

STE11 is a protein kinase required for cell-type-specific transcription and signal transduction in yeast

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The *STE11* gene of *Saccharomyces cerevisiae* is one of several genes required for mating between two haploid cell types of this yeast. Its product is required for response to a signal that causes arrest of the mitotic cell cycle in the G₁ phase and induction of mating-type-specific genes. The nucleotide sequence of the *STE11* gene was determined. The predicted amino acid sequence shows homology to the protein kinase family. We demonstrate that the *STE11* product has kinase catalytic activity and that this activity is required for its in vivo functions.

[Key Words: Protein kinase; yeast mating response pathway; gene expression, epitope-tagging]

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The yeast *Saccharomyces cerevisiae* is an experimentally accessible eukaryotic organism for the study of events controlling cell-type determination and cell communication. The **a** and **α** haploid cells of this yeast are able to mate with each other to form an **a/α** diploid cell that is mating incompetent. Recognition between the **a** and **α** cell types initiates and coordinates events that are a precondition for cell fusion. This recognition is mediated by the peptide pheromones, **a**- and **α**-factor, which are characteristic of each cell type and their respective receptors (for review, see Cross et al. 1988; Herskowitz 1989). The signal generated by the interaction between receptor and mating pheromone causes transcriptional induction of genes required for mating, as well as for arrest of the mitotic cell cycle in the G₁ phase.

It appears that all components necessary for the response elicited by pheromone-receptor binding are the same in both cell types (Bender and Sprague 1986; Nakayama et al. 1987). Genetic approaches to dissect the signal transduction pathway have led to the identification of several genes (*GPA1/SCG1*, *STE4*, *STE5*, *STE7*, *STE11*, *STE12*, *STE18*, and *FUS3*) that specify, directly or indirectly, components of the signal pathway (Hartwell 1980; Dietzel and Kurjan 1987; Miyajima et al. 1987; Jahng et al. 1988; Nakayama et al. 1988; Whiteway et al. 1989; Elion et al. 1990). Three of the genes (*GPA1/SCG1*, *STE4*, and *STE18*) were found to encode proteins homologous to the **α**-, **β**-, and **γ**-subunits of mammalian G protein (Dietzel and Kurjan 1987; Miyajima et al. 1987; Jahng et al. 1988; Whiteway et al. 1989). Various observations suggest a model for signal transmission in

which pheromone binding to receptor promotes the exchange of GDP for GTP, resulting in dissociation of the **β,γ**-complex from the GTP bound **α**-subunit. It appears that free **β,γ**-complex transmits the signal (Blinder et al. 1989; Cole et al. 1990; Nomoto et al. 1990; Whiteway et al. 1990). Little is known about the steps in the signal pathway after the G-protein, except that the *STE12* transcription factor has been implicated as a possible nuclear target for the signal (Dolan et al. 1989; Errede and Ammerer 1989).

We have begun investigations to determine what function the *STE11* product has in the pheromone response pathway. Mutation of the *STE11* gene prevents mating and responses to pheromone, including arrest of the mitotic cell cycle in the G₁ phase and induction of cell-type-specific gene expression (Hartwell 1980; McCaffrey et al. 1987; Trueheart et al. 1987). The *STE11* gene product is also required for basal expression from a cell-type-specific activator sequence (Errede et al. 1981). It has been difficult to place the point of action for *STE11* in the signal pathway, because in its absence there is a reduction in the basal-level expression of the pheromones (15- to 100-fold), receptors (5-fold), and G-protein **α**-subunit (2-fold) (Fields et al. 1988; Nakayama et al. 1988). However, we can infer that its function is required for signal transmission after the G-protein because *ste11* mutants block the constitutive G₁ arrest caused by loss of function of the G-protein **α**-subunit (Nakayama et al. 1988). In this study we provide evidence of a *STE11*-dependent protein phosphorylation. This biochemical evidence supports the view that *STE11* is a protein kinase. In vivo analysis of a *STE11* catalytic residue substitution mutation supports the view that its kinase activity is required for its function in the mating response pathway.

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Results

STE11 nucleotide sequence

We determined the nucleotide sequence of the *STE11* gene. The 3.1-kb *HindIII*–*Clal* interval (Fig. 1A, a) from the plasmid pSTE11.1 was used for sequence determination (Chaleff and Tatchell 1985). We report 2578 nucleotides of sequence from this interval for which we have data on both strands (Fig. 2). The DNA sequence was translated in all six frames. The longest open reading frame (ORF) extends from position 32 to position 2266, where it terminates with a TGA translation termination codon. No consensus sequences for RNA splice sites in yeast were detected (Langford and Gallwitz 1983).

STE11 mRNA

The 5' end of the *STE11* mRNA was determined by S1 analysis. The major *STE11* transcript maps to position 86 or 87 with minor transcripts mapping to positions 59

or 60, 68 or 69, 91 or 92, and 98 or 99. The signal from the protected DNA probe was proportional to the amount of *STE11*⁺ RNA in the reaction (Fig. 3A, lanes 1 and 2). No signal was observed for reactions performed with RNA from the isogenic *ste11Δ* strain (Fig. 3A, lane 3) or when RNA was absent from the nuclease digestion reactions (Fig. 3A, lane 4). We found no difference in 5' map positions or abundance of *STE11* mRNA prepared from either pheromone-treated (α -factor) cultures or untreated cultures (Fig. 3B). A TATA-box-like sequence (nucleotide positions 42–47) is present 40 nucleotides upstream from the major mRNA 5' map position. A sequence similar to the tripartite transcription termination control consensus sequence (TAG/TATGT/TTT) identified by Zaret and Sherman (1982) is present in the *STE11* sequence between nucleotides 2352 and 2390 (Fig. 2). On the basis of the 5' map position and the inferred 3' end, the predicted *STE11* transcription unit is close to the observed 2.4-kb size for the *STE11* RNA (Chaleff and Tatchell 1985).

STE11 translation product

The *STE11* ORF contains three ATG codons near the 5' end (located at nucleotide positions 53, 86, and 116; Fig. 2). None of these occurs in a sequence context that is a good match to the consensus associated with translational initiator regions in yeast (Cigan and Donahue 1987). The 5' map positions of *STE11* mRNAs made it possible that the ATG codons at positions 86 and/or 116 could be used for initiation of the *STE11* primary translation product. Frameshift mutations were made to determine which of these codons is necessary for a functional *STE11* product. The rationale for this approach is that a nucleotide insertion or deletion before the initiation codon should not cause a mutant phenotype, whereas an insertion or deletion after the initiation ATG should result in a *ste11* null mutation.

Two frameshift mutations were made. One mutation was an insertion of a single thymidine after nucleotide position 100 (between ATG codons at positions 86 and 116). The other mutation was a deletion of a single guanine residue at nucleotide position 377 (after the ATG codon at position 116). Plasmids carrying the different *STE11* frameshift mutations were used to transform a yeast strain with a complete *ste11* deletion (E929-6C-5). Function of the different *STE11* frameshift mutations was assessed by whether the transformed strains were able to mate with a suitable tester strain. A nucleotide inserted after the ATG at position 86 resulted in a *STE11* product that is functional. In contrast, a single nucleotide deletion after the ATG at position 116 resulted in a nonfunctional *ste11* allele. These results, together with the 5' mRNA mapping experiments, are consistent with translation initiating from the first AUG in the major *STE11* mRNA (Fig. 2). The *STE11* translation product predicted in this case begins with the methionine coded for by the ATG at nucleotide position 116. The predicted *STE11* protein is 717 amino acids in length with a calculated molecular size of 80

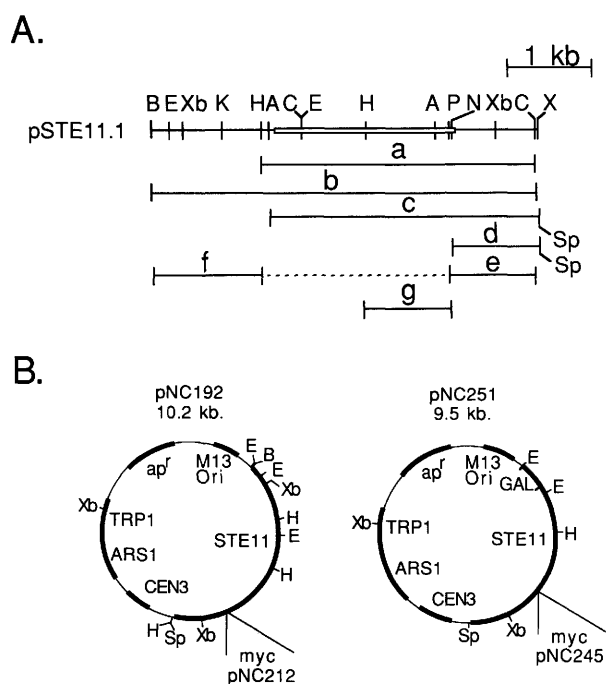


Figure 1. Representation of the *STE11* locus and *STE11* plasmids. (A) The 4.6-kb *Bam*HI–*Xho*I fragment from pSTE11.1 is shown (Chaleff and Tatchell 1985). The open box delimits the *STE11* protein-coding region. Bars indicate fragments a–g used to manipulate *STE11* as described in the text. The *Sph*I site indicated on bars c and d comes from polylinker sequences flanking *STE11* in pNC192 or pNC251. (B) The plasmid pNC192 contains the *STE11* gene and its own promoter. The plasmid pNC251 contains the *STE11* gene under control of the *GAL1,10* promoter. The ampicillin-resistance gene (*ap*^r), M13 origin of replication (M13 ori), and yeast components are indicated. The position of the myc epitope tag in plasmids pNC212 and pNC245 is also indicated. Restriction sites: (A) *Acc*I; (B) *Bam*HI; (C) *Clal*; (E) *Eco*RI; (H) *Hind*III; (K) *Kpn*I; (N) *Nco*I; (P) *Pst*I; (Sp) *Sph*I; (X) *Xho*I; (Xb) *Xba*I.

*Hind*III 10 30 50 * *70 * 90 * * *Acc*I 110
 AAGCTTTCGCCCGGATGGCTTTAAACAGTAGAAATATTCATTTTACACATGCGATAAAGAGAGACACACCTTAATAAAGCTAGTATGATAAAGATCACCCTAGACGAAATATACATGGA
 K I F I F T H M H K E R P L N K A S M I R S P V D E I Y M E
 130 150 170 190 210 230
 ACAGACACAAACAGCAGAGGCCACTGACTTACTTAATGCGTACGAAAAGACCAACGATTACCTTTTGTCCAGTATTTCTGGAGGAAATAGGATGCCACTCAATACCTGGATAGCTTAT
 Q T Q T A E G T D L L I G D E K T I N D L P F V Q L F L E E I G C T T Q Y L D S F I
 250 270 290 310 330 350
 TCAGTCCAACTTCTCAGAGAAGAAATTAAGTATCTCGACAGGATATCCCTCATTGCTTTGGCTGAAACAAAATAGGAGACAGACTCAAAAATTTTAAGGAAGTCAAAAATCGTTCCA
 Q C N L V T E E E I K Y L D K D I L I A L G V N K I G D R L K I L R K S K S F Q
 370 390 410 430 450 470
 GAGAGATAAACGATTGAACAGGTGAATGATGAAAACCTGATGGAAAAGTAAGCTCTCTATCCAGCTGCTACGATGAATTCAGAAATGATTCCTGAAAAGCACTGCTTAT
 R D K R I E Q V N R L K N L M E K V S S L S T A T T L S M N S E L I P E K H C V I
 490 510 530 550 570 590
 ATTATCTTAAACGATGCTTCCGCTAAGAAAGTAAATGTAATCGTTCTTAATGCGATTCTATTAGAAAAGGCTAATCAGAAGATTGCCACATGAATTATGCCACAACTCCAA
 F I L M D G S A K K V N V N G C F N A D S I K K R L I R R L P H E L L A T N S M
 610 630 650 670 690 710
 TGGAGAAGTAACTAAATGCTCCAAAGTATGATGCTTGTCTAGATATACCAAGAACGACTGCTATGCTATATGACGCTGGAATAGTCACTATTTGCCCAAAATGATCGTGT
 G E V T T K M V Q Y D V F V L D Y T K N V L H L L Y D V E L V T I C H A N D R V I
 730 750 770 790 810 830
 TGAGAAAATGCTAAATTTTGTTCCTCAAGACCAACCAAGTGAATGAACTATATCCACATCCAAAAGCTATCTAAGAACGTTGAGTGCATTGACCCAGTTGGCCCACTCTC
 E K N R L I F V S K D Q T F S D K A I S T S K K L Y L R T L S A L S Q V G P S S
 850 870 890 910 930 950
 GTCAAAATTTGTTGGCACAGAACAGGGATTTCGCATAACAATGCTCAAGCGAACTCCGGATCGACACACAGAAAAGGACAGAAATAGACAGATTTTTAATCAGAGCCCTCTAGCGA
 S N L L A Q N K G I S H N N A E G K L R I D M T E K D R I R Q I F N Q R P P S E
 970 990 1010 1030 1050 1070
 ATTATTTCTACCAATTTGCCCGGATATTTCCCTCATACAGACATGAAGCGGTTGCAAAAGACCGATGAGTCAATTTCCGCAATCAGCAGGCTAAGCATTGCTCAAGAAAGACCTTT
 F I S T N L A G Y F P H T D M K R L Q K T M R E S F R H S A R L S I A Q R R P L
 1090 1110 1130 1150 1170 1190
 AACTGCAGAAATCAATAATATCGGTGACATCTATTGAAACCTCAAGCGCTGTTGATATGCCCCCTATTACAGGATTAGATCAGACAAAGATTAAACAGCTAACTGACACATAAAAT
 S A E S N N I T G L I L K H S N A V D M A L T Q G L I D Q T R L S K L D T T K I
*Hind*III 1230 1250 1270 1290 1310
 TCCGAGCTTGGCCATAAAGGCCAGAGATAATGATGCCATATCTAACCTAGTAACTAATAGCTGAGTCTGTGAGAAAGAGATCAGCATTTCTTTGGGAGGACAGTGCAT
 P K L I A H K R P E D N D A I S N Q L E L L S V E S G E E E D H D F F G E D S D I
 1330 1350 1370 1390 1410 1430
 TGTTCATTACCGACGAAAATTTGCCACGCCCAAGAAATGCTTAAAGGTGCTTGCATTGGATCAGCCAGTTTGGGAGTGTACTTGGCCATGAATGCTCACACTGTTGAACTAATGGC
 V S L P T K I A T P K N W L K G A C I G S G S F G S V Y L G M N A H T G E L M A
 1450 1470 1490 1510 1530 1550
 AGTAAAGTGGCAGATAAAAATTAATACATGCTGCTCCCAAGACCAATAAACAGCCATCTGATGAGATAATGAGCAGGAGGAACAACAGAGAAAATAGAAAGATGCTGG
 V K Q V T I K N W N I G V P T D M N K Q A W S D E N N N E Q E E Q E K I E D V G G
 1570 1590 1610 1630 1650 1670
 GCGGTAAATCATCCAAAACCAATCAAAATATTCACAGAAAGATGGTGTGATGCTTTACAGCAATGAATATTTATGAAGGAGTACATGAGAACATTTGTTACTTATTATGCTGC
 A V S H P K T N Q M I H R K M V D A L Q H E M H L L K E L H H E N I V T Y Y G A
 1690 1710 1730 1750 1770 1790
 TTCCTAAGAAAGCCGAAATTTAATATTTTCTGAAATACGTTCTCGGGGCTTCCGTTTCCCTCATGCTGAATAATACCGTCCATTGAGGAATCACTGATTACTAATTTCACTAGGCA
 S Q E G G N L N I F L E Y V P G G S V S S M L N W Y G P F E E S L I T N F T R Q
 1810 1830 1850 1870 1890 1910
 AATACTGATTCGGCTTCCGATTTGCTATAGAAAGACATTAATCAGACGATATCAAGGCTGCAAAATTTTATGATGATATCAAAAGGCTCCGCTAAAATTAATGATTGGTATTTCAAA
 I L I G V A Y L H K K W I I H R D I K G A N I L I D I K G C V K I T D F G I S K
 1930 1950 1970 1990 2010 2030
 AAAATATCACCTTTGAATAAAAACAAAATAAGAGAGCTTCTTTCGAAAGTCCGCTATTCTGATGTCACAGAGGCTGCTCAAAACAGCCCTACTACTGCTAAAGCCGATATATGGTC
 K L S P L N K K Q G S V F W M S P E V V K Q T A T T A T T A K A D I W S
 2050 2070 2090 2110 2130 2150
 TACAGGATGCTGCTCATTGAAATGTTTACCGGTAAGCATCTTCCGAGATTTTCTCAAATGCAAGCCGATCTCAAATAGGCACAAACAGCCCGGAGATACCTTCCGCGCTAC
 T G C V V I E M F T G K H P F P D F S Q M Q A I F K I G T N T T P E I P S W A T
 2170 2190 2210 2230 2250 2270
 GTCAGAAGAAAGAAATTTCTTAAAGAAAGCAATTCAGTTGGATTATCAATACAGCCCTAGTCCCTTGAATTTGAAATGCTGACAGATCCATGGCTGGATGCACACATAATTTGAAAAGGGCTAC
 S E G K N F L R K A F E L D Y Q Y R P S A L E L L Q H P W L D A H I I
 2290 2310 2330 2350 2370 2390
 TTATTAATCTTTTATGGCACTAAAGTCTCTGCGCGATTTTTATCAAATAATACATATATATCAATTTAGTGACATTTATTGAAATATCTGCTATTACCGGTTTTTTTTTTCA
 2410 2430 2450 2470 2490 2510
 ATTACGCTGAATTAAGTAATCATAAATCACAATTTGAAAAGAAAGCAAACTAGATGACGGTATTGATTCTCATACCCCTGGAAATAAATCAAGTAAGATAATTCGTATAAAAAATCC
 2530 2550 2570
 GACCGCACAGATGGAAGCAGGAAGACAGCCCTTCTTTGCGAGCTGATATCAAACC

Figure 2. Nucleotide and predicted protein sequence of the *STE11* gene. The nucleotide sequence of the coding strand is shown. The amino acid sequence for the complete ORF (nucleotide positions 32–2266) is shown below the nucleotide sequence. The initiating methionine (codon positions 116–118) for the predicted protein is underlined. A consensus TATA element (TATTTA) at nucleotide positions 42–47 is overlined. The 5' map positions for the *STE11* mRNA are denoted by asterisks (*) above the nucleotide sequence at positions 59 or 60, 68 or 69, 86 or 87, 91 or 92, and 98 or 99. The transcription termination control sequence (TAG/TATCT/TTT) between nucleotides 2352 and 2390 is underlined. Certain restriction sites are indicated: *Hind*III (positions 1 and 1205), *Acc*I (positions 101 and 2038), *Cl*aI (position 440), *Eco*RI (position 445), *Pst*I (position 2234), and *Nco*I (position 2243).

kD. These data do not indicate whether the AUG at position 86 could be used in the case of the minor mRNAs that map to nucleotide positions 59 or 60 and 68 or 69. Even if this longer translation product is made, it is not an essential species for *STE11* function because the frameshift mutation at nucleotide position 100 has no effect on mating ability. The position 100 frameshift mutation similarly rules out the possibility that a *STE11* product initiating with the ATG codon at position 53 could be essential for mating.

The predicted *STE11* protein is related to protein kinases

The FASTA algorithm of Pearson and Lipman (1988) was used to compare the predicted *STE11* protein to the NBRF protein data base (Release 17, January 1989). The search revealed that the carboxy-terminal region (amino acid positions 413–717) of the predicted *STE11* protein shows striking homology to the catalytic domain of protein kinases. The *STE11* kinase domain has the 15-

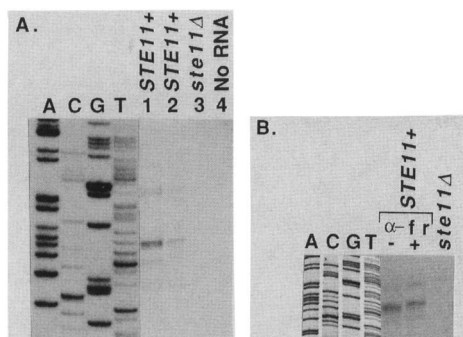


Figure 3. *STE11* 5' mRNA map positions as determined by S1 analysis. (A) (Lanes 1 and 2) Protected products from S1 nuclease digestion reactions using 50 μ g and 20 μ g of total RNA from *STE11*⁺ strain E929-6C; (lane 3) control reaction using 50 μ g of total RNA from *ste11* Δ strain E929-6C-20; (lane 4) mock reaction with no RNA. RNAs were hybridized to the 200-nucleotide probe prepared with oligonucleotide STE11.75 (Table 2). (B) (Lanes *STE11*⁺) Protected products from S1 nuclease digestion reactions using 30 μ g of RNA isolated from cultures of *STE11*⁺ strain E929-6C either without α -factor (-) or after 2-hr exposure to 5 μ M α -factor (+); (lane *ste11* Δ) control reaction using 30 μ g of RNA prepared from *ste11* Δ strain E929-6C-5. RNAs were hybridized to the 1100-nucleotide probe prepared with oligonucleotide STE11.18 (Table 2). Lanes A, C, G, and T in A and B depict dideoxy sequencing products using either labeled oligonucleotide STE11.75 or STE11.18, respectively, as a primer and pNC193 DNA as template. These ladders show the nucleotide sequence of the noncoding strand.

amino-acid residues that are conserved in the catalytic domain for at least 62 of the 65 protein kinases compared by Hanks et al. (1988; Fig. 4). *STE11* has amino acid sequences in subdomains VI (DIKGAN) and VIII (GSVFWMSPE) with high similarity to the sequences DLKPEN and GT/SXXY/PXAPE, respectively, which are indicators of the serine/threonine family of protein kinases (Hanks et al. 1988).

An alignment of the *STE11* kinase catalytic domain was made with that of the three other predicted protein kinases (*STE7*, *KSS1*, and *FUS3*), which have roles in the mating response pathway (Fig. 4) (Teague et al. 1986; Courchesne et al. 1989; Elion et al. 1990). The alignment indicates that *STE11* is not more closely related to this group than to other protein kinases. Outside of the 15 conserved residues characteristic of protein kinases, there are only 18 additional residues conserved among this group. *STE11* is notably more similar to *STE7* in the region of subdomains IV and V (38% identity) than to *FUS3* or *KSS1* (12% identity to each). When the entire catalytic domain is compared, *STE11* is not significantly more similar to *STE7* than to the other kinases. For the catalytic domain overlap region of 255 amino acids, the *STE11* protein showed 27% identity to *STE7*, 24% identity to *FUS3*, and 22% identity to *KSS1*. This is in contrast to the *KSS1* and *FUS3* protein kinases, which are more closely related to each other (54% identity) than to other protein kinases.

Immunological detection of *STE11*

We fused the coding sequence of a known epitope to the *STE11* gene to allow detection of the *STE11* product. The chosen epitope consisted of 10 amino acids from human c-myc for which a specific monoclonal antibody (Myc1-9E10) was available (Evan et al. 1985; Munro and Pelham 1987). An in-frame fusion of the epitope-coding fragment was made after codon 717 of *STE11*. The resulting fusion product, designated *STE11M*, added an 11-amino-acid peptide (EQKLISEEDLN) to the carboxyl terminus of the normal *STE11* protein. The *STE11M* allele carried on a centromeric plasmid (pNC212) was functional when tested for complementation of the mating defect caused by a *ste11* deletion in the recipient strain (E929-6C-5).

We established that the monoclonal antibody Myc1-9E10 specifically detected the tagged *STE11M* protein produced in yeast by immunoblot analysis (data not shown). Because of the low abundance of the *STE11M* protein, this detection required enrichment of the protein by fractionation of significant amounts of extract. To simplify detection procedures and biochemical analyses described below, we overproduced the *STE11M* protein by placing the fusion gene under control of the *GAL1,10* promoter (Fig. 1B, pNC245). An analogous *GAL1,10-STE11* construction (Fig. 1B, pNC251) was made so that we would have a control for overproduction of *STE11* protein in a form that does not react with the Myc1-9E10 antibody. The overproduced *STE11M* protein was easily detected by immunoblot analysis of extracts fractionated by SDS-PAGE (Fig. 5, *STE11M*). Under the same conditions, no yeast protein was detected using the control extract (Fig. 5, *STE11*) or with extract from yeast with a complete *ste11* deletion (Fig. 5, no *STE11*). The Myc1-9E10 antibodies also allowed specific detection of the *STE11M* protein by immunoprecipitation (see below).

STE11 kinase domain mutation

An *in vitro* immune complex assay would allow us to determine whether the *STE11* gene product is a protein kinase (Collett and Erikson 1978). We wanted to have a catalytically inactive protein available as a negative control to establish whether any protein phosphorylation observed in this assay was due to *STE11* activity. The lysine at *STE11* position 444 corresponds to the conserved lysine that is essential for catalytic activity of known protein kinases (Hanks et al. 1988 and references therein). We constructed an arginine substitution mutation at lysine 444 of *STE11M* by site-directed mutagenesis. This mutation was chosen because an arginine substitution at the analogous position of other protein kinases has been shown to abolish kinase catalytic activity (Booher and Beach 1986; Celenza and Carlson 1989). Moreover, the arginine substitution is conservative and not expected to cause instability of the resulting protein.

The *STE11M-R*⁴⁴⁴ protein was expressed from the

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STE11	413	KNWLKGCIG	SGSFGSVYLG	MNAHTGELMA	VKQVEIKNNN	IGVPTDNNKQ	ANSDENNEQE	EQQEKIEDVG
STE7	189	QDLVQLGKIG	AGNSGTVVKA	LHVDPDSKIVA	KKTIPVEQNN	ST.....IIN	QLVRELSIVK	NVK.....
FUS3	11	SDFQLKSLIG	EGAYGVVCSA	THKPTGEIVA	IKKIEPFDKP	LF.....AL	RTLREIKILK	HF.....
KSS1	11	SQYKLVLDIG	EGAYGTVCSA	IHKPSGIVKA	IKKIQPFSSK	LF.....VT	RTIREIKLLR	YFH.....
		-----G	-G-G-V-	-----A	-K-	-----	-----E	-----
			I		II		III	
STE11	483	AVSHPKTNQN	IHRKMVDALQ	HEMNLKELH	HENIVTYYGA	SQEG...GNL	NIFLEYVPGG	SVSSMLNNG
STE7	247P	HENIITFYGA	YYNQHINNEI	IILMEYSDCG	SLDKILSVYK
FUS3	67K	HENIITIFNI	QRPSDFENFN	EVYIIQELMQ	T.DLHRVIST
KSS1	68E	HENIISILDK	VRPVSIDKLN	AVYLVEELME	T.DLQKVINN
					HENI			
					IV		V	
STE11	550	P.....FEESL	ITNFTRQILI	GVAYLHKKNI	IHRDIKGANI	LIDIKGCVKI	TDFGISKKLS
STE7	288	RFVQRGTVSS	KKTWFNELTI	SKIAYGVLNG	LDHLYROYKI	IHRDIKPSNV	LINSKGQIKL	CDFGVSKKLI
FUS3	107	QM.....LSDDH	IQYFYQTLR	AVKVLHGSNV	IHRDLKPSNL	LINSNCDLKV	CDFGLARIID
KSS1	108	QNSGFST...LSDDH	VQYFTYQILR	ALKSIHSAQV	IHRDIKPSNL	LLNSNCDLKV	CDFGLARCLA
						IHRD-K-N-	L-----K-	-----DEF-
					VI		VII	
STE11	606	PLNK.....	.KQNKRASLQ	GSVFWMSPEV	V.KQTATTAK	ADIWSTGCVV	IEMFTGKHPF	PDF...SQMQ
STE7	358	NSIAD.....TFV	GTSTYMSPER	I.QGNVYSIK	GDVWSLGLMI	IELVTGEFPL	GGHN...DTP
FUS3	164	ESAADNSEPT	GQQSGMTEYV	ATRWYRAPEV	MLTSAKYSRA	MDVWSCGCIL	AELFLRRPIF	PGRDYRHQLL
KSS1	170	SSSD...SRE	TLVGFMTYEV	ATRWYRAPEI	MLTFQEYTTA	MDIWSCGCIL	AEMVSGKPLF	PGRDYHHQLW
				-----PE-	-----	-D-WS-G-	-E-----	-----
			VIII			IX		
STE11	665	AIFKIGTNTT	PE.IPSW...ATSE	GKNFLRKAFF	LDYQYRPSAL	
STE7	412	DGILDLLQRI	VN.EPSPRLP	KDRI.....YSKE	MTDFVNRCCI	KNERERSIIE	
FUS3	234	LIFGIIGTTPH	SDNDLRCTES	PRAREYIKSL	PMPYAAPLEK	MFRP...VNP	GIDLLQRLMV	FDPAKRITAK
KSS1	237	LILEVLGTPS	FE.DFNQIKS	KRAKEYIANL	PMPRPLPWET	VWSKTDLNDP	MIDLLDKMLQ	FNPDKRISAA
			X				XI	-----R-----
STE11	705	ELLQHPWLDA	HII*.....
STE7	459	ELLHDLIMK	YVSPSKDDKF	RHWCRIKSK	IKEDKRIKRE	ALDRAKLEKK	QSERSTH*..
FUS3	302	EALRHPYLQT	YHDPNDEPEG	EPIPPSFFEF	DHKKEALTTK	DLKLIWNEI	FS*.....
KSS1	306	EALRHPYLAM	YHDPSEPEY	PPLNLDDEFW	KLDNKIMRPE	EEEEVPIEML	KDMLYDELMM	TME*
		E-L-H-----	-----	-----	-----	-----	-----	-----

Figure 4. Alignment of the conserved protein kinase domain of STE11 with STE7, FUS3, and KSS1. Arabic numerals at the beginning of each line specify the amino acid position for each protein. Roman numerals beneath the sequences refer to subdomains of the protein kinase catalytic domain. The protein alignments and subdomain designations follow the criteria described in Hanks et al. (1988). Residues conserved among the four kinases are shown below the protein sequences. Those residues conserved in 62 of the 65 kinases compared in Hanks et al. (1988) are underlined. The sequences are STE11 (Fig. 2), STE7 (Teague et al. 1986), FUS3 (Elion et al. 1990), and KSS1 (Courchesne et al. 1989).

GAL1,10 promoter (pNC245-R444) in a *ste11* deletion yeast strain (E929-6C-5). The yeast strain that expresses the STE11M-R⁴⁴⁴ protein was not able to mate with an appropriate tester strain. We showed that the same amount of the STE11M (lane 5) and STE11M-R⁴⁴⁴ (lane 3) proteins were present in immune complexes from equivalent amounts of corresponding yeast extracts (Fig. 6A). No yeast protein was detected with the control immune complex performed with the untagged STE11 protein (Fig. 6A, lane 1). These results confirm our expectations (1) that the arginine substitution protein is non-functional and (2) that it is stable.

In vitro kinase assays

Kinase assays were performed directly on immune complexes prior to fractionation and blot analysis of the immune complex proteins (see Fig. 6A). An autoradiograph of the immunoblot detected those proteins present in the immune complex that were ³²P-phosphorylated during the kinase reaction. The autoradiograph shows that a number of phosphoproteins of varying size were generated during assays with all immune complexes (Fig. 6C, lanes 1, 3, and 5). Notably, phosphoproteins

were present at the position of the STE11M or STE11M-R⁴⁴⁴ proteins (Fig. 6C, lanes 5 and 3). There was no phosphoprotein at this position in the control with the untagged STE11 protein (Fig. 6C, lane 1). These results indicate that one or more contaminating kinases are nonspecifically adsorbed with the immune complexes and that both the STE11M and STE11M-R⁴⁴⁴ proteins serve as a substrate. Unfortunately, this result precludes any conclusion as to whether STE11 undergoes autophosphorylation. Nonetheless, the autoradiograph provided evidence for a STE11-dependent phosphorylation. A 78-kD phosphoprotein (p78) was observed among the fractionated proteins from the immune complex with the functional STE11M but not from the complex with the mutant STE11M-R⁴⁴⁴ protein (Fig. 6C, lanes 5 and 3).

It is most likely that the p78 phosphoprotein observed in this assay is an endogenous yeast protein that coprecipitates with STE11. First, it is present whether immune complexes are adsorbed with protein A-Sepharose beads or *Staphylococcus aureus* cells (data not shown). Second, it does not appear to be a protein that cross-reacts with the Myc1-9E10 antibodies because it is not observed on immunoblots of yeast proteins (Figs. 5B and 6A). The presence of p78 has been inferred only from

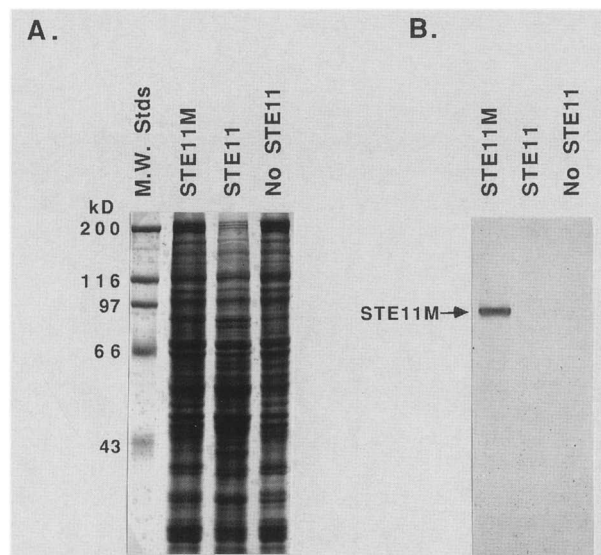


Figure 5. Immunological detection of the STE11M protein. (A) Protein extract was prepared from a *ste11Δ* strain (E929-6C-20) expressing STE11 (pNC251), STE11M (pNC245), or no STE11 (no plasmid). Each extract (100 μg) was separated by SDS-gel electrophoresis and stained with Coomassie blue. Molecular weight standards are myosin, rabbit muscle phosphorylase b, bovine serum albumin, and hen egg white ovalbumin. (B) Immunoblot showing duplicates of samples in A. An arrow indicates the position of the STE11M protein.

its appearance as a phosphoprotein in the protein kinase assay. We were unable to detect p78 directly by using immune precipitates prepared from ³⁵S-labeled protein extracts or by silver stain procedures (data not shown). Therefore, we could not directly rule out the possibility that p78 is a yeast protein whose expression might be dependent on a functional STE11. For this reason additional experiments were performed to ensure that p78 was present in both the STE11M and STE11M-R⁴⁴⁴ extracts used for immune complex formation.

An immune complex assay was performed with STE11 extract that had been mixed with STE11M-R⁴⁴⁴ extract. In this experiment, the STE11 extract would supply the 78-kD polypeptide for coprecipitation with the nonfunctional but immune-reactive STE11M-R⁴⁴⁴ protein. Even though p78 was present in the mixed extract, it was not observed as a phosphoprotein with the nonfunctional STE11M-R⁴⁴⁴ immune complex kinase reaction (Fig. 6C, lane 2). The p78 phosphoprotein was observed in the control experiment where STE11 extract was mixed with STE11M extract (Fig. 6C, lane 4). This control provides evidence that the STE11 extract did not inadvertently inhibit phosphorylation of p78 in the mixing experiment.

A second set of experiments took advantage of the knowledge that there are no known genes whose expressions are STE11-dependent without also being dependent on the STE12 transcription factor (Errede et al. 1981; Fields et al. 1988). If we establish that p78 is present in a *ste12* deletion strain, we can rule out the

possibility that p78 expression is STE11-dependent with reasonable assurance. Immune complex kinase assays were done with extracts prepared from a *ste12* deletion strain (E929-6C-7) expressing either the STE11M or the STE11M-R⁴⁴⁴ protein. Analysis of the fractionated immune complex proteins from these assays showed that equal amounts of the STE11M and the STE11M-R⁴⁴⁴ proteins were present (Fig. 6B, lanes 1 and 2). The p78 phosphoprotein was observed for kinase reactions with the normal STE11M protein (Fig. 6D, lane 2). This result provides direct evidence that expression of p78 is not dependent on the STE12 transcription factor and, by inference, is independent of STE11 function. Yet p78 is not observed as a phosphoprotein for the immune complex assay with the kinase domain mutant STE11M-R⁴⁴⁴ protein (Fig. 6D, lane 1). It should be noted that the functional but untagged STE11 protein is expressed from the genome in the *ste12* deletion strain used here. Therefore, p78 is present in this extract regardless of the deduction that p78 expression is independent of STE11 function. By two independent methods, we have ensured the presence of p78 but have not observed it as a phosphoprotein in kinase assays with the nonfunctional STE11M-R⁴⁴⁴ protein. This result establishes that phosphorylation of p78 is STE11-dependent and provides biochemical support for the view that STE11 is a protein kinase.

Protein kinases require a divalent cation for activity, and different kinases have different cation specificities. We investigated the divalent cation requirement for STE11 kinase activity. Parallel immune complex assays were performed in the presence of 5 mM Mg²⁺, 5 mM Mn²⁺, 5 mM Ca²⁺, or 5 mM Zn²⁺. Autoradiographs of the SDS-PAGE-fractionated proteins were compared visually for the presence and intensity of the signal that corresponds to the 78-kD polypeptide (data not shown). Mn²⁺ appeared to be the preferred ion for kinase activity, followed by Mg²⁺ and Zn²⁺. Ca²⁺ did not support any STE11 catalytic activity.

STE11 kinase activity is not regulated by pheromone induction and is not restricted to the mating-competent cell types

The STE11 gene product is required for mating and for response to pheromone. It is not essential for other cellular processes because cells with a complete deletion of the gene are viable. Therefore, STE11 function appears to be very specific, and one might expect that its expression or activity could be restricted to the cell types and stages where its action is required. It has already been shown that STE11 mRNA is present in all cell types (Chaleff and Tatchell 1985). We have shown that STE11 mRNA is not increased in abundance when cells are treated with pheromone (Fig. 3B). These results indicate that STE11 expression is not regulated by the steady-state abundance of its mRNA.

Immune complex kinase assays were performed to investigate the possibility that STE11 kinase activity may be enhanced or repressed under pheromone-inducing

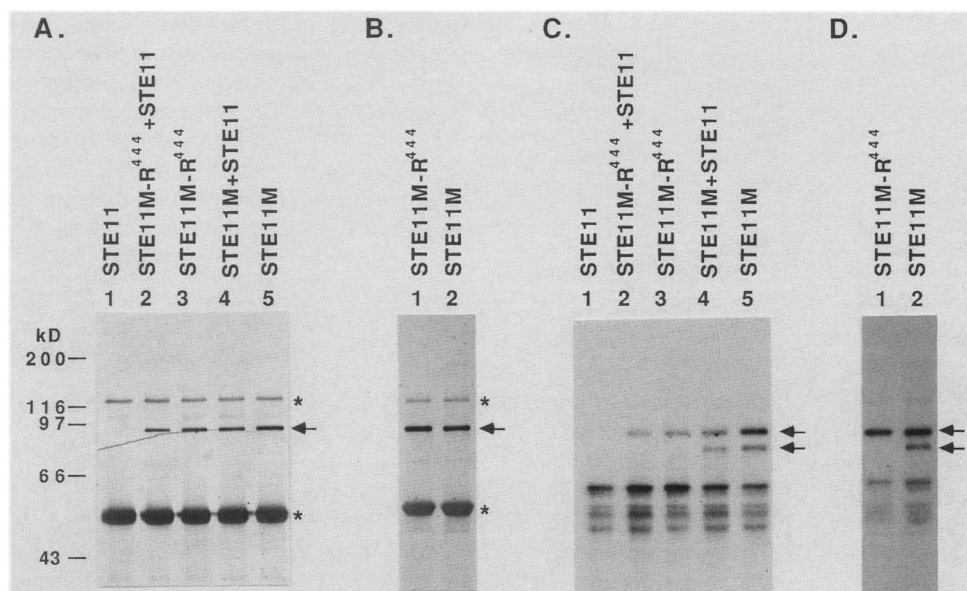


Figure 6. Immune complex kinase assays. (A and B) Immunoblots of immune complexes formed with Myc1-9E10 antibody and protein extracts as specified below. An arrow indicates the position of the STE11M and STE11M-R⁴⁴⁴ proteins. Asterisks (*) show the positions of IgG(H+L) and IgG(H). (C and D) Autoradiographs of the immunoblots in A and B, respectively. The upper arrow indicates the position of phosphorylated STE11M-R⁴⁴⁴ and STE11M; the lower arrow indicates the position of the phosphorylated 78-kD protein p78. (A and C) Protein extracts from transformed *ste11Δ* (E929-6C-5) strains as follows: (Lane 1) STE11 (pNC251); (lane 2) STE11 (pNC251) mixed with STE11M-R⁴⁴⁴ (pNC245-R444); (lane 3) STE11M-R⁴⁴⁴ (pNC245-R444); (lane 4) STE11 (pNC251) mixed with STE11M (pNC245); (lane 5) STE11M (pNC245). (B and D) Protein extracts from transformed *STE11+ ste12Δ* (E929-6C-7) strains as follows: (lane 1) STE11M-R⁴⁴⁴ (pNC245-R444); (lane 2) STE11M (pNC245).

conditions. Immune complex assays were performed with extracts prepared from cultures treated with α -factor. The same amount of STE11M was immunoprecipitated from each extract (Fig. 7A). Little difference in STE11M kinase activity was observed on the basis of its ability to phosphorylate p78 (Fig. 7B). This result suggests that STE11 kinase activity is not regulated by pheromone induction. This conclusion assumes that systems that may activate or inhibit STE11 under conditions of pheromone induction do not become limiting with respect to the amount of STE11 produced under control of the *GAL1,10* promoter.

We also investigated the possibility that STE11 activity as a kinase is restricted to mating-competent cell types. Extracts were prepared from mating-incompetent a/α diploid cells that overproduce the STE11M or STE11M-R⁴⁴⁴ protein. Both proteins were present in immune complexes from the respective diploid cell extracts (Fig. 7C). Autoradiographs of the proteins fractionated from the kinase assay showed that p78 was phosphorylated in reactions with the STE11M protein but not the inactive STE11M-R⁴⁴⁴ protein (Fig. 7D). These results indicate that STE11 kinase activity is not absolutely restricted to the haploid cell type.

STE11 kinase activity is required for its functions in vivo

We wished to determine whether STE11 function as a kinase is required for both G₁ arrest and the transcriptional controls that are a prerequisite for mating. To

make these tests, a *MATa* yeast strain with a complete deletion of the *ste11* gene (E929-6C-28) was transformed with a centromeric plasmid that either does not express STE11 (pNC160), that expresses the normal STE11 protein (pNC193), or that expresses the catalytically inactive STE11-R⁴⁴⁴ protein (pNC193-R444). The transformed strains were first compared for ability to mate by the standard patch test. The *ste11Δ* transformed strain that did not express STE11 (E929-6C-28 pNC160) and the strain that produced the STE11-R⁴⁴⁴ protein (E929-6C-28 pNC193-R444) were completely mating defective (Table 1).

These three strains were then compared for their ability to become arrested in the G₁ phase by treatment with pheromone. The culture expressing the STE11 protein behaved toward pheromone in the characteristic fashion (Fig. 8). After a 2-hr exposure to pheromone, cells arrested at the G₁ stage of the cell cycle, as evidenced by the accumulation of unbudded cells. The culture accumulated unbudded cells (80%) for 4 hr. After this time cells began to recover from pheromone-induced arrest, and numbers of unbudded cells in the cultures decreased. In contrast, the culture without any STE11 and the culture expressing the STE11-R⁴⁴⁴ kinase mutation maintained the same low percentage of unbudded cells in the culture (25–30%) throughout the time course of the experiment (Fig. 8).

We made use of two different reporter genes to determine whether the STE11-R⁴⁴⁴ kinase mutation would affect STE11-dependent transcription. The recipient strain used for these comparisons carries the *CYC7-H2*

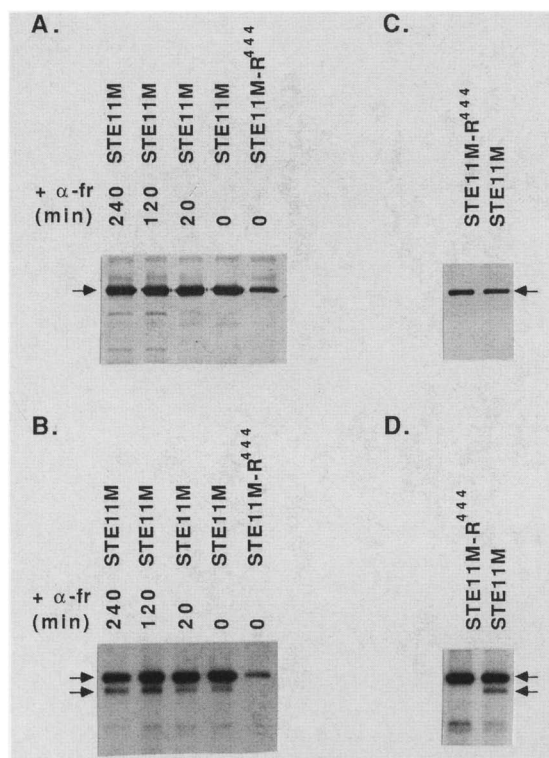


Figure 7. In vitro kinase activity of STE11 is not regulated by pheromone or cell type. (A and C) Immunoblots of immune complex kinase reactions performed with protein extracts as specified below. An arrow indicates the position of the STE11M and STE11M-R⁴⁴⁴ proteins. (B and D) Autoradiographs of immunoblots shown in A and C, respectively. The upper arrow indicates the position of the phosphorylated STE11M-R⁴⁴⁴ and STE11M proteins; the lower arrow indicates the position of the phosphorylated 78-kD protein, p78. (A and B) Protein extracts from transformed *ste11Δ* (E929-6C-28) strains expressing STE11M (pNC245) or STE11M-R⁴⁴⁴ (pNC245-R444) proteins. For pheromone induction, the culture expressing the STE11M protein (E929-6C-28, pNC245) was treated with 5 μ M α -factor, and protein extracts were prepared at indicated intervals after induction. (C and D) Protein extracts were from *MATa/MATα* diploid (E929-6C-32) strains expressing STE11M (pNC245) or STE11M-R⁴⁴⁴ (pNC245-R⁴⁴⁴) proteins.

gene as a reporter for basal levels of cell-type-specific gene expression and the *FUS1-lacZ* gene as a reporter for pheromone-inducible gene expression. The *CYC7-H2* allele is an insertion of a Ty1 element in the 5'-non-coding region of *CYC7*, which is the structural gene for iso-2-cytochrome *c*. The inserted Ty1 element causes iso-2-cytochrome *c* expression to come under mating-type control and to be dependent on a functional STE11 product (Errede et al. 1981). The STE11-R⁴⁴⁴ kinase mutation was defective in supporting basal levels of cell-type-specific transcription as measured by iso-2-cytochrome *c* production from the *CYC7-H2* reporter (Table 1). Expression of *FUS1* in yeast is pheromone inducible, and this induction requires a functional STE11 product (McCaffrey et al. 1987). The 44-bp pheromone-inducible upstream activating sequence (UAS) from the

FUS1 gene was cloned upstream of the *Escherichia coli lacZ* gene, which encodes β -galactosidase. The STE11-R⁴⁴⁴ kinase mutation was defective in supporting pheromone-inducible transcription as measured by β -galactosidase activity from the *FUS1-lacZ* reporter (Table 1). The results of the above experiments show that strains expressing the kinase domain mutant protein were defective for both pheromone-induced G₁ arrest and transcriptional activation functions. Because a single amino acid substitution that abolishes kinase activity is responsible for these defects, we conclude that STE11 kinase activity is crucial for each of these in vivo functions.

We also investigated the possibility that the *ste11-R444* allele may have a dominant negative phenotype. A strain with a normal chromosomal copy of *STE11* (E929-6C-16) was transformed with the centromeric plasmid that overproduces the catalytically inactive STE11-R⁴⁴⁴ protein (pNC245-R444). There was no interference with mating ability in the transformed strain even though the STE11-R⁴⁴⁴ protein was produced in large excess (E929-6C-16 pNC245-R444; Table 1). The more sensitive and quantitative assay for pheromone-inducible expression of the *FUS1-lacZ* reporter was also applied. A fivefold reduction in the amount of β -galactosidase was observed with extracts from the strain that overproduces the non-functional STE11-R⁴⁴⁴ protein (E929-6C-16 pNC245-R444) compared with the wild-type strain without overproduction of a STE11 protein (E929-6C-16). Therefore, it appears that the STE11-R⁴⁴⁴ protein may partially sequester components that become limiting for the mating response. Taken together, these data indicate that the *ste11-R444* allele has only a weakly dominant negative phenotype.

Discussion

Protein phosphorylation is required for signal transmission and response to pheromone

The predicted protein sequence and biochemical evidence presented here support the view that STE11 is a protein kinase. On the basis of genetic criteria, STE11 function is required on the pheromone-induced signal pathway at a point after the G protein and coincident with or prior to the STE12 transcription factor (Nakayama et al. 1988; Dolan and Fields 1990). It follows that a STE11-dependent protein phosphorylation is required for signal transmission between these two components. There are two alternative models by which the STE11 kinase could fulfill this role. According to one model, the STE11 kinase could be transiently activated to transmit the induction signal by phosphorylation of a component on the pathway. In an alternative model, a STE11-dependent phosphorylation is a precondition for function or presence of a component that receives and transmits the pheromone-induced signal by a STE11-independent process. The immune complex assay we applied did not reveal a change in STE11 kinase activity under pheromone-inducing conditions. At face value, this result favors the second model for STE11 function

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Table 1. Mating and transcriptional activation assays

Strain	Plasmid	Relevant genotype	Mating ^a	Iso-2-cytochrome <i>c</i> ^b	β-Galactosidase ^c	
					- α-fr	+ α-fr
E929-6C-28	none	<i>ste11Δ</i>	-	5	10	11
E929-6C-28	pNC160	<i>ste11Δ</i>	-	ND	ND	ND
E929-6C-28	pNC193	<i>ste11Δ, pSTE11+</i>	+	18	3	1035
E929-6C-28	pNC193-R444	<i>ste11Δ, pste11-R444</i>	-	5	9	8
E929-6C-16	none	<i>STE11+</i>	+	20	64	4692
E929-6C-16	pNC245	<i>STE11+, pGALSTE11M</i>	+	ND	37	5821
E929-6C-16	pNC245-R444	<i>STE11+, pGALste11M-R444</i>	+	ND	8	1367

(ND) Not determined.

^aMating competence (+) or mating incompetence (-) was determined by the patch test.^bOne unit corresponds to the amount of cytochrome *c* produced in standard *cyc1 CYC7+* yeast strains.^cUnits of β-galactosidase are (1000 × ΔA₄₂₀)/(min × mg extract).

on the response pathway. However, an in vitro kinase assay could be insensitive to in vivo regulatory mechanisms. Therefore, the question of whether the STE11 kinase transmits the pheromone-induced signal or is necessary to establish the function of some other signal transmission component remains unresolved.

Three other genes (*STE7*, *KSS1*, and *FUS3*) whose functions are implicated in the mating signal pathway have protein sequences predicting that they encode protein kinases. So far, STE11 is the only one for which biochemical evidence of a protein phosphorylation function has been provided. Each of the four kinases is likely to have a different role in the response pathway. On the basis of the phenotype of null mutants, it is clear that the activity of the KSS1 kinase is required for recovery from pheromone-induced G₁ arrest, whereas FUS3 has a predominant role in establishing pheromone-induced G₁ arrest. The STE7 and STE11 kinases are required for pheromone-inducible transcription, G₁ arrest, and mating. Even though the null mutant phenotypes are the same for the STE7 and STE11 kinases, it is unlikely that the two have redundant functions. First, the sequences of these two kinases are significantly different. This would exclude the possibility that they are isozymes or members of the same kinase subfamily. Second, we have found that the mating defect caused by a deletion of *ste7* is not relieved by overproduction of the STE11 kinase and vice versa (N. Rhodes and Z. Zhou, unpubl.).

Possible targets for STE11 kinase

Resolution of the precise role that the STE11 kinase plays in the signal process will be aided by identification of its physiological targets and a knowledge of their biochemical functions. One possible physiological substrate of the STE11 kinase is the p78 protein that becomes phosphorylated in the immune complex kinase assay. Other protein kinases have been found to immunoprecipitate in complexes with physiologically relevant polypeptides. For example, the Cdc2 kinase immunoprecipitates in a complex with a functionally relevant substrate (Booher et al. 1989). If p78 is a relevant

substrate, it represents an as-yet-unidentified component of the pheromone response pathway. The 78-kD mobility is too slow for the predicted size of the α-subunit (54 kD), β-subunit (47 kD), or γ-subunit (13 kD) of G-protein, the STE7 protein kinase (55 kD), or the FUS3 kinase (40 kD) (Teague et al. 1986; Dietzel and Kurjan 1987; Miyajima et al. 1987; Whiteway et al. 1989; Elion et al. 1990). The 78-kD mobility is faster than that observed for the STE12 (116 kD) or STE5 (115 kD) polypeptides (B. Errede, unpubl.; M. Hasson, R. Freedman, J. Schultz, and J. Thorner, in prep.). Even though none of the known components of the response pathway was implicated as a target of STE11 by the immune complex assay, some of these are logical candidates. For example, the STE12 transcription factor, the STE7 kinase, and the STE5 protein are each phosphoproteins (B. Errede, unpubl.; Z. Zhou, unpubl.; M. Hasson, R. Freedman, J. Schultz, and J. Thorner, in prep.).

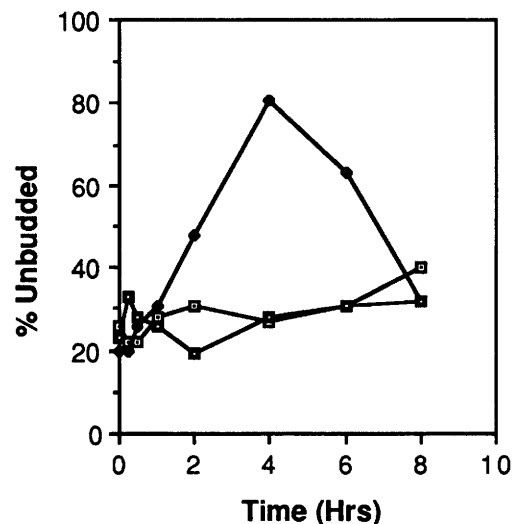


Figure 8. Pheromone-induced G₁ arrest. Cultures of exponentially growing E929-6C-20 cells carrying plasmids pNC160 (□), pNC193 (◆), or pNC193-R444 (■) were treated with α-factor (5 μM). Aliquots were removed at appropriate intervals, and the percentage of unbudded cells was determined.

Materials and methods

DNA sequence determinations

The *STE11* DNA sequence was determined by the chain-termination method according to "shotgun cloning" and sequencing procedures (Sanger et al. 1977; Bankier and Barrell 1983). DNA sequence data were assembled using DBSYSTEM programs on a VAX-11/780 computer (Staden 1982). In several regions, data for only one strand were obtained from the random cloning and sequencing strategy. Specific oligonucleotides were synthesized and used as sequencing primers for completion of the DNA sequence on both strands.

Plasmids

The *STE11* centromeric plasmids pNC192 (Fig. 1B) and pNC193 were constructed by cloning the 4.6-kb *Bam*HI–*Xho*I fragment (b) from pSTE11.1 (Fig. 1A) into the *Bam*HI–*Sal*I sites of the yeast shuttle vectors pNC160 and pNC161, respectively (Chaleff and Tatchell 1985; Rhodes et al. 1990). Fragment b includes the *STE11* gene and promoter sequences. The pNC192 and pNC193 plasmids are identical except that the *STE11* gene is in opposite orientations in relation to the M13 origin of replication on the vector. The two plasmids allow single-stranded DNA to be obtained for either the coding or noncoding strands of *STE11*. A construction was made with the *GAL1,10* promoter to allow overproduction of the STE11 protein. A 685-bp *Eco*RI–*Bam*HI fragment containing the *GAL1,10* promoter from pBM150 was cloned into the *Eco*RI–*Bam*HI site of pNC160 (Johnston and Davis 1984; Rhodes et al. 1990). The *Hind*III site in the polylinker of the vector was destroyed by end-repair with Klenow polymerase and religation. The *STE11* gene was cloned into the *Bam*HI(end-repaired)–*Sph*I site of this vector as the *Acc*I(end-repaired)–*Sph*I fragment (c) (Fig. 1A). The resulting plasmid is designated pNC251 (Fig. 1B).

The plasmids pNC203 and pNC202 carry the *ste11Δ6* and *ste11-Δ6::URA3* alleles, respectively. Construction of pNC203 involved the following steps. The *STE11* 3'-flanking region was isolated as the 1-kb *Pst*I–*Clal*(end-repaired) fragment (e) from pSTE11.1 (Fig. 1A) and cloned into the *Pst*I–*Acc*I(end-repaired) sites in pUC119 (Vieiria and Messing 1987). This fragment was transferred as a *Sph*I–*Bam*HI fragment into the *Sph*I–*Bam*HI sites in pNC98 (Company and Errede 1987). The *STE11* 5'-flanking region was isolated as the 1.3-kb *Bam*HI(end-repaired)–*Hind*III fragment (f) from pSTE11.1 (Fig. 1A) and cloned into the *Hind*III(end-repaired)–*Eco*RI sites of the previous construction. The 1.2-kb end-repaired–*Hind*III fragment containing the *URA3* gene from pLG669-Z was isolated and cloned into the *Hpa*I site of pNC203 to give pNC202 (Guarente and Ptashne 1981). All end-repair steps were accomplished by filling in of 5' overhanging ends with Klenow polymerase.

The YRp7MAT α plasmid used for *MAT* gene replacement was provided by K. Tatchell and has been described (Tatchell et al. 1981). The *FUS1-lacZ* reporter gene was provided by G. Ammerer on the integrating plasmid pGA1716. An oligonucleotide with *Xho*I-compatible ends and a sequence corresponding to the *FUS1* UAS (nucleotide positions 3036–3074; Trueheart et al. 1987) was inserted into the *Xho*I site of pLGΔ178 (Guarente et al. 1984). To give the integrating plasmid pGA1716, sequences encompassing the 2- μ m origin of replication were deleted by digestion with *Hind*III and religation.

All plasmid constructions and DNA manipulations were performed as described (Maniatis et al. 1982). Enzymes were pur-

chased from U.S. Biochemicals, New England Biolabs, and Bethesda Research Labs.

Site-directed mutagenesis

Two *STE11* frameshift mutations were constructed. A single base pair insertion at *STE11* nucleotide position 100 and a single base pair deletion of *STE11* nucleotide position 377 were generated with mutagenic oligonucleotides STE11.26 and STE11.23, respectively (Table 2). pNC193 single-stranded DNA served as template for each of the mutagenesis reactions. The respective products of the mutagenesis were designated pNC193.100 and pNC193.377. The mutations were confirmed by DNA sequence determinations using the corresponding sequencing primers STE11.27 and STE11.18 (Table 2).

A fusion of the entire *STE11*-coding sequence with an oligonucleotide encoding the myc epitope tag (EQKLISEEDLN) was constructed by site-directed mutagenesis. The oligonucleotide used to insert the epitope-coding sequences (STE11.39; Table 2) includes the last four codons of *STE11* (nucleotide positions 2255–2266), 11 codons for the peptide tag followed by the *STE11* stop codon and 3'-noncoding sequences (nucleotide positions 2267–2280). Single-stranded DNA from pNC192 served as the template for oligonucleotide-primed DNA synthesis. The insertion of the epitope-encoding sequences was confirmed by DNA sequence determination using oligonucleotide STE11.40 (Table 2) as the sequencing primer. The resulting construction is designated pNC212 (Fig. 1B). pNC245 carries the *STE11*–myc fusion under control of the *GAL1,10* promoter. pNC245 was constructed by inserting the *Nco*I–*Sph*I fragment (d) (Fig. 1A) from pNC212 into the *Nco*I–*Sph*I site of pNC251 (Fig. 1B).

A lysine (amino acid position 444) to arginine substitution in the kinase catalytic domain of STE11 was constructed by use of mutagenic oligonucleotide STE11.29 (Table 2). pNC193 single-stranded DNA served as template for the mutagenesis reaction. The product of the mutagenesis reaction was designated pNC193-R444. The substitution mutation was confirmed by DNA sequence determination using the oligonucleotide primer STE11.30 (Table 2). The substitution mutation under control of the *GAL1,10* promoter (pNC245-R444) was constructed by cloning the 1.0-kb *Hind*III–*Nco*I fragment (g) (Fig. 1A) from pNC193-R444 into the *Hind*III–*Nco*I site of pNC245 (Fig. 1B).

Oligonucleotides (Table 2) for these studies were made by automated synthesis at the oligonucleotide facility in the Department of Pathology at the University of North Carolina. Single-stranded template DNA was produced by the method of Kunkel (1985) with the addition of the helper phage M13K07 (Vieira and Messing 1987). Mutagenic oligonucleotides were annealed to the template, and second strand synthesis was performed as described (Zoller and Smith 1982). The resulting double-stranded DNA was transformed into *E. coli* strain DH5 α F' (Bethesda Research Laboratories). The desired mutations were identified by DNA sequence of random *E. coli* isolates. The phenotype of at least two independent isolates for each mutation was tested in yeast.

Yeast genetic procedures

The LiCl procedure was used for yeast transformations (Ito et al. 1983). Gene replacements were performed according to the method of Rothstein (1983). Unless specified, media and genetic procedures were as described in Sherman et al. (1979).

Pheromone inductions were performed by use of α -factor (synthesized in the UNCCH-NIEHS Protein Chemistry Lab) at a final concentration of 5 μ M in medium that was buffered to pH 4.5 as described (Fink and Styles 1972). Pheromone-induced G₁ arrest was assayed by using exponentially growing cells exposed to α -factor. Aliquots were removed at appropriate in-

Table 2. Oligonucleotides

Oligonucleotide	Sequence (5'-3') ^a	Strand ^b	Nucleotide homology ^c
STE11.18	AATATAACACAGTGC	–	469–483
STE11.23	ATTCACCTGTTAATCCGTTT	–	368–389
STE11.26	TTCGTCTACCAGGTGATCTTA	–	90–109
STE11.27	CTCTGCTGTTTGTGT	–	125–139
STE11.29	CTCCACTTGCNTTACTGCC	–	1438–1456
STE11.30	TTGTTGTCTGTGGGAAC	–	1478–1494
STE11.39	GCACACATAATTGAGCAAAAAGCTCATTTC TGAAGAGGACTTGAATTGAAAAAGGGCTAC	+	2255–2280
STE11.40	GGATTATCAATACAGGC	+	2200–2217
STE11.75	CTCCAGAAATAACTGCACAA	–	186–205

^aMutagenic base(s) are underlined. (N) A mixture of the four nucleotides.

^b(+) Coding strand of STE11; (–) noncoding strand.

^cNumbers indicate positions in the STE11 gene.

intervals and fixed in 5% formaldehyde, and cells were scored under a microscope as budded or unbudded. At least 200 individual cells were scored for each culture.

Mating competence was scored by the patch test (Sprague and Herskowitz 1981).

Yeast strains

The strain E929-6C (*MAT α cyc1, CYC7-H2, can1, leu2-3,112, trp1- Δ 1, ura3-52*) was used for the construction of the isogenic derivatives described below. The strains KZ8-1D (*MAT α cyc1-1, CYC7, his4, ura1*) and KZ8-5C (*MAT α cyc1-1, CYC7, his4, ura1*) were used as tester strains for determining mating phenotypes.

The *ste11* deletion strain E929-6C-5 (*ste11- Δ 6 :: URA3*) was constructed from E929-6C by use of the 2.6-kb *Xba*I fragment from pNC202 for gene replacement. A second *ste11* deletion strain without the *URA3* marker, E929-6C-20 (*ste11- Δ 6*), was constructed from E929-6C-5 by use of the 1.4-kb *Xba*I fragment from pNC203 for gene replacement. The *ste11- Δ 6 :: URA3* allele in E929-6C-5 and *ste11- Δ 6* allele in E929-6C-20 were confirmed by Southern blot analysis. Strains with the *FUS1-lacZ* reporter gene, E929-6C-16 (*STE11⁺ FUS1-lacZ*) and E929-6C-28 (*ste11- Δ 6 FUS1-lacZ*) were constructed from E929-6C and E929-6C-20, respectively, by integration of pGA1716. The plasmid pGA1716 was linearized with *Apa*I to target its integration to the *ura3-52* locus of the respective recipient strains. The diploid strain E929-6C-32 (*MAT α /MAT α LYS2⁺/lys2*) was made by crossing strain E929-6C to E929-6C-15 (*MAT α lys2*). First, an isogenic *MAT α* derivative of E929-6C was constructed by gene replacement using the 4.2-kb *Hind*III *MAT α* fragment from the plasmid YRp7MAT α . A *Lys*[–] derivative of this *MAT α* strain was selected on α -amino adipate (Chattoo and Sherman 1979). The lesion causing the *Lys*[–] phenotype was shown to be a *lys2* mutation by complementation analysis with a known *lys2* strain. Construction of the *ste12* deletion strain E929-6C-7 (*STE11⁺ ste12- Δ 1 :: URA3*) has been described (Company and Errede 1988).

RNA analyses

RNA was prepared from cultures of E929-6C and E929-6C-20 in YPD medium. Cultures were grown to a density of 1×10^7 to 2×10^7 cells/ml at 30°C. RNA from pheromone-induced cells was prepared from E929-6C cultures in buffered YPD medium that had been exposed to 5 μ M α -factor for 2 hr (Fink and Styles 1972). Total RNA was prepared according to the glass-bead lysis and extraction method (Broach et al. 1979).

Probe DNA for S1 protection analysis was prepared either

with oligonucleotide STE11.75 or oligonucleotide STE11.18 (Table 2). The oligonucleotides were end-labeled using polynucleotide kinase and [γ -³²P]ATP. The labeled primers were annealed to pNC193 template DNA and extended with Klenow polymerase. In the case of STE11.75, a 200-nucleotide probe was obtained by digestion with *Hind*III. In the case of STE11.18, a 1.1-kb nucleotide probe was obtained by digestion with *Kpn*I. The probe DNAs were isolated from their corresponding mixtures after fractionation on a denaturing polyacrylamide gel (Dretzen et al. 1981). S1 nuclease reactions were performed according to established procedures (Greene and Struhl 1987). The size of the protected fragments was determined by electrophoresis on 8% denaturing polyacrylamide gels. A reference DNA sequence ladder was provided by DNA sequence reaction products obtained with pNC193 template DNA and the appropriate ³²P-end-labeled primer (McGraw 1984).

Preparation of yeast protein extracts

Cultures requiring selection of plasmids were grown to mid-log phase in selective medium. Other cultures were grown in complete synthetic medium. Galactose (2%) was used as the carbon source where overproduction of the STE11 protein required induction of the *GAL1,10* promoter. Pheromone (α -factor)-induced cultures were grown as described above with aliquots of the same culture taken at various intervals after treatment with α -factor. Protein extracts were prepared as described by Company et al. (1988). A 40% ammonium sulfate fraction was used for the experiments presented in this work. All protein concentrations were determined by use of the Bio-Rad protein assay reagent kit.

Immunoprecipitations and kinase assays

Immunoprecipitations and kinase assays were performed as described by Celenza and Carlson (1989). For our purpose, 500 μ g of protein extract was incubated with 10 μ g of the mouse monoclonal antibody Myc1-9E10 at 4°C (Evan et al. 1985). The antibody–protein complexes were precipitated by adsorption to *Staphylococcus aureus* cells (Sigma) or protein A–Sepharose beads (Sigma). The kinase assays were performed directly on the washed, adsorbed immune complexes by incubation with kinase assay buffer in the presence of [γ -³²P]ATP. After the reaction, proteins were released from the adsorbed complex by boiling in SDS-PAGE buffer, fractionated by SDS-PAGE, and transferred to nitrocellulose. The nitrocellulose filters were subjected to immunoblot analysis for detection of immunoprecipitated proteins prior to autoradiography (see below).

Immunoblot analysis

Whole-cell extracts or immune-precipitated proteins were fractionated on a 10% SDS-polyacrylamide gel and electroblotted onto nitrocellulose paper (Laemmli 1970; Towbin et al. 1979). The Myc1-9E10 antibody (2 μ g/ml) was used to detect the electroblotted STE11M and STE11M-R⁴⁴⁴ proteins. The Promega Protoblot immunoblotting system with goat anti-mouse IgG conjugated to alkaline phosphatase (0.1 μ g/ml) was used to detect the primary antibody by colorimetric methods. Immunoblotting and color development were performed according to procedures provided with the Promega Protoblot system.

Assays for reporter gene products

Enzymatic activity of β -galactosidase was measured by use of whole-cell protein extracts prepared 3 hr after induction with α -factor. Approximately 10^8 cells were pelleted and washed with 1 ml of Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgCl₂, 50 mM 2-mercaptoethanol) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) (Miller 1982). The washed cells were suspended in 200 μ l of Z buffer with 1 mM PMSF. Cells were lysed by vigorous vortexing with glass beads (0.45–0.50 μ m diameter) for 8 min at 4°C. The supernatant from a 5-min centrifugation was removed to a clean tube, and protein concentration was determined. The β -galactosidase assays were performed by use of 10 μ g of protein extract in 120 μ l of Z buffer containing 2.2 mM *O*-nitrophenyl- β -D-galactopyranoside. The change in absorbance at 420 nm with time was followed using a V_{\max} kinetic microtiter plate reader (Molecular Devices).

The relative amount of iso-2-cytochrome *c* was determined spectroscopically from intact yeast cells that had been grown under derepressed conditions for cytochrome *c* content (Errede et al. 1981).

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