphorylation experiments were also performed on immune complexes of STE7 protein. Under these conditions, dephosphorylation did not proceed to completion, presumably owing to protection of areas of STE7 protein by the polyclonal antibody. Immunoblot analysis of STE7 protein under these conditions reveals the accumulation of STE7 phosphorylation forms of intermediate electrophoretic mobility (Fig. 5, lane 5). This result indicates that the slow-migrating STE7 phosphorylation forms arise from multiple phosphorylations and argues against the possibility that each form represents a unique singly phosphorylated species.

STE7 is phosphorylated rapidly in the presence of mating pheromone

Having demonstrated the occurrence of STE7 phospho-

Figure 5. Treatment of the STE7 protein with calf intestinal phosphatase in the absence or presence of STE7 polyclonal antibody. The effect of phosphatase treatment on whole-cell extracts (left) or immunoprecipitates of the STE7 protein (right) was assayed by immunoblot analysis as described in Materials and methods. Whole-cell extracts were prepared from a W303-1A transformant expressing STE7 with pYGU-STE7 grown in the presence of galactose for 8 hr, either in the absence of pheromone (left), or subsequently treated with 2.0 μ M mating factor for 5 min (right). Extracts were either mock treated (lanes 1,4), treated with 12 units of calf intestinal phosphatase (lanes 2,5), or treated with phosphatase in the presence of phosphatase inhibitors (lanes 3,6). The arrows indicate the position of unphosphorylated STE7 protein; the asterisks (*) indicate that of the multiply phosphorylated form of the STE7 protein. Positions of molecular mass markers (kD) run in another lane of the same gel are indicated.

Figure 6. Effect of pheromone on the phosphorylation of STE7 protein. Whole-cell extracts (5 μ g of protein per lane) were prepared, and immunoblot analysis was performed as described in Materials and methods. Each strain expressed STE7 from pYGU-STE7. Cultures of W303-1A (lanes 1,2) or the isogenic protease-deficient derivative YBC102 (lanes 3,4) were grown in the presence of galactose for 4 hr to induce STE7 expression. Pheromone was either not added (lanes 1,3) or added to a final concentration of 2.0 μ M (lanes 2,4). These cultures were incubated for 4 min at 30°C and centrifuged and frozen within 5 min of the addition of pheromone. The arrows indicate the position of unphosphorylated STE7 protein; the asterisks (*) indicate that of the multiply phosphorylated form of the STE7 protein. Positions of molecular mass markers (kD) run in another lane of the same gel are indicated.

rylation forms in the absence of pheromone, we investigated the impact of pheromone on the distribution of these forms. STE7 protein was expressed in both the wild-type strain W303-1A and an isogenic protease-deficient derivative YBC102. Treatment with α -factor-mating pheromone for 5 min caused the accumulation of slower-migrating phosphorylation forms of STE7 protein and the disappearance of the fastest-migrating form (Fig. 6). The phosphorylation forms were revealed most clearly in extracts derived from protease-deficient cells (lanes 3,4). Similar results were obtained with the wild-type strains YPH499 and KMY211-A. Experiments by others have shown that both the transcriptional induction of genes such as *FUS1* (McCaffrey et al. 1987) and the phosphorylation of STE12 protein (Song et al. 1991) occur within 5 min of the addition of pheromone. Alteration of the phosphorylation state of STE7 protein is therefore correlated with mating pathway activation.

STE11 Δ N expression potentiates STE7 phosphorylation

Having shown that the STE11 gene product functions upstream of the STE7 gene product in the mating pathway and, further, that phosphorylation of STE7 protein is correlated with mating pathway activation, we asked whether the dominant STE11 Δ N allele affects the distribution of STE7 phosphorylation forms. Coexpression of both STE11 Δ N and STE7 protein resulted in nearly com-

plete conversion of STE7 protein to multiply phosphorylated species (Fig. 7, lane 3). This result provides further evidence that STE7 phosphorylation is correlated with signal transmission and that STE11 Δ N initiates the mating pathway upstream of STE7.

STE7 phosphorylation forms are present in strains lacking other components in the mating pathway

The mating response requires the concerted action of several components, including four putative protein kinases (Chaleff and Tatchell 1985; Courchesne et al. 1989; Elion et al. 1990, 1991; Rhodes et al. 1990). To assess the dependence of STE7 phosphorylation forms on the activity of other components in the mating pathway, we expressed STE7 protein in strains isogenic to the wild-type strain KMY211-A, lacking the *STE4*, *STE5*, *STE11*, or *STE12* gene. In addition, we expressed STE7 protein in strains isogenic to strain YPH499, lacking either the *FUS3* or *KSS1* gene, or both. Immunoblot analysis revealed the presence of both unphosphorylated and multiply phosphorylated forms of STE7 protein in all of the deletion strains tested (Fig. 8). The other immunoreactive species (Fig. 8A, lane 3; Fig. 8B, lanes 1–5) are also present in extracts derived from cells grown in medium containing sucrose (Fig. 8B, lane 1, and data not shown) and, thus, are not STE7-dependent. To confirm the lack of impact on STE7 phosphorylation observed with YSC-A Δ ste11, the *STE11* gene was also deleted from the chromosome in both YPH499 and W303-1A. STE7 was expressed in these alternate Δ ste11 strains, and immunoblot analysis revealed unphosphorylated and multiply phosphorylated forms of STE7 protein that were indistinguishable from the forms observed in extracts derived

from the wild-type parent strains in the absence of pheromone (data not shown). Therefore, the other components of the mating pathway, including STE11, are not necessary for the generation of multiply phosphorylated forms of STE7 protein; nonetheless, these other components may affect the phosphorylation state of STE7 during signal transduction.

Overproduction of STE7 protein does not activate STE12-dependent transcription

Considering that multiply phosphorylated forms of STE7 are present in the absence of pheromone and that their amounts are increased by pheromone treatment, we asked whether overproduction of STE7 protein would activate the mating pathway. To examine this possibility, activation of STE12-dependent gene expression by overproduction of STE7 protein was compared to activation by pheromone treatment. The β -galactosidase reporter plasmid pUZ4 (Fig. 1C) was used to monitor STE12-dependent gene expression. Overproduction of STE7 in strains KMY211-A, YPH499, or W303-1A had no effect on STE12-dependent transcription in the absence of pheromone (Table 2). In addition, STE7 overexpression in the absence of pheromone did not initiate the phenotypes associated with pheromone-dependent activation of the mating pathway (data not shown). Finally, strains overproducing STE7 respond normally to pheromone stimulation, displaying high levels of STE12-dependent transcription (Table 2).

We also investigated whether overproduction of STE7 protein could suppress the mating defect of a strain lacking the *STE11* gene. In addition, we performed the complementary experiment of expressing wild-type STE11 protein with pYGU-STE11 in an isogenic strain lacking the *STE7* gene. No diploids were formed in any of the 12 independent patch mating experiments that were performed with each strain (data not shown), demonstrating that the mating defect was not suppressed in either strain. This result is consistent with observations of others in similar experiments with high-copy plasmids (Dolan and Fields 1990), and provides further evidence that the kinases play unique roles in the mating pathway.

Construction and activities of a STE7 site-directed mutant

Finally, we investigated whether the presumed protein kinase activity of STE7 protein is required for signal propagation. A lysine at position 220 in STE7 protein corresponds to the conserved lysine found in the vast majority of serine/threonine kinases (Hanks et al. 1988). This residue was changed to an arginine, and the mutant *STE7* gene was fused to the *GAL1* promoter in pSE936 (Elledge et al. 1991) to create pYGU7-A220 (Fig. 1B). Similar substitutions in other kinases have been shown to impair or eliminate catalytic activity (Snyder et al. 1985; Booher and Beach 1986; Kamps and Sefton 1986; Celenza and Carlson 1989; Rhodes et al. 1990). The resulting protein, ste7-A220, was expressed in YSC-

Figure 7. Expression of the STE11 Δ N protein potentiates the phosphorylation of STE7 protein. The phosphorylation forms of the STE7 protein under conditions of STE11 Δ N coexpression was revealed by immunoblot analysis. Whole-cell extracts (5 μ g/lane) were prepared, and immunoblot analysis was performed as described in Materials and methods. The strain W303-1A was transformed with either pYGU-STE7 (lanes 1,2) or both pYGU-STE7 and pYGL-11 Δ N (lane 3). All three transformants were initially grown in medium containing 2% sucrose. For experiments presented in lanes 2 and 3, cells were centrifuged, resuspended in medium containing 2% galactose, and grown for an additional 8 hr. Positions of molecular mass markers (kD) run in another lane of the same gel are indicated at right.

Figure 8. Immunoblot analysis of the STE7 protein expressed in strains lacking mating pathway components. (A) Whole-cell extracts (5 μ g of protein per lane) were prepared from derivatives of the wild-type strain KMY211-A. Each strain expressed STE7 protein using pYGU-STE7. Immunoblot analysis was performed as described in Materials and methods. Extracts were derived from the following strains. (Lane 1) YSC-A Δ ste4, lacking the *STE4* gene; (lane 2) YSC-A Δ ste5, lacking the *STE5* gene; (lane 3) YSC-A Δ ste11, lacking the *STE11* gene; (lane 4) YSC-A Δ ste12, lacking the *STE12* gene. The arrow indicates the position of unphosphorylated STE7 protein; the asterisk (*) indicates that of the multiply phosphorylated form of STE7 protein. The additional cross-reactive species present in extracts derived from YSC-A Δ ste11, with an apparent mass of 67 kD, is present in control extracts derived from YSC-Aste11 cells grown in sucrose (data not shown). Positions of molecular mass markers (kD) run in another lane of the same gel are indicated at right. (B) Whole-cell extracts (5 μ g of protein per lane) were prepared from derivatives of the wild-type strain YPH499. Each strain expressed STE7 protein with pYGU-STE7. Immunoblot analysis was performed as described in Materials and methods. Extracts were derived from the following strains. (Lane 1) YPH499, wild-type strain; (lane 2) YPH499- Δ fus3, lacking the *FUS3* gene; (lane 3) YPH499- Δ kss1, lacking the *KSS1* gene; (lane 4) YPH499- Δ fus3 Δ kss1, lacking both the *FUS3* gene and the *KSS1* gene. Arrow indicates the position of unphosphorylated STE7 protein; the asterisk (*) indicates that of the multiply phosphorylated form of STE7 protein. The additional cross-reactive species are not STE7 dependent and are present in control extracts of each strain grown in sucrose (lane 1 and data not shown). Positions of molecular mass markers (kD) run in another lane of the same gel are indicated at left.

A Δ ste7 for 6 hr, and the strain was tested for its ability to mate with the α -tester strain DC17 in qualitative patch mating experiments. This strain mated very poorly, yielding only 1–16 individual diploid colonies within the patch after 96 hr of growth on selective media (Fig. 9A). In contrast, expression of the native STE7 protein in YSC-A Δ ste7 gave dense patches within 36 hr. Expression of pYGU7-A220 in YSC-A Δ ste7 for long periods of time (24 hr) increased the efficiency of mating two- to three-fold, but the mating efficiency remained far below wild-type levels (data not shown). No diploids were observed with YSC-A Δ ste7 patches that did not express STE7 protein. In addition, YSC-A Δ ste7 transformants expressing ste7-A220 protein did not display any pheromone-dependent activation of STE12-dependent transcription.

These experiments indicate that STE7 protein kinase activity is important for efficient mating. The low level of mating provided by ste7-A220 might be the result of residual kinase activity or a secondary function such as complex formation.

To determine the relationship of the multiply phosphorylated form of STE7 protein to STE7 catalytic activity, the phosphorylation state of ste7-A220 protein in the strain YSC-A Δ ste7 was examined. Immunoblot analysis established that ste7-A220 protein is present primarily in an unphosphorylated form (Fig. 9B, lane 2). The multiply phosphorylated, slow-migrating species were not present when ste7-A220 was expressed, although a species of low immunoreactivity did appear, migrating at $\sim$ 73 kD, slightly faster than the prominent multiply

Table 2. Effect of overproduction of wild-type STE7 or STE11 protein on mating and STE12-dependent transcription

Strain	Deletion	Expression plasmid pYGU-	Mating ^a	β -Galactosidase ^b		
				sucrose	galactose	galactose + 2 μ M pheromone
W303-1A	none(WT)	STE7	ND	7	7	235
W303-1A	none(WT)	STE11	ND	7	7	ND
YPH499	none(WT)	STE7	+++	2	2	358
YPH499	none(WT)	STE11	+++	2	2	ND
KMY211-A	none(WT)	STE7	+++	<1	<1	82
KMY211-A	none(WT)	STE11	+++	<1	<1	ND
YSC-A Δ ste7 ^c	Δ ste7	STE7	+++	<1	<1	54
YSC-A Δ ste7 ^c	Δ ste7	STE11	-	<1	<1	ND
YSC-A Δ ste11 ^c	Δ ste11	STE7	-	<1	<1	ND
YSC-A Δ ste11 ^c	Δ ste11	STE11	+++	<1	<1	ND

(ND) Not determined.

^aMating tests were performed by the patch method as described in Materials and methods. (+++) Indistinguishable from wild-type. Dense patches form within 36 hr of replica plating. (-) No diploids form with 96 hr of replica plating.

^b β -Galactosidase assays, with the reporter plasmid pUZ4 (Fig. 1C), are in Miller units as described in Materials and methods.

^cKMY211-A strain background

Figure 9. Phosphorylation state and mating ability of a *ste7* mutant. (A) The ability of mutant *ste7*-A220 protein to restore mating ability to a strain lacking the *STE7* gene was determined. Patches represent diploids growing on minimal media plates, the result of successful mating of transformants of the strain YSC-AΔ*ste7* with a lawn of the mating tester strain DC17. Patch 1 expressed *STE7* wild-type protein from pYGU-*STE7*; patches 2–4 expressed *ste7*-A220 from pYGU7-A220; patch 5 contained the parental plasmid pYES-1 and thus did not express any plasmid-dependent *STE7* protein. (B) The phosphorylation forms of *ste7*-A220 were revealed by immunoblot analysis. Whole-cell extracts (5 μg/lane) were prepared, and immunoblot analysis was performed as described in Materials and methods. All extracts were derived from transformants of the strain YSC-AΔ*ste7*. (Lane 1) A pYGU7-A220 transformant grown in medium containing 2% sucrose; (lane 2) a pYGU7-A220 transformant expressing *ste7*-A220 protein; (lane 3) a pYGU-*STE7* transformant expressing wild-type *STE7* protein. Positions of molecular mass markers (kD) run in another lane of the same gel are indicated at left.

phosphorylated bands observed with wild-type *STE7* protein. In addition, overexpression of *ste7*-A220 in the wild-type strains KMY211A and W303-1A gave immunoreactive species identical in mobility to those produced in YSC-AΔ*ste7* (data not shown), indicating that the presence of the wild-type *STE7* has little or no effect on the phosphorylation state of overexpressed *ste7*-A220 protein.

Discussion

We have discovered a dominant allele of the *STE11* kinase whose expression causes constitutive activation of the mating pathway. Characterization of this dominant mutation shed light on the structure and function of the *STE11* gene product and clarified its relationship to other mating pathway components. The dominant mutation lacks the amino-terminal 351 amino acids present in wild-type *STE11* protein but retains the conserved kinase subdomains in the remaining carboxy-terminal 366 amino acids (Hanks et al. 1988; Rhodes et al. 1990). In the absence of pheromone, the mutant *STE11* protein

(*STE11*ΔN) activates the mating pathway to a level equal to or greater than that in the presence of pheromone, suggesting that the amino terminus of *STE11* acts as a regulatory region that inhibits the activity of the carboxy-terminal catalytic domain. During signaling, upstream components in the mating pathway may interact with this regulatory domain directly or generate a second messenger that influences the function of the regulatory domain.

We have used *STE11*ΔN to determine the functional order of several components of the mating pathway whose epistatic relationships were previously unknown. *STE11*ΔN expression restores a significant level of mating ability to strains lacking either the *STE4* or *STE5* gene but not to strains lacking the *STE7* or *STE12* genes. We conclude that the *STE4* and *STE5* gene products function upstream of the *STE11* gene product, and the *STE7* and *STE12* gene products function downstream of the *STE11* protein. These results, along with those of others (Dietzel and Kurjan 1987; Miyajima et al. 1987; Blinder et al. 1989; Dolan and Fields 1990), are consistent with an order of execution (in MATa cells) as follows: α-factor, *STE2*, *GPA1*(*SCG1*), *STE4*, *STE5*, *STE11*, *STE7*, *STE12*, transcriptional induction. Although this order assumes that the action of the gene products are sequential, more complex interactions are also consistent with the data (Fig. 10).

Our experiments also shed light on the point of action of the *FUS3* and *KSS1* kinases in the signaling pathway. Expression of the dominant allele of *STE11* induced high levels of *STE12*-dependent transcription in strains lacking the *FUS3* or the *KSS1* gene but not in a strain lacking both the *FUS3* and *KSS1* genes. This result is consistent with *FUS3* and *KSS1* serving redundant roles, in concert with, or following the action of, *STE11* protein. Alternatively, these kinases could be required for the synthesis or activity of a pathway component.

We have observed both unphosphorylated and multiply phosphorylated forms of *STE7* protein and have investigated both the origin and significance of these forms. In the absence of pheromone, multiply phosphorylated forms of *STE7* protein are present in strains lacking each of the kinases involved in the mating pathway, including the Δ*fus3*Δ*kss1* double deletion strain. This result requires that multiply phosphorylated forms of *STE7* arise from *STE7* autophosphorylation and/or the direct phosphorylation of *STE7* by a novel kinase. Experiments with the *STE7* mutant *ste7*-A220 indicate that autophosphorylation may play an important role: The vast majority of *ste7*-A220 protein is unphosphorylated, and the scarce *ste7*-A220 phosphorylated form does not comigrate with the multiply phosphorylated species observed with wild-type *STE7* protein. Components upstream in the pheromone response pathway might influence the rate of *STE7* autophosphorylation during signal transmission by one of several mechanisms, including the generation of second messengers or the direct phosphorylation of *STE7* protein by an upstream kinase. Alternatively, a novel kinase may phosphorylate *STE7* protein multiple times and be potentiated by another path-

Figure 10. Order of action of mating pathway gene products. This schematic representation of the order of action of the mating pathway gene products reflects the data presented here and the data of others [Dietzel and Kurjan 1987; Miyajima et al. 1987; Blinder et al. 1989; Dolan et al. 1989]. Although the execution point of the FUS3 and KSS1 kinases appears to be downstream of the STE11 kinase, its precise location remains undefined (brackets). The model does not imply that the interactions are necessarily direct, nor does it exclude the possibility of more complex interactions.

way component. Experiments with purified STE7 protein *in vitro* are needed to establish the possible role of STE7 autophosphorylation in signal propagation.

Three lines of evidence indicate that STE7 phosphorylation plays a role in pheromone response: (1) Although both unphosphorylated and multiply phosphorylated forms of STE7 protein are present in the absence of pheromone activation, multiply phosphorylated forms of STE7 protein accumulate upon exposure to mating pheromone; (2) this accumulation is temporally competent, occurring on the same time scale as other responses to pheromone such as transcriptional induction of the *FUS1* gene [McCaffrey et al. 1987] and the phosphorylation of the transcriptional activator STE12 [Song et al. 1991]; and (3) expression of the dominant allele of *STE11* causes nearly quantitative conversion of STE7 protein to multiply phosphorylated forms.

The correlation of STE7 phosphorylation with mating pathway activation can be interpreted in at least two ways. One interpretation is that multiply phosphorylated forms of STE7 protein are active signal transducers, but the unphosphorylated form of STE7 inhibits signal transduction. The role of upstream components is to induce the conversion of STE7 protein from the unphosphorylated to multiply phosphorylated species, thus relieving the antagonistic action of unphosphorylated STE7. This interpretation is consistent with our obser-

vation that overproduction of STE7 protein does not activate the mating pathway, eliminating the possibility that an increase in the amount of multiply phosphorylated STE7 protein alone can initiate signaling. Alternatively, the repertoire of multiply phosphorylated forms of STE7 in pheromone-stimulated cells may differ from the forms present in unstimulated cells. Thus, there may be unique phosphorylation forms of STE7 protein generated in the presence of pheromone that could not be distinguished in the immunoblot analysis experiments presented here.

A second interpretation is that phosphorylation of STE7 protein is part of the adaptive response to pheromone [Ciejek and Thorner 1979; Sprague and Herskowitz 1981; Courchesne et al. 1989; Cole and Reed 1991; Irie et al. 1991]. According to this interpretation, the phosphorylation of STE7 is important in down-regulating or otherwise altering STE7 protein kinase activity. This model requires the involvement of unphosphorylated STE7 protein in signal propagation, with multiply phosphorylated forms of STE7 having altered substrate specificities or impaired catalytic activities. Further experiments on STE7 phosphorylation, both *in vivo* and *in vitro*, are needed to distinguish between these and other potential models.

Materials and methods

Yeast strains and genetic methods

All strains used in this study (Table 3), except mating testers, are either derivatives of W303-1A (gift of S. Fields) KMY211-A (gift of K. Matsumoto), or YPH499 (gift of P. Hieter). CB023 is a W303-1A derivative lacking the three major vacuolar proteases [Brenner and Fuller 1992]. The protease-deficient strain lacking the *BARI*(*SST1*) gene, YBC102, was prepared from CB023 by one-step gene replacement [Rothstein 1983] with the *SalI*-*EcoRI* fragment of pJGsst1 [Reneke et al. 1988]. Selection of *ura3* recombinants was accomplished with 5'-fluoro-orotic acid (5'-FOA) [Boeke et al. 1984], and replacement was verified by Southern blot analysis [Southern 1975]. Strains lacking the *STE4*, *STE5*, *STE7*, *STE11*, and *STE12* genes are all derivatives of KMY211-A and were prepared and kindly provided by K. Sugimoto. Confirmation of their construction (mating complementation and Southern blot analysis) was performed in collaboration with K. Sugimoto. The derivative lacking the *STE4* gene, YSC-AΔste4, was prepared by one-step gene replacement with the 3.9-kb *PstI*-*PstI* fragment of p4-121 (gift of V. MacKay). Correct integrants were confirmed by complementation of their mating defect with the wild-type gene with pL19 [Whiteway et al. 1990]. The derivative lacking the *STE5* gene, YSC-AΔste5, was prepared by one-step gene replacement with pSF32 (gift of V. MacKay). Correct integrants were confirmed by complementation of the mating defect with the wild-type *STE5* gene with pHO22 (gift of K. Sugimoto). The derivative lacking the *STE7* gene, YSC-AΔste7, was prepared by one-step gene replacement with pNC113 (constructed by B. Errede). Correct integrants were confirmed by complementation of the mating defect with the wild-type gene with pYGU-STE7 (see below) and Southern blot analysis. The derivative lacking the *STE11* gene, YSC-AΔste11, was prepared by one-step gene replacement with the 2.6-kb *XbaI* fragment from pNC202 [Rhodes et al. 1990]. Correct integrants were confirmed by complementation of the mating defect with the wild-type gene using pYGU-STE11 (see be-

Table 3. Yeast strains used in this study

Strains	Genotype	Source
KMY211-A	<i>a his3 leu2 ura3 trp1</i>	K. Matsumoto
YSC-Δste4	<i>a his3 leu2 ura3 trp1 ste4::LEU2^a</i>	this study, with K. Sugimoto
YSC-Δste5	<i>a his3 leu2 ura3 trp1 ste5::LEU2^a</i>	this study, with K. Sugimoto
YSC-Δste7	<i>a his3 leu2 ura3 trp1 ste7::LEU2^a</i>	this study, with K. Sugimoto
YSC-Δste11	<i>a his3 leu2 ura3 trp1 ste11::ura3^a</i>	this study, with K. Sugimoto
YSC-Δste12	<i>a his3 leu2 ura3 trp1 ste12::LEU2^a</i>	this study, with K. Sugimoto
YPH499	<i>a his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 ade2-101 lys2-801^b</i>	D. Ma and J. Thorner
YPH499-Δfus3	<i>a his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 ade2-101 lys2-801 fus3::LEU2^b</i>	D. Ma and J. Thorner
YPH499-Δkss1	<i>a his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 ade2-101 lys2-801 kss1::HIS3^b</i>	D. Ma and J. Thorner
YPH499-Δfus3Δkss1	<i>a his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 ade2-101 lys2-801 fus3::LEU2 kss1::HIS3^b</i>	D. Ma and J. Thorner
W303-1A	<i>a his3-15 leu2-3,112 ura3-1 trp1-1 ade2-1 can1-100</i>	S. Fields
YBC102	<i>a pep4::HIS3 prb1::LEU2 prc1::HISG bar1::HISG can1-100 ade2-101 trp1-1 ura3 his3-15 leu2-3,112^c</i>	this study
NNY19	<i>a his3 leu2 trp1 lys2 FUS1-lacZ::LEU2</i>	K. Matsumoto laboratory
DC14	<i>a his1</i>	R. Fuller
DC17	<i>α his1</i>	R. Fuller

^aKMY211-A strain background.^bYPH499 strain background.^cW303-1A strain background.

low). *ura3* revertants were selected by growth on 5'-FOA plates (Boeke et al. 1984). The derivative lacking the *STE12* gene, YSC-Δste12, was prepared by one-step gene replacement with the *SphI*-*SacI* fragment of pSUL16 (Fields and Herskowitz 1987). Correct integrants were confirmed by complementation of the mating defect with the wild-type *STE12* gene using pOF22 (Dolan and Fields 1990) and by Southern blot analysis.

All media were prepared as described in Sherman et al. (1979). Transformations of yeast strains were accomplished by the use of LiCl (Ito et al. 1983). Mating tests were performed using a modification of the patch test method (Sprague and Herskowitz 1981). Patch tests involving the expression of proteins from the *GAL1* promoter were performed by growing patches on appropriate plates containing minimal medium and 2% sucrose. Matings were performed by replica-plating the patches to plates containing rich media (YP) and 2% galactose, with mating tester (strain DC17) lawns, and growing for 8 hr at 30°C before replica-plating to selective plates containing minimal medium and 2% glucose. Experiments involving mating pheromone were done with α-mating factor purchased from Peninsula Laboratories (no. 8360).

Plasmids

The plasmid pBSCR-STE7 containing the *STE7* gene in Bluescript (BSCR, Stratagene) was prepared by cloning the 1.7-kb *STE7* *BanII*-*HindIII* fragment from pYNS7 (gift of K. Sugimoto) into the *XhoI*-*HindIII* polylinker sites of BSCR and repairing the 5'-coding region with a 56-bp/48-bp *XhoI*-*BanII* oligonucleotide. This oligonucleotide also created an *NdeI* site at the initiator codon flanked by an *EcoRI* site. The bacterial expression plasmid pET-STE7 was prepared by cloning the 1.8-kb *NdeI*-*BamHI* fragment from pBSCR-STE7 into the *NdeI*-*BamHI* site in pET11-A (Novagen). The galactose-inducible yeast overproduction vector pYGU-STE7 was prepared by cloning the 1.8-kb *KpnI*-*NotI* fragment from pBSCR-STE7 into the *KpnI*-*NotI* site of pYES-1(2μ origin, *URA3*, *GAL1* promoter; Invitrogen).

Site-directed mutagenesis of *STE7*, replacing the lysine at position 220 with an arginine, was performed by direct oligonucleotide replacement of the 42-bp/34-bp *AflIII*-*BstXI* fragment of *STE7* with a double-stranded oligonucleotide containing a single-base-pair substitution (AAA → AGA). The dephosphorylated 2.3-kb *AflIII*-*BstXI* fragment of pBSCR-STE7, the 1.3-kb *AflIII* fragment of pBSCR-STE7, and the phosphorylated double-stranded oligonucleotide were ligated to form pBC7-A220. The construction was confirmed by sequencing with the dideoxy chain termination method (Sanger et al. 1977). The site-directed mutant was placed under control of the galactose-inducible *GAL1* promoter by ligation of the 1.8-kb *XbaI*-*XhoI* *STE7* fragment from pBC7-A220 to the 7.8-kb vector *XbaI*-*XhoI* fragment of pSE936 (Elledge et al. 1991), resulting in pYGU7-A220.

The *STE11* gene was cloned from yeast genomic DNA by the polymerase chain reaction (PCR) procedure (Saiki et al. 1988) with *Taq* thermostable DNA polymerase (Cetus) and the oligonucleotide primers 5'-CCCCGAATTCGTCGACCATATGGAACAGACACAAACAGCAGA-3' and 5'-CCCGGATCCTCTAGATGATTACTTAATCAGCGTAATTGA-3'. A 2.4-kb DNA, the sole PCR product produced, was digested with *BamHI* and *XbaI* and cloned into the *BamHI*-*XbaI* site of pSE936 (Elledge et al. 1991) to give the galactose-inducible expression vector pYGU-STE11. Four independent PCR clones were tested in the mating and transcription experiments described in the text.

The plasmid containing the amino-terminal truncation of *STE11* under the control of the *GAL1* promoter was isolated from a genetic screen by S. Ramer (S. Ramer and R. Davis, in prep.). A plasmid with a pSE936 (Elledge et al. 1991) backbone (*URA3*, *CEN4*, *GAL1* promoter), which hybridized with a *STE11* probe, was provided by S. Ramer for further analysis in the present study. This *STE11*-containing plasmid was found to contain a deletion in the 5'-coding region of *STE11* and was therefore named pYGU-11ΔN. Sequencing (Sanger et al. 1977) with an oligonucleotide primer corresponding to nucleotides

764–788 of the *GAL1* promoter (Johnston and Davis 1984) determined the 5' end of this *STE11* allele. The sequence of the junction between the *GAL1* promoter and *STE11* is 5'-(AAACCTCTA)[GCCGAATTCCTCGAGCTACGTCAGGG]<AAGGCTAAGC>-3', with (), [], and < > representing the 3' end of the *GAL1* promoter, the DNA linker, and the 5' end of the *STE11* gene, respectively. The last nucleotide of the *GAL1* promoter included is nucleotide 851 (Johnston and Davis 1984), and the first nucleotide of *STE11* present is nucleotide 1051 (Rhodes et al. 1990). A *LEU2* version of this plasmid (pYGL-*STE11ΔN*) was prepared by cloning the 1.8-kb *EcoRI*-*XbaI* fragment of pYGU-11ΔN into the *SacI*-*XbaI* polylinker sites of pMW20LEU2 (*CEN4 LEU2 GAL1/10* promoter; kindly provided by H. Ziegler and M. Walberg) by use of the phosphorylated oligonucleotide 5'-AATTAGCT-3' to repair the *EcoRI*-*SacI* overhang.

STE11ΔN was overproduced in *E. coli* with the glutathione S-transferase fusion vector pGEX-3X (Pharmacia). *STE11ΔN* was modified by PCR to contain a *Bam*HI site at the 5' end and a *Bgl*II site at the 3' end. This fragment was cloned in-frame into the *Bam*HI site of pGEX-3X to form pGEX-*STE11ΔN*.

The reporter plasmid pUZ4 (*CEN4 TRP1*) containing three consensus-binding sites for the transcription factor *STE12* upstream of the β -galactosidase gene was prepared with the following double-stranded oligonucleotides (gift of K. Sugimoto): 5'-CTAGATGAAACAAACATGAAACATCTGATGAAACAC3' and 5'-TCGAGTGTTCATCAGATGTTTCATGTTTGTTCAT-3'. This oligonucleotide was hybridized and cloned into the *XhoI*-*XbaI* site of pCZΔ (Buchman and Kornberg 1990) to create pCZ4. pUZ4 is a vector containing a pUN20 backbone (*TRP1 CEN4 SUP11*), the 3' end of the *lacZ* gene from pMC1871, and the *XbaI*-*Clal* fragment of pCZ4 containing the *STE12* UAS, the *CYC1* promoter, and the 5' end of the *lacZ* gene.

Purification of *STE7* protein

BL21[DE3] cells (Novagen) containing the lysozyme-expressing plasmid pLysS (Novagen) were transformed with pET11A-*STE7*. The transformant was grown at 37°C in LB medium (2 liters) containing 100 μ g/ml of ampicillin and 25 μ g/ml of chloramphenicol to an OD₆₀₀ of 0.7. The culture was induced with 0.5 mM isopropylthiogalactoside and grown for an additional 2 hr. The cells were harvested by centrifugation at 5000g for 10 min, washed with 100 ml of 20 mM HEPES (pH 7.5), and suspended in 100 ml of buffer A [20 mM HEPES at pH 7.5, 10% glycerol, 10 mM β -mercaptoethanol, 1 mM EDTA, 2 μ g/ml of chymostatin, 2 μ M pepstatin A, 0.6 μ M leupeptin, 2 mM benzamide, 1 mM phenylmethylsulfonyl fluoride (PMSF)], containing 400 mM NaCl. The suspension was lysed by sonication at 4°C and centrifuged at 10,000g for 10 min. The pellet, containing the majority of *STE7* protein, was suspended in 5 ml of buffer A containing 0.5% Triton X-100, incubated at 4°C for 10 min, and centrifuged at 10,000g for 10 min at 4°C. The pellet was washed four additional times in the same manner and suspended in 1 ml of buffer A containing 50 mM NaCl. The resulting *STE7* protein was judged to be ~80% pure by SDS-PAGE electrophoresis.

Purification of *STE11* protein

XL1 cells (Stratagene) were transformed with pGEX-*STE11ΔN*. The *STE11* fusion protein was induced and purified by the same protocol as was the *STE7* protein. The resulting *STE11* fusion protein was judged to be ~90% pure by SDS-PAGE electrophoresis.

Antisera

Approximately 2 mg of purified *STE7* was subjected to electrophoresis in a 10% SDS-polyacrylamide gel (Laemmli 1970). The gel was stained with Coomassie brilliant blue R-250, and a slice containing *STE7* was removed. A rabbit was initially immunized with 0.5 mg of *STE7* and then boosted every 21 days with 0.25 mg of *STE7* protein for a total of four boosts. The antisera used in this study were taken 10 days after the second boost. All antibody preparations were performed by BabCO (Berkeley, CA). Essentially the same protocol was employed for the preparation of *STE11* polyclonal antisera, by use of 2 mg of recombinant *STE11*-GST fusion protein.

Immunoblot analysis

For the analysis of *STE7* phosphorylation in strains containing pYGU-*STE7*, whole-cell extracts were prepared as described below for β -galactosidase assays, except buffer A also contained phosphatase inhibitors (5 mM sodium fluoride, 5 mM sodium phosphate, 10 mM sodium pyrophosphate, 10 mM sodium molybdate, 1 mM EGTA). Whole-cell extracts (5 μ g/gel lane) were subjected to electrophoresis in a 10% SDS-polyacrylamide gel and transferred to nitrocellulose filters (Towbin et al. 1979). The *STE7* polyclonal antibody (1 : 1000 dilution) was used as the primary antibody, and Bio-Rad goat anti-rabbit IgG conjugated to alkaline phosphatase (1 : 2000 dilution) was used as the secondary antibody. A similar protocol, omitting the phosphatase inhibitors, was followed for the analysis of *STE11* protein.

Phosphatase treatments

Whole-cell extract (5 μ g of protein in ~1 μ l) was diluted with 8.5 μ l of phosphatase buffer (100 mM Tris-HCl, at pH 9.6, 2 mM MgCl₂, 0.1 mM ZnCl₂), followed by the addition of 12 units of calf intestinal phosphatase (Boehringer Mannheim; 24 U/ μ l) to all but mock-treated samples. Samples containing phosphatase inhibitors were supplemented with 1 μ l of a 10 $\times$ phosphatase inhibitor cocktail (final concentrations, 5 mM sodium fluoride, 5 mM sodium phosphate, 10 mM sodium pyrophosphate, 10 mM sodium molybdate, 5 mM EDTA, 5 mM EGTA). Mixtures were incubated for 1 hr at 37°C, terminated with 10 μ l of SDS gel loading buffer, and boiled for 2 min before gel electrophoresis.

The immune complexes of *STE7* used for dephosphorylation experiments were prepared as follows: Whole-cell extract (100 μ g of protein) was diluted to a final volume of 200 μ l in buffer B (20 mM Tris-HCl at pH 7.5, 1 mM EDTA, 5% glycerol, 0.5 mM dithiothreitol, 2 μ g/ml of chymostatin, 2 μ M pepstatin A, 0.6 μ M leupeptin, 2 mM benzamide, 1 mM PMSF) containing 100 mM NaCl, and *STE7* polyclonal antibody (4 μ l; 1 : 500 final dilution) was added followed by a 2-hr incubation on a rotator at 4°C. Protein A beads [50 μ l of a 10% solution in buffer B (with 50 mM NaCl and 0.2 mg/ml of BSA)] were added and incubated for an additional hour. The suspension was centrifuged at 4000g for 2 min, and the pellet was washed twice with 0.5 ml of buffer B containing 1 M NaCl and 0.5% Triton X-100, with each rinse separated by a 10-min incubation and a centrifugation at 4000g. The final wash was performed with buffer B containing 50 mM NaCl and no Tris. Dephosphorylation experiments (10 μ l final volume per assay) were performed as indicated above on the immune complexes attached to the protein A beads. Following two washes with 0.5 ml of buffer B containing 1 M NaCl, the beads were suspended in 20 μ l of 2 $\times$ SDS gel loading buffer, and boiled for 2 min before gel electrophoresis.

β -Galactosidase assays

Activation of pheromone-inducible genes was quantified by

β -galactosidase assays with the reporter plasmid pUZ4 (*CEN4 TRP1 ARS*). Strains containing pUZ4 (alone or in combination with galactose-inducible plasmids) were grown in 50 ml of synthetic medium containing the appropriate amino acids, 2% sucrose, and 0.1% glucose to an OD₆₀₀ of 0.5. The cell suspension was centrifuged at 5000g for 10 min, washed with sterile water, resuspended in 100 ml of rich medium (YP) containing 2% galactose, and grown for an additional 8 hr. Cells were harvested and suspended in 1.0 ml of buffer B containing 400 mM NaCl. The cells were disrupted by beating with glass beads for 9 min at 4°C. The extract was centrifuged at 13,000g for 10 min, and the supernatant was centrifuged again at 13,000g for 10 min. β -Galactosidase activities, determined as described by Miller (1972), are given in units per microgram of protein in the whole-cell extract.

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