Mutational Analysis of *STE5* in the Yeast *Saccharomyces cerevisiae*: Application of a Differential Interaction Trap Assay for Examining Protein-Protein Interactions

Carla Inouye,*[,1] Namrita Dhillon,*[,1,2] Tim Durfee,[†,1] Patricia C. Zambryski[†] and Jeremy Thorner*

**Department of Molecular and Cell Biology, Division of Biochemistry and Molecular Biology and [†]Department of Plant and Microbial Biology, University of California, Berkeley, California 94720*

Manuscript received May 6, 1997
Accepted for publication June 27, 1997

ABSTRACT

Ste5 is essential for the yeast mating pheromone response pathway and is thought to function as a scaffold that organizes the components of the mitogen-activated protein kinase (MAPK) cascade. A new method was developed to isolate missense mutations in Ste5 that differentially affect the ability of Ste5 to interact with either of two MAPK cascade constituents, the MEKK (Ste11) and the MEK (Ste7). Mutations that affect association with Ste7 or with Ste11 delineate discrete regions of Ste5 that are critical for each interaction. Co-immunoprecipitation analysis, examining the binding *in vitro* of Ste5 to Ste11, Ste7, Ste4 (G protein β subunit), and Fus3 (MAPK), confirmed that each mutation specifically affects the interaction of Ste5 with only one protein. When expressed in a *ste5*Δ cell, mutant Ste5 proteins that are defective in their ability to interact with either Ste11 or Ste7 result in a markedly reduced mating proficiency. One mutation that clearly weakened (but did not eliminate) interaction of Ste5 with Ste7 permitted mating at wild-type efficiency, indicating that an efficacious signal is generated even when Ste5 associates with only a small fraction of (or only transiently with) Ste7. Ste5 mutants defective in association with Ste11 or Ste7 showed strong interallelic complementation when co-expressed, suggesting that the functional form of Ste5 *in vivo* is an oligomer.

IN the yeast *Saccharomyces cerevisiae,* the binding of mating pheromone to its receptor activates the associated G protein, resulting in release of the G$\beta\gamma$ complex (Ste4–Ste18). This step initiates subsequent events that prepare haploid cells for fusion (reviewed in SPRAGUE and THORNER 1992). These downstream events require a signal transduction pathway, in which both cytoplasmic and membrane-associated constituents (CHENEVERT *et al.* 1994; SIMON *et al.* 1995; ZHAO *et al.* 1995; LEBERER *et al.* 1996; LYONS *et al.* 1996; PRYCIAK and HARTWELL 1996) lead to alterations in cell polarity and morphology (reviewed in CID *et al.* 1995) and to activation of nuclear components, thereby inducing changes in gene expression and arresting cells in the G1 phase of the cell cycle (reviewed in SCHULTZ *et al.* 1995). The regulatory processes that occur in the nucleus require the activity of an evolutionarily conserved mitogen-activated protein kinase (MAPK) cascade (reviewed in BARDWELL *et al.* 1994; HERSKOWITZ 1995) comprised of the following: Ste11, a MAPK kinase kinase (MEKK); Ste7, a MAPK kinase (MEK); and, Fus3 and Kss1, two MAPKs partially redundant in function. When Fus3 and Kss1 become activated via phosphorylation (GARTNER *et al.* 1992; MA *et al.* 1995), they in turn phosphorylate and stimulate both Ste12 (ELION *et al.* 1993), a transcription factor responsible for the induction of pheromone-induced genes (FIELDS and HERSKOWITZ 1985; HAGEN *et al.* 1991), and Far1, a Cdk inhibitor of the G1 cyclin-bound forms of the protein kinase, Cdc28 (TYERS and FUTCHER 1993; PETER and HERSKOWITZ 1994). The function of the entire pheromone response pathway requires a novel protein, Ste5 (reviewed in ELION 1995).

The *STE5* gene product (LEBERER *et al.* 1993; MUKAI *et al.* 1993; PERLMAN *et al.* 1993) is a 917-residue polypeptide that shares similarity to Far1 over two limited regions (~50 and ~20 amino acids, respectively), but possesses no extensive homology to any other protein in current databases. Overexpression (HASSON 1992; AKADA *et al.* 1996) or mutational activation (HASSON *et al.* 1994) of Ste5 stimulates the mating pathway in the absence of pheromone, suggesting that its function in signal transmission may be rate-limiting. Moreover, the hyperactive alleles of *STE5* partially suppress the mating defects of *ste4*Δ and *ste18*Δ mutants, but not *ste11*Δ, *ste7*Δ, *fus3*Δ *kss1*Δ, or *ste12*Δ mutants, arguing that Ste5 functions downstream of the G$\beta\gamma$ complex, but upstream of or interdependently with the components of the MAPK cascade (HASSON *et al.* 1994). Genetic and biochemical evidence indicates that Ste5 physically associates with the Ste4 component of G$\beta\gamma$. First, Ste5 was isolated initially as a multi-copy suppressor of a *ste4*^{ts}

Corresponding author: Jeremy Thorner, Department of Molecular and Cell Biology, Division of Biochemistry and Molecular Biology, Room 401, Barker Hall, University of California at Berkeley, Corner of Hearst and Oxford Streets, Berkeley, CA 94720-3202.
E-mail: jthorner@mendel.berkeley.edu

[1]These authors contributed equally to this work.

[2]*Present address:* Laboratory of Molecular Embryology, National Institute of Child Health and Human Development, National Institutes of Health, Building 18T, Room 106, Bethesda, MD 20892-5431.

mutation, which did not suppress a *ste4* null mutation (MACKAY 1983). Second, the effects of the hyperactive *STE5* alleles are significantly more potent in a *ste4-3*^{ts} mutant than in a *ste4*Δ background (HASSON *et al.* 1994); correspondingly, stimulation of mating by overexpression of normal Ste5 requires the presence of Ste4 (HASSON *et al.* 1994; AKADA *et al.* 1996). Conversely, *ste5* missense alleles were identified by virtue of their "synthetic" sterile phenotype when combined with conditional *ste4* mutations, implying that the two proteins functionally interact (AKADA *et al.* 1996). Finally, at least some Ste4 is present in a stable complex with Ste5, as judged by co-immunoprecipitation of these proteins from cell extracts (WHITEWAY *et al.* 1995).

Ste5 also functionally interacts with all of the protein kinases of the pheromone signaling pathway. The *STE5* gene was isolated as a multi-copy suppressor of *ste20* mutants (LEBERER *et al.* 1993), suggesting that Ste5 functions downstream of Ste20. *STE20* encodes a Ser-/Thr-specific protein kinase (RAMER and DAVIS 1993; WU *et al.* 1995) related to mammalian p65^{PAK} (MANSER *et al.* 1994) and appears to act early in the mating pathway at (or near) the function of G$\beta\gamma$ (LEBERER *et al.* 1992; HASSON *et al.* 1994; AKADA *et al.* 1996). Ste20 may serve as a MEKK kinase (MEKKK) to initiate the MAPK signaling cascade, although it has not been demonstrated that Ste20-mediated phosphorylation of Ste11 directly activates this MEKK (NEIMAN and HERSKOWITZ 1994; WU *et al.* 1995). Although initial epistasis analysis (LEBERER *et al.* 1992; STEVENSON *et al.* 1992) suggested that Ste5 might act upstream of the MAPK module, activated alleles of *STE11* or *STE7* only weakly suppress *ste5* mutants (STEVENSON *et al.* 1992; YASHAR *et al.* 1995), and overexpression of the MAPKs does not allow *ste5*Δ cells to mate (HASSON 1992), suggesting a more complex function for Ste5. Indeed, using the two-hybrid method to assess protein-protein interaction, Ste5 was found to associate *in vivo* with Ste11, Ste7, Kss1 and Fus3 (CHOI *et al.* 1994; MARCUS *et al.* 1994; PRINTEN and SPRAGUE 1994), and deletion analysis showed that distinct regions of Ste5 appear to be required for its interaction with each of these components. Physical association of Ste5 with some of these components in cell extracts has been confirmed by biochemical methods. For example, Ste11 and Ste5 bind sufficiently tightly to remain as a complex upon density gradient sedimentation (CHOI *et al.* 1994), and Fus3-Ste5 complexes can be co-immunoprecipitated (KRANZ *et al.* 1994). On the basis of these findings, it was proposed that Ste5 acts as a scaffold that juxtaposes the MAPK cascade components facilitating efficient signaling (reviewed in ELION 1995).

Structure-function analysis of Ste5 to date has utilized large deletion mutations, at least some of which perturb more than one function of the protein and may affect as yet unknown properties of the protein. To assess the biological significance of the various interactions of Ste5 with other proteins, missense mutations would be especially useful. Here we describe a differential interaction trap scheme that we used to identify single amino acid substitutions that specifically affect Ste5 association with only one of its demonstrated partners, either Ste11 or Ste7. Such point mutants can abolish the ability of *STE5* to complement a *ste5*Δ mutation, demonstrating for the first time that these discrete protein-protein interactions are essential for Ste5 function *in vivo*. The phenotype of one of these alleles suggests that the association of Ste5 with Ste7 may only be transient. Moreover, we found that *ste5* mutants severely crippled in their ability to associate with either Ste11 or Ste7 show strong interallelic complementation when co-expressed, a genetic hallmark of multimeric proteins. Hence, Ste5 presumably oligomerizes *in vivo*, and this self-association is likely to be necessary for the function of Ste5 in signal transmission.

MATERIALS AND METHODS

Bacterial and yeast strains: *Escherichia coli* strains SURE (Stratagene) and DH5α(F$^-$ *endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1*) were used for routine plasmid manipulations, and MH1066 (Δ*lacX74 hsr*$^-$ *rpsL pyrF:Tn5 leuB600 trpC9830 galE galK*) was used for recovery of library plasmids from yeast. The following *S. cerevisiae* strains were constructed. A *lys2* derivative of a W303 haploid (*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 lys2*Δ*::hisG trp1-1 ura3-1*) was transformed with an *Xba*I fragment containing a *ste5* deletion-insertion allele (*ste5*Δ*::LYS2*) that was excised from plasmid pLYSEA. Lys$^+$ prototrophs were selected, scored for a sterile phenotype, and authentic transplacement of the *STE5* locus was further verified by Southern hybridization analysis. One of the resulting *ste5*Δ strains was designated BYB69. A Gal$^+$-revertant of a protease-deficient haploid, BJ2168 (*MAT***a** *GAL leu2 prb1-1122 pep4-3 prc1-407 trp1 ura3-52*) (JONES 1991), was transformed with pCJ99 that had been cut with *Bgl*II to target integration to the *STE5* locus. pCJ99 (see next section) is a derivative of the *URA3*-marked integrating vector, YIplac211, and contains a genomic fragment from which nearly all of the *STE5* coding region has been deleted. Ura$^+$ prototrophs were selected; subsequently, segregants from which the integrated plasmid had been excised were selected by growth on medium containing 5-fluoro-orotic acid (5-FOA; American Bioorganics, Inc.). The Ura$^-$ colonies were scored for a sterile phenotype, and proper transplacement was verified by PCR. One of the resulting *ste5*Δ strains was designated BYB84. Strain YCJ4 (*MAT***a** *his3-*Δ*200 leu2-3,112 trp1-901 ura3-52 can*^{R} *gal80-538 gal4-542 ade2::*P_{GAL}*-URA3 lys2::lexA*$_{op}$*-lacZ*) was prepared as follows. YM954 (*MAT***a** *his3-*Δ*200 leu2-3,112 trp1-901 ura3-52 can*^{R} *gal80-538 gal4-542 ade2-101 lys2-801*), kindly provided by M. JOHNSTON, Department of Genetics, Washington University, St. Louis, MO, was first transformed with *ADE2* DNA and Ade$^+$ prototrophs (white colonies) were selected. The Ade$^+$ derivative was transformed with *LYS2* DNA (BARNES and THORNER 1986), and Ade$^+$ Lys$^+$ colonies were selected. The *GAL1-URA3* reporter was introduced into this strain by integration of the following construct: 879-bp *Mun*I-*Mun*I fragment from the 3′-end of the *ADE2* gene, 825-bp *Eco*RI-*Bam*HI fragment containing the *GAL1-10* promoter, 985-bp *Bam*HI-*Hin*dIII fragment containing the *URA3* coding sequence, and 1022-bp *Hin*dIII-*Hin*dIII fragment from the 5′-end of the *ADE2* gene. Ade$^-$ transformants (red colonies) were picked, and correct integration at the *ADE2* locus was confirmed by Southern hybridization analysis. The *lacZ* re-

porter was introduced by integration of a construct composed of the following: 469-bp *Spe*I-*Nco*I fragment from the 5′-end of the *LYS2* gene, 4.5-kb *Nco*I-*Sma*I fragment from pSH18ΔSpe (kindly provided by ROGER BRENT, Department of Molecular Biology, Massachusetts General Hospital, Boston, MA) containing the *GAL1* promoter with *lexAop* sites inserted upstream of *lacZ*, and 930-bp *Eco*RV-*Hin*dIII fragment of the 3′-end of the *LYS2* gene. This construct was introduced along with a plasmid expressing a LexA-Gal4 fusion, and filter replicas of the resulting transformants were assayed using 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside (X-gal) indicator to identify clones expressing β-galactosidase. Furthermore, Southern hybridization analysis was used to confirm authentic integration at the *LYS2* locus.

Plasmids: All plasmids were constructed using standard molecular biology techniques (SAMBROOK *et al.* 1989). Constructs prepared from DNA fragments generated via amplification using PCR were verified by the dideoxynucleotide sequencing method. *Pfu*I DNA polymerase (Stratagene) was used for all PCR reactions, except where indicated otherwise. YCpLG and YCpUG (kindly provided by L. BARDWELL, this laboratory) were derived from the centromere-based plasmids, YCplac111 and YCplac33 (GIETZ and SUGINO 1988), respectively, and contain the 685-bp *GAL1,10* promoter inserted as a *Bam*HI-*Eco*RI fragment. A construct, $(His)_6$MycSTE5, in which the *STE5* coding sequence was fused in-frame to a DNA encoding a $(His)_6$ tract and a c-Myc epitope tag was generated by PCR with appropriate synthetic oligonucleotide primers to yield a coding strand sequence with a *Bam*HI site (underlined) at its 5′-end: 5′-GGATCCATATGATG$(CAT)_6$G CCATCGAAGAACAAAAGTTGAATTCTGAAGAAGATTTG TTGAGAAAGAGAGATGCCGAA-3′. This modified version of *STE5*, which contains a genomic *Bam*HI site 265 bp downstream of the natural TAG stop codon, was excised as a 3.3-kb *Bam*HI-*Bam*HI fragment and inserted into pUC18, generating pCJ3. A *Sph*I-*Sph*I fragment, which contains the *ADH1* promoter, DNA encoding Gal4 activation domain (Gal4AD), and the *ADH1* terminator, was excised from pGAD10 (Clontech, Inc.) and inserted into pUC18, yielding pGAD18. PCR was used to produce another derivative of *STE5* that has a modified 5′-end with a *Bam*HI site (underlined) and the sequence 5′-GGATCCTGGAAACTCCTACAGAC-3′; the resulting product was inserted as a *Bam*HI-*Bam*HI fragment into the *Bgl*II site in pGAD18, downstream of and in-frame with the sequences that encode the Gal4AD, yielding pCJ67. The *ADH1* promoter- and terminator-containing *Sph*I-*Sph*I segment in pGAD10 was replaced with the *Sph*I-*Sph*I fragment from pCJ67 (which encodes the Gal4AD-Ste5 fusion driven by the *ADH1* promoter and terminator), generating pCJ68. The *Bam*HI-*Bam*HI fragment containing the $(His)_6$MycSTE5 construct DNA was excised from pCJ3 and inserted (in the correct transcriptional orientation) into the *Bam*HI site in YCpUG, yielding YCpUG-$(His)_6$MycSTE5. To produce plasmids expressing tagged versions of each of the *ste5* alleles, *Asp*718-*Xho*I inserts excised from each of the mutant DNAs were substituted for the corresponding segment in YCpUG-$(His)_6$MycSTE5. YCp33-PHMSTE5 was constructed in several steps. First, the native *STE5* promoter was amplified by PCR using a genomic clone as the template and suitable primers, resulting in a product that spanned the *Xba*I site at the 5′-end of the gene and included, at its 3′-end, a *Bam*HI site just downstream of the normal start codon. This product was inserted into the vector, YCplac33, that had been cleaved with *Xba*I-*Bam*HI, yielding YCp33-P. The *Bam*HI-*Bam*HI fragment from pCJ3 that encodes the $(His)_6$MycSTE5 derivative was then inserted in-frame into the *Bam*HI site, yielding YCp33-PHMSTE5. To produce plasmids expressing each of the *ste5* alleles, the corresponding *Bam*HI-*Bam*HI fragments from the YCpUG-$(His)_6$MycSTE5 derivatives were inserted into YCp33-P. pCJ99 was constructed by first removing the *Asp*718 site in the vector, YIplac211, by digesting with *Asp*718, filling-in with T4 DNA polymerase, and religating, to yield YIplac211ΔAsp. The 3.7-kb genomic *Xba*I-*Bam*HI fragment containing the *STE5* gene was subcloned into the corresponding sites in YIplac211ΔAsp; the resulting plasmid was then digested with *Asp*718 and *Xho*I to remove essentially all of the *STE5* coding region, and the ends so created were converted to flush ends by incubation with T4 DNA polymerase and religated. A *Sal*I-*Sac*I fragment, which contains both the *STE4* and *STE18* genes expressed divergently from the *GAL1,10* promoter, was excised from pRS316.GAL-STE4/18 (SONG *et al.* 1996) and inserted into YCplac22 that had been digested with *Sal*I and *Sac*I, generating pCJ125. An *Nde*I-*Bam*HI fragment containing the *STE7* coding region was excised from pYGUSTE7 (CAIRNS *et al.* 1992) and inserted into pAS1 (DURFEE *et al.* 1993) that had been digested with *Nde*I and *Bam*HI, yielding pAS-STE7. A *Bam*HI-*Hin*dIII fragment containing *STE7* was excised from pNC279 (kindly provided by BEVERLY ERREDE, Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, NC) and inserted into YCpLG that had been digested with *Bam*HI and *Hin*dIII, yielding YCpLG-STE7. The 5′-end of the *STE11* gene was modified using standard PCR methods to introduce a *Bam*HI site (underlined) just upstream of the translation initiation codon (italic), 5′-GGG ATCCAT*ATG*-3′. Sequences encoding an influenza virus hemagglutinin (HA) epitope, 12CA5, and a *Bam*HI site (underlined), 5′-TACCCATACGACGTCCCAGACTACGCT TAGGATCCC-3′, were inserted in-frame immediately following codon 402 of the modified *STE11* coding sequence. The 1.25-kb *Bam*HI-*Bam*HI fragment containing the resulting construct (STE11ΔC·HA) was inserted into pLEX202PL (kindly provided by ROGER BRENT, Department of Molecular Biology, Massachusetts General Hospital, Boston, MA) that had been digested with *Bam*HI, generating pLex-Ste11ΔC. The 5′-end of the *STE11* gene in pSTE11.1 (RHODES *et al.* 1990) was modified using PCR to introduce a *Bam*HI site immediately upstream of the initiation codon (as described immediately above). A *Bam*HI-*Xba*I fragment containing the modified *STE11* gene was excised and inserted into YCpLG that had been cleaved with *Bam*HI and *Xba*I, generating YCpLG-STE11. pLYSEA is described elsewhere (HASSON *et al.* 1994) and was constructed by RICHARD FREEDMAN (this laboratory). YEpT-Fus3 is described elsewhere (BARDWELL *et al.* 1996) and was constructed by DOREEN MA (this laboratory).

Isolation of *ste5* mutants: To generate mutations in *STE5*, pCJ67 was used as the template for PCR amplification under standard conditions with Taq DNA polymerase (Perkin-Elmer) and the following primers: Gal4-AD, 5′-ATCAAAGTG GGAATATTGCTGATAGC-3′; and AdhT, 5′-GCATGCCGG TAGAGAGAGGTGTGG-3′. Competent cells were prepared from YCJ4 containing pAS-STE7 and pLEX-Ste11ΔC-HA by pregrowing them under selective conditions in SCGlc medium lacking histidine and tryptophan, and then diluting them into 800 ml of prewarmed YPGlc medium and growing them for two generations (until A_{600nm} reached ~0.8). A sample (20 μg) of pGAD10 that had been linearized by cleavage with *Bgl*II and a sample (20 μg) of the PCR-generated mutagenized DNA were co-transformed into the YCJ4 cells using the lithium acetate method. The resulting library of pGAD10 derivatives containing potentially mutant *STE5* sequences was generated by gap repair *in situ* (MUHLRAD *et al.* 1992). One half of the transformation mixture was plated on 10 large Petri dishes containing SCGlc medium lacking histidine, tryptophan, leucine and uracil, and including X-gal; the other half was plated on 10 large Petri plates containing SCGlc medium lacking histidine, tryptophan and leucine, and in-

cluding both 5-FOA and X-gal. The plates were incubated at 30° for 3–5 days.

Quantitation of β-galactosidase activity: Cultures of yeast cells carrying *lacZ* reporter constructs were grown in an appropriate selective medium to an A_{600nm} of 0.8–1.0, and then prepared and assayed for β-galactosidase activity as described in detail elsewhere (DURFEE *et al.* 1993) using as the substrate, chlorophenol-red-β-D-galactopyranoside (CPRG; Boehringer Mannheim).

Immunoprecipitations: Cells of strain BYB84 carrying plasmids expressing various genes under control of the *GAL* promoter were pre-grown under appropriate selective conditions in SCRaf medium to an A_{600nm} of 0.6. Gene expression was induced by adding galactose to a final concentration of 2% and incubating the cultures for an additional 2–3 hr. Cells were harvested by centrifugation, washed, and lysed by vigorous vortex mixing with glass beads in cold lysis (L) buffer (20 mM Tris-HCl pH 7.2, 12.5 mM potassium acetate, 4 mM $MgCl_2$, 0.5 mM EDTA, 5 mM sodium bisulphite, 0.1% Tween-20) containing 12.5% glycerol, 1 mM dithiothreitol, and additional protease inhibitors (2 μg/ml leupeptin, 2 μg/ml pepstatin A, 1 mM benzamidine, 2 μg/ml aprotinin, and 1 mM AEBSF). The crude extracts were clarified by centrifugation in a microfuge for 10 min at 4°. Samples (1 mg total protein) were diluted to 600 μl in L buffer containing 1% bovine serum albumin, and a suspension of Protein A + Protein G-agarose beads (15 μl; Oncogene Science) was added. The mixtures were agitated on a rollerdrum for 30 min at 4°, and then the beads were collected by centrifugation in a microfuge for 5 min. The supernatant solution was transferred to a fresh microfuge tube, to which was added another sample (20 μl) of the Protein A/G-agarose beads and 1 μl of mouse ascites fluid containing the anti-c-Myc monoclonal antibody, 9E10 (EVAN *et al.* 1985). The mixtures were incubated on the rollerdrum for 2 hr at 4°, and then the beads were collected, washed three times with 1 ml each of ice-cold L buffer, and resuspended in SDS-PAGE sample buffer. The bound immune complexes were released by boiling for 5 min, and the eluted material was resolved by SDS-PAGE. The resulting gel was electrophoretically transferred onto an Immobilon-P membrane filter (Millipore) using a semi-dry transfer cell (Bio-Rad). Immunoblotting was performed with the appropriate primary antibodies, followed by appropriate horse radish peroxidase-conjugated secondary antibodies, and then visualized by a chemiluminescence detection system (NEN).

Mating assays: Patch mating tests were performed by replica-plating patches of cells onto a lawn of appropriate mating type tester strains on YP medium containing as the carbon source either 2% galactose + 0.2% sucrose or 2% glucose (depending on the promoter controlling *STE5* expression). After incubation overnight at 30°, the mating plates were replica-plated onto plates containing synthetic minimal medium selective for diploids and incubated at 30° until growth was clearly visible. Quantitative mating assays were performed according to SPRAGUE (1991). Briefly, the *MAT***a** strains to be tested were pregrown in an appropriate selective medium containing either 2% raffinose or 2% glucose (depending on the promoter controlling *STE5* expression) to mid-exponential phase. For strains carrying galactose-inducible *STE5* constructs, cells were pregrown in raffinose medium and expression was induced by addition of 2% galactose for 60 min before initiation of the mating test. To initiate mating, 0.2 ml of 10-fold serial dilutions of the *MAT***a** strains were mixed, in triplicate, with 0.6 ml of the *MATα* tester strain, DC17, which was pregrown to mid-exponential phase in YPGal/Suc or YPGlc medium, as appropriate. Samples (0.4 ml) of these mixtures were plated on medium lacking the appropriate supplements to select for diploids (and containing either Gal/Suc or Glc, as appropriate) and incubated at 30° for 36–40 hr. Corresponding dilutions of the *MAT***a** strains were also plated on YPGlc to determine the input number of viable haploid cells. Mating efficiencies were calculated as the ratio of the number of diploid cells formed over the total number of input haploids.

RESULTS

Rationale and strategy for development of the differential interaction trap approach: The biological relevance of a given protein-protein interaction can be established by studying the effects of specific mutations that affect only the association in question and no other activities of a protein. It is frequently difficult, however, to identify among randomly generated mutations those that have such a differential effect on the properties of a gene product. We have devised and implemented a generally applicable method that allows the interactions of a single protein with two of its targets to be monitored simultaneously in the same cell. To test the feasibility of this approach, we have utilized some of the tools already developed for two-hybrid assays (FIELDS and SONG 1989; MENDELSOHN and BRENT 1994; BAI and ELLEDGE 1996) to delineate specific residues responsible for the interaction of *S. cerevisiae* Ste5 with two of its known partners, Ste11 and Ste7 (Figure 1). We have dubbed this approach the "differential interaction trap" method.

The recipient strain (YCJ4) is transformed with three separate plasmids. Each plasmid encodes a chimeric protein: one expresses a fusion of Ste5 to the transcriptional activation domain (AD) of the Gal4 transactivator, another expresses a fusion of Ste7 to the Gal4 DNA-binding domain (Gal4), and the third expresses a fusion of Ste11 (lacking its C-terminal kinase domain) to the LexA DNA-binding domain (LexA). To provide transcriptional read-outs unique to each interaction, the recipient strain harbors two reporter constructs: the *URA3* gene driven by UAS*GAL* (integrated at the *ADE2* locus), and the *E. coli lacZ* gene under control of the *lexA* operator (integrated at the *LYS2* locus). Thus, interaction between AD-Ste5 and each of the DNA-binding domain fusions (Gal4-Ste7 and LexA-Ste11) should yield a cell that is phenotypically Ura^+, β-galactosidase-positive, and unable to grow on a medium containing 5-FOA (BOEKE *et al.* 1984) (Figure 1A).

To confirm the validity of this method, the plasmid (pGAD-Ste5) expressing the AD-Ste5 fusion, or an empty vector control (pGAD10), was introduced into YCJ4 cells carrying both the plasmid (pAS-Ste7) expressing the Gal4-Ste7 fusion and the plasmid (pLex-Ste11ΔC) expressing the LexA-Ste11 fusion. As expected, cells expressing all three chimeric proteins were Ura^+, β-galactosidase-positive, and 5-FOA-sensitive; control cells expressing just the two DNA-binding domain fusions were Ura^-, β-galactosidase-negative, and 5-FOA-resistant (Figure 2). Two additional controls were per-

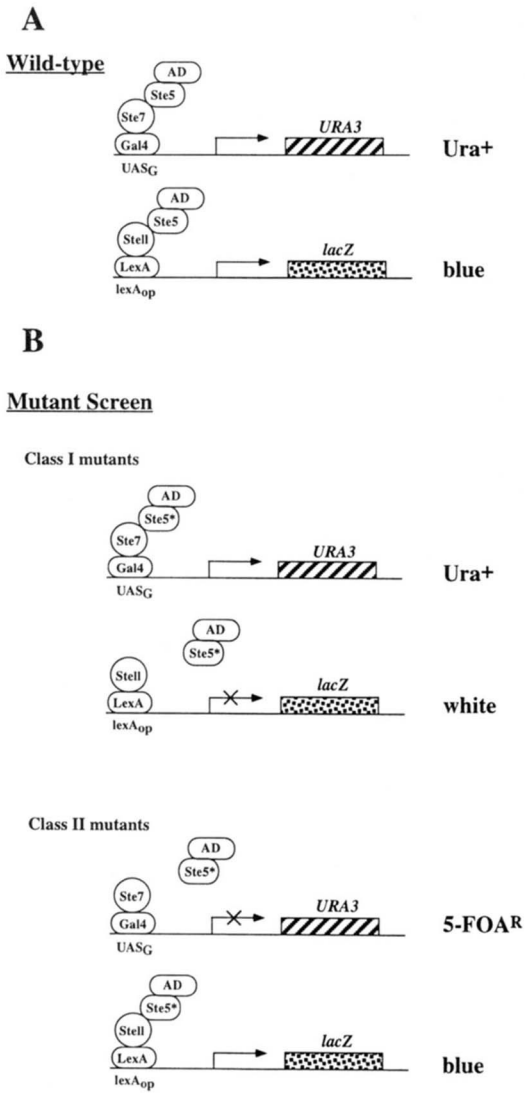

FIGURE 1.—Design of the differential interaction trap method for isolating *ste5* alleles defective in association with a specific protein. (A) YCJ4 cells transformed with plasmids (pAS-Ste7, pLex-Ste11ΔC, and pGAD-Ste5) encoding three fusion proteins, Gal4-Ste7, LexA-Ste11, and AD-Ste5, are phenotypically Ura$^+$ and β-galactosidase$^+$ due to the Ste5-Ste7 and the Ste5-Ste11 interactions, respectively. (B) Two types of mutants can be detected when randomly mutagenized populations of Ste5 are screened: Class I *ste5* alleles, which can associate with Ste7 (and, hence, are Ura$^+$), but cannot interact with Ste11 (and, thus, are β-galactosidase$^-$); and Class II *ste5* alleles, which can bind to Ste11 (and, thus, remain β-galactosidase$^+$), but cannot associate with Ste7 (and, hence, are Ura$^-$ and 5-FOA-resistant).

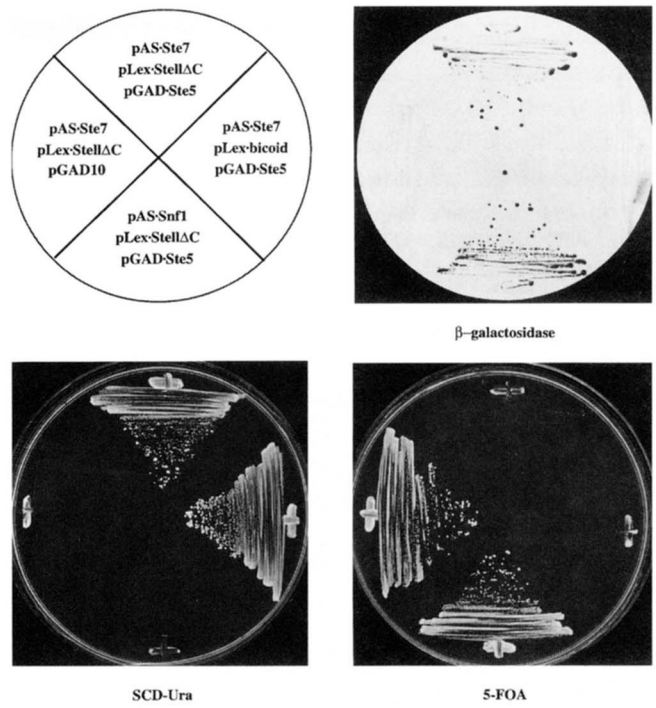

FIGURE 2.—Implementation of the differential interaction trap method. Strain YCJ4 reliably detects Ste5-Ste7 and Ste5-Ste11 interactions. Strain YCJ4 was transformed with the indicated plasmid combinations (top left) and the resulting transformants were as sayed for the following: β-galactosidase production (top right) using a colony lift assay (BREEDEN and NASMYTH 1985), growth on selective medium lacking uracil (bottom left), and growth on selective medium containing 5-FOA (bottom right).

formed. First, the pGAD-Ste5 plasmid was introduced into YCJ4 cells that harbored the pAS-Ste7 plasmid and a plasmid (pLex-bicoid) expressing a fusion of the Drosophila homeobox-containing transcription factor, bicoid, to the LexA DNA-binding domain. These cells were Ura$^+$ (and 5-FOA-sensitive), but β-galactosidase-negative, indicating that AD-Ste5 did not interact with LexA-bicoid, as expected, and that the presence of this noninteracting chimera in the cell did not interfere with the interaction between the AD-Ste5 and Gal4-Ste7 fusions (Figure 2). Second, the pGAD-Ste5 plasmid was introduced into YCJ4 cells that harbored the pLex-Ste11ΔC plasmid and a plasmid (pAS-Snf1) expressing a fusion of the *S. cerevisiae* Snf1 protein kinase to the Gal4 DNA-binding domain. The resulting transformants were Ura$^-$ (and 5-FOA-resistant), but β-galactosidase-positive, demonstrating that AD-Ste5 does not interact with an irrelevant protein kinase fused to the Gal4 DNA-binding domain and that the presence of this noninteracting chimera did not perturb the interaction between the AD-Ste5 and LexA-Ste11 fusions (Figure 2). Thus, both the Ste5-Ste7 and the Ste5-Ste11 interaction, or the lack thereof, could be monitored independently within the same cell.

Isolation of *ste5* point mutants: To isolate mutants, DNA containing the entire *STE5* gene (and flanking pGAD vector-derived sequences) was randomly mutagenized by PCR. The resulting amplification products, together with appropriately linearized pGAD-Ste5 vector, were then introduced into YCJ4 cells already carrying the Gal4-Ste7 and LexA-Ste11 fusions. In this procedure, the pGAD-Ste5 plasmids expressing the pool of mutant AD-Ste5 proteins are generated *in situ* via a gap repair mechanism (MUHLRAD *et al.* 1992). Two informative classes of mutants could then be readily detected

(Figure 1B). Class I Ste5 mutants that prevent binding to Ste11, but not to Ste7, were identified by plating ~30,000 transformants and selecting for Ura^+ transformants on a medium lacking uracil (but otherwise selective for all three plasmids) and then screening for the lack of β-galactosidase production on the same medium, which also contained X-gal indicator dye (MILLER 1972). Out of ~2000 total Ura^+ colonies, eight were no longer β-galactosidase-positive (indicative of Ste5 mutants capable of interacting with Ste7, but not with Ste11). All eight of the pGAD-Ste5 plasmids recovered from these cells reproducibly conferred a Ura^+, β-galactosidase-negative, 5-FOA-sensitive phenotype in YCJ4.

The reciprocal Class II Ste5 mutants that prevent binding to Ste7, but not to Ste11, were identified by plating ~30,000 transformants and selecting for Ura^- colonies on a medium containing 5-FOA (but otherwise selective for all three plasmids) and then screening for continued expression of β-galactosidase. Out of ~5000 total 5-FOA-resistant colonies, 37 were still β-galactosidase-positive (as expected for Ste5 mutants incapable of binding to Ste7, but able to associate with Ste11). Eight of the 37 recovered plasmids reproducibly led to a 5-FOA-resistant (and Ura^-) and β-galactosidase-positive phenotype in YCJ4.

The nucleotide sequence of the entire coding region was determined for each of the 16 *ste5* isolates obtained. Eleven of the mutants contained three or fewer base pair changes. These alleles are listed in Table 1; their positions within Ste5 are depicted schematically in Figure 3. The mutant *ste5* DNAs were recovered and retested for interaction with Ste11 and Ste7 using the standard two-hybrid method in YCJ4 and in another reporter strain (Y190) (BAI and ELLEDGE 1996), respectively. Six Class I mutations, including two alleles that represent single amino acid substitutions, abolished interaction with Ste11 in both reporter strains (Table 1). The Class I mutations cluster within a 52-amino acid segment (residues 463–514) in the middle of the Ste5 protein (Figure 3A) and represent a much more restricted region than that (residues 336–586) implicated previously in Ste11 binding on the basis of deletion analysis (CHOI *et al.* 1994). This region is predicted to form an α-helix-loop-β-sheet structure; mutations that fall in each of these putative substructures were isolated. Four of the six Class I mutants also contained an additional base pair change that affects residues 57–71; however, Ste5 constructs containing only these amino-terminal mutations showed no qualitative difference in association with Ste11 as compared to wild-type Ste5 (data not shown), consistent with the modest (twofold) reduction in Ste11 binding observed for amino-terminal truncations of Ste5 (CHOI *et al.* 1994). Since the amino-terminal substitution mutations alone do not impair Ste5 interaction with Ste11, yet were isolated repeatedly, this small region of the Ste5 amino-terminus may enhance Ste5 affinity for Ste11. The two Class I alleles, *ste5-358* and *ste5-405*, that have single amino acid changes (F514L and I504T, respectively) were analyzed in greater detail (see below).

Class II mutations that affect interaction with Ste7, but not with Ste11 (Table 1), cluster at the C-terminal end of Ste5 (between residues 744–895) (Figure 3A). Again, these mutations delineate a much more restricted region of the Ste5 protein than the 331-amino acid segment (residues 586–917) defined as the Ste7 interaction site by deletion analysis (CHOI *et al.* 1994). This region of Ste5 is predicted to be primarily α-helical and is extremely negatively charged (40 acidic and only six basic residues), suggesting that Ste5 association with Ste7 may be mediated largely via electrostatic forces. Two of the five Class II alleles (*ste5-317* and *ste5-305*) are single amino acid changes (D746G and R895G, respectively) that alter the regions immediately outside of the strongly acidic segments, suggesting that they might affect the positioning of the negatively charged α-helices (Figure 3B). Two of the Class II alleles (*ste5-308* and *ste5-316*) also have an additional amino acid change outside the C-terminal acidic region. Since each of these changes lies in a very different region of the protein, they are presumably adventitious and probably do not contribute to the phenotype observed (although the effect of these individual alterations alone has not yet been tested). Only one Class II allele (*ste5-336*) appeared to be completely defective for association with Ste7 in both reporter strains (Table 1). Interestingly, this tight allele contains two alterations (V763A S861P), which lie on opposite sides of the acidic domain. The other Class II *ste5* alleles obtained also appeared to be completely defective for interaction with Ste7 under the conditions of the original selection and upon retesting in YCJ4; however, when re-examined for β-galactosidase activity quantitatively in Y190, these other alleles displayed a weak, but detectable, association with Ste7 (Table 1). Thus, both the tight Class II allele (*ste5-336*) and one of the representative weaker alleles (*ste5-317*) that contains a single amino acid substitution (D746G) were chosen for further characterization.

Biochemical analysis of the *ste5* point mutants: To confirm the properties of the altered Ste5 proteins by an independent biochemical method, two Class I and two Class II mutants were tested for their ability to bind Ste11 and Ste7 *in vitro* using co-immunoprecipitation from cell extracts. Because Ste5 is a rather unstable and inabundant protein (HASSON 1992; HASSON *et al.* 1994; C. INOUYE and N. DHILLON, unpublished observations), *STE5* and each of the four *ste5* alleles (*ste5-317, ste5-336, ste5-358* and *ste5-405*) were fused downstream of and in-frame with sequences encoding a $(His)_6$ tract and a c-Myc epitope tag and expressed from the *GAL1* promoter on a low-copy vector (YCpLGHM). These plasmids were introduced by transformation into a *ste5*Δ strain (BYB84), either alone or together with a second plasmid that expresses either Ste7 or Ste11 under *GAL1*

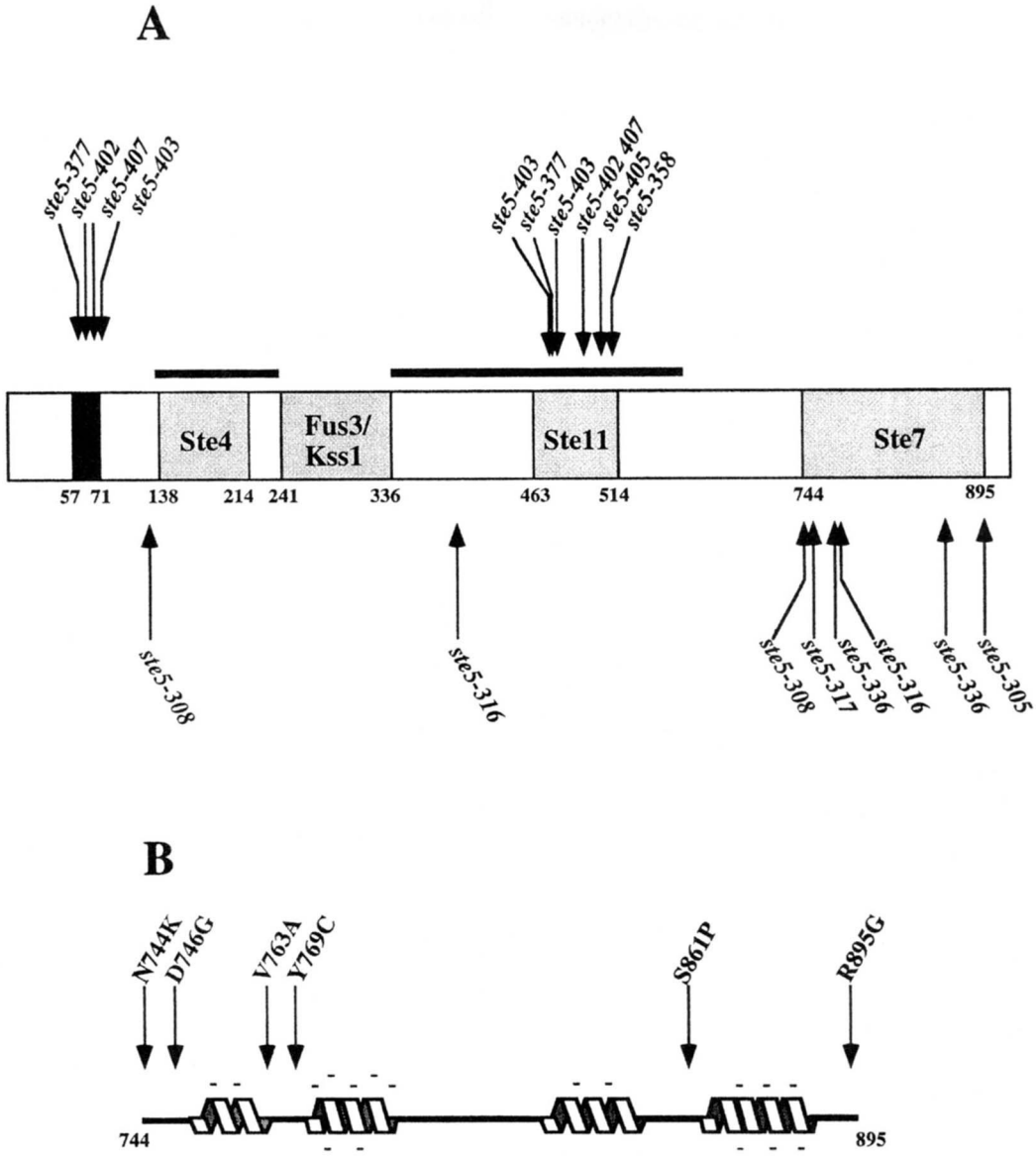

FIGURE 3.–Location of the *ste5* point mutations within the Ste5 polypeptide. (A) Schematic diagram of the 917-residue Ste5 protein (open bar); numbers below the bar represent residue positions. Regions implicated in the binding of Ste11, Ste7, Fus3 and Kss1, either derived from this study or from previous deletion analysis (see text for details), are shown as shaded boxes. Region implicated in Ste4 binding is described elsewhere (INOUYE *et al.* 1997). A region potentially involved in reinforcing Ste11 binding is represented by the filled box. Segments implicated in oligomerization (YABLONSKI *et al.* 1996) are indicated by the solid lines above the bar. Locations and allele numbers of Class I mutants, which do not bind Ste11, are given above the bar; locations and allele numbers of Class II mutants, which fail to interact with Ste7, are shown below the bar. (B) Predicted secondary structure of the Ste7-binding region of Ste5 and the location of the Class II alleles. Predicted α-helices and highly net negatively charged regions are shown.

promoter control. After brief induction by growth of the cells in galactose medium for 2 hr, extracts of the cultures were subjected to immunoprecipitation with the anti-c-Myc monoclonal antibody, 9E10 (EVAN *et al.* 1985); the resulting immunoprecipitates were resolved by SDS-PAGE, blotted, and bound proteins detected using antibodies specific to each protein.

Each of the four mutant Ste5 proteins was immunoprecipitated in amounts indistinguishable from that of wild-type Ste5 (Figure 4, bottom panels), regardless of what other proteins were co-expressed in the same cell. The Class I mutants, Ste5(I504T) and Ste5(F514L), co-immunoprecitated the same amount of Ste7 as wild-type Ste5 (Figure 4A) but were unable to associate detectably with Ste11 (Figure 4B). Both of the Class II mutants co-immunoprecipiated the same amount of Ste11 as wild-type Ste5 (Figure 4B), and the product of the tightest Class II allele, Ste5(V763A S861P), bound Ste7 only slightly above the background level (Figure 4A). Consistent with the residual interaction detected

TABLE 1

Amino acid substitutions and binding properties of Ste5 mutants

Allele	Mutation(s)[a]	Interaction with[b]	
		Ste7	Ste11
ste5-305	R895G	−/+	+
ste5-308	L128P N744K	−/+	+
ste5-316	F382L Y769C	−/+	+
ste5-317	D746G	−/+	+
ste5-336	V763A S861P	−	+
ste5-358	F514L	+	−
ste5-377	R57K T465A	+	−
ste5-402	R60G S497G	+	−
ste5-403	S71P M463T V489A	+	−
ste5-405	I504T	+	−
ste5-407	S62G S497G	+	−

[a] Amino acid changes at the indicated positions in the Ste5 polypeptide are given in the single-letter code.

[b] Based on two-hybrid assays performed in reporter strain Y190 (BAI and ELLEDGE 1996).

using the two-hybrid assay (Table 1), the product of the other Class II allele, Ste5(D746G), displayed a clearly reduced (but still readily detectable) association with Ste7. Since Ste5 and each potential partner were overexpressed, the interactions observed by this method are, if anything, an overestimate of the strength of these associations in the cell.

These findings were further corroborated by quantitative assessment of the strength of these interactions *in vivo* using the two-hybrid method (Table 2). When normalized to the amount of β-galactosidase expression supported by the interaction of wild-type Ste5, the interaction of the Class I mutants, Ste5(I504T) and Ste5(F514L), with Ste11 was decreased to nearly $^1/_{20}$th of the wild-type value, whereas their interaction with Ste7 was essentially unaffected. Conversely, Class II mutants interacted with Ste11 similarly, or perhaps slightly better, than wild-type Ste5. Interaction of the weaker Class II Ste5(D746G) variant with Ste7 was decreased to $\sim^1/_{10}$th of the value observed with wild-type Ste5, whereas the tight Class II Ste5(V763A S861P) form displayed no detectable interaction with Ste7 above the background (Table 2). Thus, there was remarkably good agreement between the quantitative two-hybrid analysis and the results obtained by both the differential interaction trap method and the co-immunoprecipitation procedure, even though the two-hybrid approach can often be applied only qualitatively (ESTOJAK *et al.* 1995).

Specificity of the *ste5* point mutants: As summarized above, Ste5 can interact with the MAPKs, Fus3 and Kss1, and with the G protein β subunit, Ste4, in addition to Ste11 and Ste7. If the effects of the *ste5* alleles isolated by the differential interaction trap method are truly confined to the Ste5-Ste11 and Ste5-Ste7 interactions, then the ability of the Ste5 mutant proteins to associate with other proteins should be unperturbed. To confirm the specificity of the *ste5* alleles, the ability of the Ste5 mutant proteins to associate with Fus3 and Ste4 was examined by co-immunoprecipitation from extracts of a *ste5*Δ strain expressing either Ste5 or each of the Ste5 mutants and either an empty vector or plasmids expressing either *FUS3* or *STE4*. Each of the four mutant Ste5 proteins was immunoprecipitated in amounts indistinguishable from that of wild-type Ste5, regardless of whether Fus3 or Ste4 was co-overexpressed in the same cell (Figure 5, bottom panels). The products of both the Class I and Class II alleles retained the ability to interact with Fus3 (Figure 5A) and with Ste4 (Figure 5B) at levels comparable to wild-type Ste5. In addition, all four Ste5 mutant proteins bound to both the faster and slower migrating forms of Ste4, which are known to be un-/hypo- and hyperphosphorylated Ste4 isoforms, respectively (COLE and REED 1991). These results demonstrate that the mutants isolated using the differential interaction trap method are highly specific and are only defective in the interaction for which they were chosen.

Genetic analysis of the *ste5* point mutants: To address whether point mutations that disrupt the individual interactions of Ste5 with either Ste11 or Ste7 have any consequences for the ability of Ste5 to perform its function in the pheromone response pathway, the representative Class I and Class II alleles were tested for their ability to complement the mating defect of a *MAT***a** *ste5*Δ strain (BYB69). For this purpose, each of the *ste5* alleles was inserted into a low-copy centromere-containing vector under control of the authentic *STE5* promoter. The mating efficiency of the resulting transformants was determined using a quantitative mating assay, as described in MATERIALS AND METHODS. Both of the Class I mutants that affect Ste5 association with Ste11 showed a reduction in mating proficiency of at least 10^{-2}–10^{-3} compared to wild-type Ste5 (Table 3). Moreover, as judged by a patch mating method on plates, both of the Class I alleles did not yield any diploid colonies (Figure 6). Likewise, the product of the strongest Class II allele, Ste5(V763A S861P), which appears unable to bind Ste7, showed a clear mating defect as judged either by the quantitative mating assay (Table 3) or on plates (Figure 6). Remarkably, however, the product of the weaker Class II allele, Ste5(D746G), which has only residual (but detectable) affinity for Ste7 when assessed by the differential interaction trap method (Table 1), by co-immunoprecipitation (Figure 4A), or by quantitative two-hybrid analysis (Table 2), appeared to fully support conjugation when assessed by the quantitative mating assay (Table 3). This result suggests that only a weak (or transient) interaction between Ste5 and Ste7 may be sufficient for signaling to occur.

Since point mutations that disrupt the individual interactions of Ste5 with either Ste11 or Ste7 were iso-

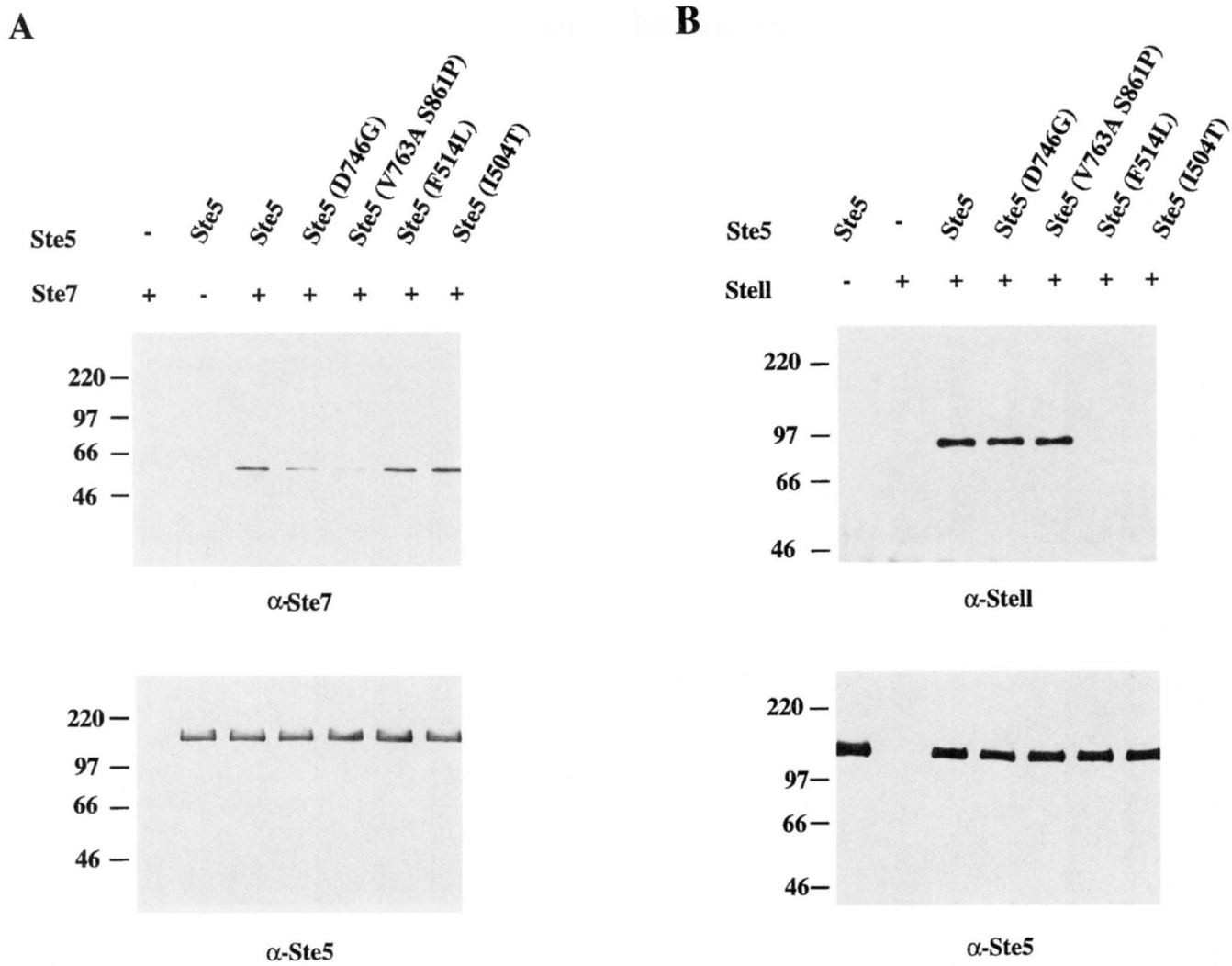

FIGURE 4.—Class I and Class II *ste5* mutants are defective for Ste11 or Ste7 binding *in vitro*. (A) Strain BYB84 expressing either a vector control (−), Myc-tagged wild-type Ste5, or Myc-tagged versions of each of the indicated *ste5* point mutants, and co-expressing either Ste7 or a vector control, were lysed and subjected to immunoprecipitation with an anti-c-Myc monoclonal antibody, 9E10. After resolution of the immune complexes on SDS-PAGE and blotting to a nylon membrane, the proteins present were analyzed by probing with rabbit polyclonal anti-Ste7 antibodies (generous gift of B. CAIRNS, Department of Biochemistry, Stanford University) (top panel) or with rabbit polyclonal anti-Ste5 antibodies (bottom panel), whose preparation and characterization is described in detail elsewhere (N. DHILLON, C. INOUYE and J. THORNER, unpublished results). A small amount of Ste7 nonspecifically adsorbs to the anti-c-Myc monoclonal antibody (lane 1). (B) Interaction with Ste11 was measured as in A, except that the Ste7 expression plasmid was replaced with YCpLG-Ste11 and a suitable vector control (−). The resulting blots were probed (top panel) with a rabbit polyclonal anti-Ste11 antiserum (CAIRNS *et al.* 1992) (bottom panel).

lated, it was of interest to determine whether the Class I and Class II mutations could complement each other in *trans*, when co-expressed in the same cell. Such interallelic (also called intragenic) complementation can be positive or negative, and is a genetic test that is considered diagnostic for the interaction between subunits of a multimeric protein (ZABIN and VILLAREJO 1975; JENNESS and SCHACHMAN 1983). An appropriate empty vector (YCp33), or wild-type *STE5*, or each of the two representative Class I alleles were introduced into a *MAT***a** *ste5*Δ strain (BYB69) carrying either an empty vector (YCp111) or the same vector expressing the tightest Class II allele. As judged by growth on a medium selective for the presence of the plasmids, all the resulting transformants grew equivalently (Figure 6, top). This master plate was first cross-stamped with an appropriate *MAT*α mating partner (DC17) and after incubation was replica-plated to a medium selective for the resulting prototrophic diploids. As expected, none of the three *ste5* alleles alone was able to rescue the mating defect of the *ste5*Δ recipient strain, whereas the wild-type *STE5* gene did (Figure 6, bottom). Strikingly, however, when either of the two Class I alleles was combined in the same cell with the representative tight Class II allele, a readily detectable level of mating was restored. This positive interallelic complementation is

TABLE 2

Two-hybrid interactions of Ste7 and Ste11 with wild-type and mutant Ste5

DBD fusions	AD fusions	β-galactosidase activity (%)[a]
pAS·Ste7[b]	pGAD10	2.7 ± 0.6
	Ste5	100
	$Ste5^{D746G}$	13.3 ± 3.5
	$Ste5^{V763A\ S861P}$	3.4 ± 1.5
	$Ste5^{F514L}$	91.0 ± 12.5
	$Ste5^{I504T}$	94.3 ± 16.0
pLexA·Ste11ΔC[c]	pGAD10	1.3 ± 0.2
	Ste5	100
	$Ste5^{D746G}$	196 ± 37
	$Ste5^{V763A\ S861P}$	132 ± 17
	$Ste5^{F514L}$	6.3 ± 1.5
	$Ste5^{I504T}$	6.0 ± 1.7

DBD, DNA-binding domain; AD, activation domain.

[a] Nine independent transformants carrying each of the combinations of plasmids indicated were assayed for β-galactosidase activity using CPRG as the substrate. Values are averages ± SD, normalized to the value obtained for wild-type Ste5.

[b] Interactions assessed in Y190 (Bai and Elledge 1996).

[c] Interactions assessed in YCJ4.

consistent with the results of Yablonski *et al.* (1996) and strongly suggests that Ste5 self-association is required for its function.

DISCUSSION

We developed a differential interaction trap assay that allowed the identification of *ste5* point mutations that specifically affect interaction of Ste5 with a single target protein without altering its interaction with other proteins with which it is known to associate. In this approach, randomly mutagenized pools of a gene of interest are assessed for their ability to interact with two different partners simultaneously in the same cell. The ability to assay concomitantly interaction of a protein with two separate partners in the same cell has several advantages. First, previous methods for the isolation of mutations that have such a differential effect required cumbersome sequential screening of candidates using different reporter strains (see, for example, White *et al.* 1994). Second, this procedure selects against alterations of the protein of interest that grossly perturb its global folding since the ability to bind one of the two partners demands that at least some native structure be retained. Indeed, each of the Class I Ste5 mutants was unable to associate with Ste11, yet was still able to bind Ste7, Fus3, and Ste4. Likewise, the Class II Ste5 mutants no longer interacted with Ste7 normally, but still bound Ste11, Fus3, and Ste4. Third, the requirement for maintaining interaction with one partner also selects against the recovery of mutations that cause premature termination of the mutagenized protein. Indeed, despite the relatively large size of the *STE5* gene, only one of 16 mutants isolated encoded a truncated protein (C. Inouye and N. Dhillon, unpublished observations). Fourth, another advantage of requiring preservation of one interaction is that it selects against mutations that prevent expression of the mutagenized protein or that compromise the function of the attached transcriptional activation domain. Fifth, the method could be modified easily for the facile isolation of conditional mutations that affect a given protein-protein interaction. Finally, the recipient strain (YCJ4) could be used for screening activation domain-tagged expression libraries for proteins that display selective interactions, such as the ability to interact with two different partners; the ability to interact with one target, but not another; or the ability to interact with the wild-type form of a protein, but not with a mutant version of the same protein. The differential interaction trap method described here adds another tool to the repertoire of techniques available (reviewed in Allen *et al.* 1995; Phizicky and Fields 1995; White 1996) for refining our understanding of how specific protein-protein interactions contribute to biological function *in vivo*.

The *ste5* point mutations we isolated delineate more precisely the domains of Ste5 necessary for its association with Ste11 and Ste7 than previously defined by deletion analysis (reviewed in Elion 1995). Based on the position of the mutations identified, the Ste7 binding domain encompasses residues 744–895 at the C-terminus of Ste5. The altered sites bracket a region of the protein predicted to be comprised largely of highly negatively charged α-helical segments, arguing that association of Ste5 with Ste7 may be mediated primarily by electrostatic interactions. The fact that the mutated sites lie mainly outside of the acidic helices suggests that the mutations could interfere with Ste7 binding by altering the relative positioning of the putative α-helices with respect to one another, rather than by altering residues that represent side chains that are themselves in intimate contact with Ste7. In support of this suggestion, the only allele that totally abolished the ability of Ste5 to bind Ste7, as judged by every assay method applied, was a double mutant, Ste5(V763A S861P) that affects a residue on either side of the acidic region. If the Ser-to-Pro substitution in this allele lies in an α-helix or just outside of an α-helix, it might by itself change the spatial relationships in this region enough to affect Ste7 binding.

Based on the locations of the mutations identified, the Ste11 binding domain is circumscribed by residues 463–514 near the middle of the Ste5 polypeptide, a region that is not particularly charged. Moreover, mutations were isolated throughout this segment and thus should affect each of three predicted substructures in this region (α-helix-loop-β-strand), suggesting that the precise tertiary structure of this domain is critical for Ste11 binding, or that the altered residues represent

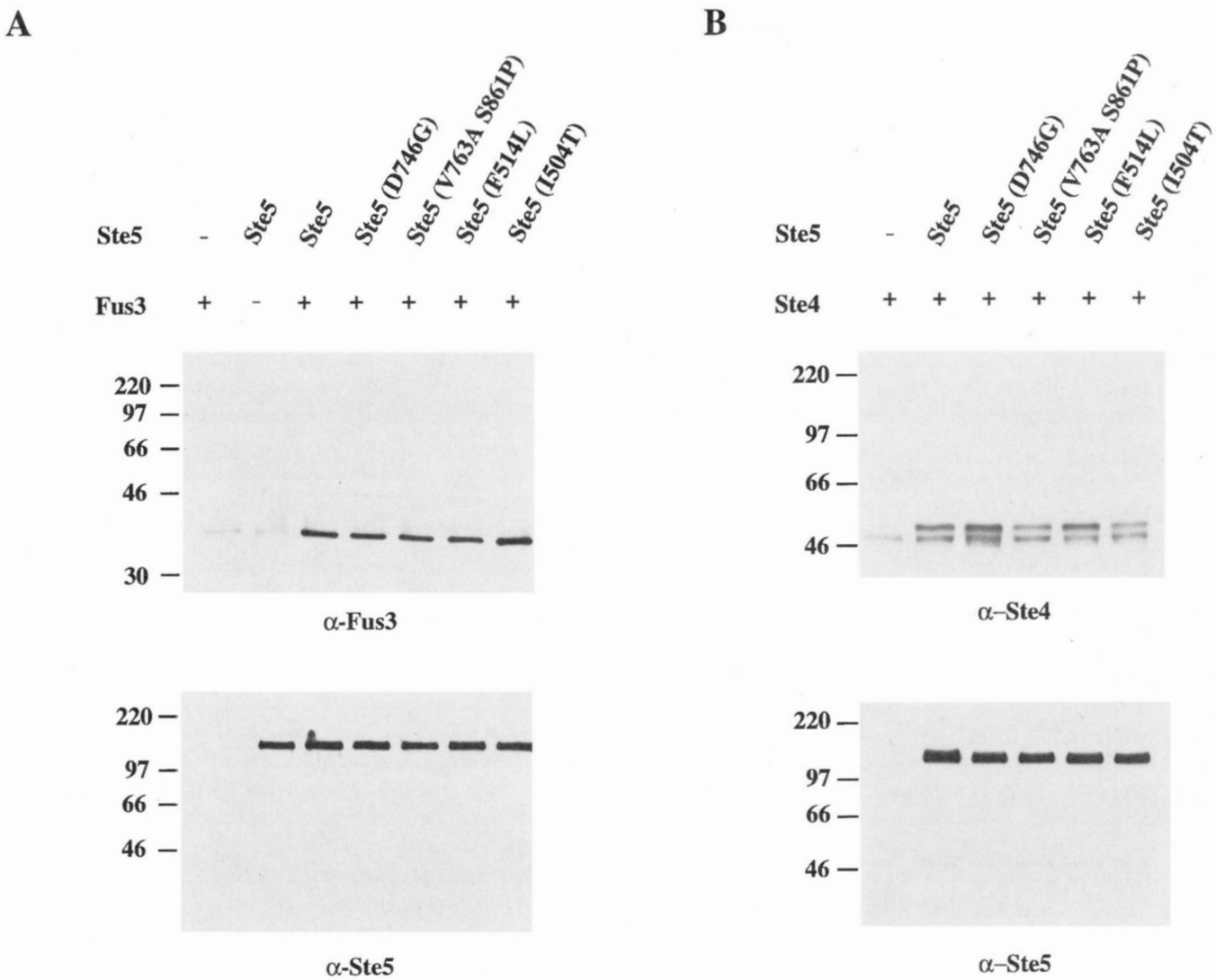

FIGURE 5.—The Class I and Class II *ste5* mutants still bind Fus3 and Ste4 normally. (A) Interaction of wild-type Ste5, and the representative Class I and Class II *ste5* mutants indicated, with Fus3 was measured as described in the legend to Figure 4, except that a Fus3 expression plasmid, YEpT-Fus3, and a suitable vector control (−) were used. The resulting blots were probed (top panel) with an anti-Fus3 antibody (BRILL *et al.* 1994) or with the polyclonal anti-Ste5 serum (bottom panel). A small amount of Fus3 nonspecifically adsorbs to the anti-c-Myc monoclonal antibody (lanes 1 and 2). (B) Interaction with Ste4 was assessed as in A, except that the Ste4 expression plasmid, pCJ125, was used. The resulting the blot was probed with a rabbit polyclonal anti-Ste4 antiserum (HIRSCHMAN *et al.* 1996) (top panel) or with anti-Ste5 antibodies (bottom panel).

side chains in intimate contact with Ste11, or both. Interestingly, four of the six Class I alleles that no longer bound Ste11 also had mutations that lie in residues 57–71 of Ste5. These alterations appear to be silent because Ste5 proteins containing only these amino-terminal mutations still interacted with Ste11. Nonetheless, the fact that these secondary mutations clustered in a single region provides a hint that this portion of the protein may serve to buttress the domain responsible for Ste5-Ste11 interaction.

In addition to more precisely demarcating the Ste11 and Ste7 interaction domains of Ste5, the point mutants also permitted us to rigorously test the hypothesis that Ste5 association with a given component of the MAPK cascade is critical to its signaling function. We found that single amino acid substitutions that abolish the ability of Ste5 to associate with Ste11, but not with Ste4 or with any other component of the MAPK cascade, were mating defective. Likewise, a double mutant that prevented association of Ste5 with Ste7, but not with Ste4 or with any of the other MAPK cascade components, was also mating defective. These findings demonstrate unequivocally the essential roles of Ste5-Ste11 and Ste5-Ste7 interactions in the signaling process under physiological conditions. We did observe, however, that gross overexpression of these *ste5* alleles restored some degree of mating to *ste5*Δ cells (C. INOUYE and N. DHILLON, unpublished obeservations). This fact may explain the appearance of hyperphosphorylated Ste4 when the *ste5* alleles were overexpressed from the *GAL1* promoter.

TABLE 3

Quantitative mating assays

Plasmid	Mating efficiency[a]	% of wild-type[b]
YCp33	$<10^6$	0
YCp · *STE5*	0.42 ± 0.07	100
YCp · *STE5(D746G)*	0.38 ± 0.07	91
YCp · *ste5(V763A S861P)*	$1.0 \times 10^{-3} \pm 3.9 \times 10^{-4}$	0.2
YCp · *ste5(F514L)*	$4.3 \times 10^{-3} \pm 1.1 \times 10^{-4}$	1.0
YCp · *ste5(I504T)*	$8.2 \times 10^{-4} \pm 4.6 \times 10^{-5}$	0.2

[a] Mating efficiency of a *MAT***a** *ste5*Δ strain (BYB69) carrying each of the indicated plasmids was calculated by dividing the number of diploid colonies formed after mating by the number input haploids. Values are averages ± SD of at least three independent assays, each run in triplicate.

[b] Normalized to the mating efficiency of cells expressing the wild-type *STE5* gene from the centromere-containing plasmid.

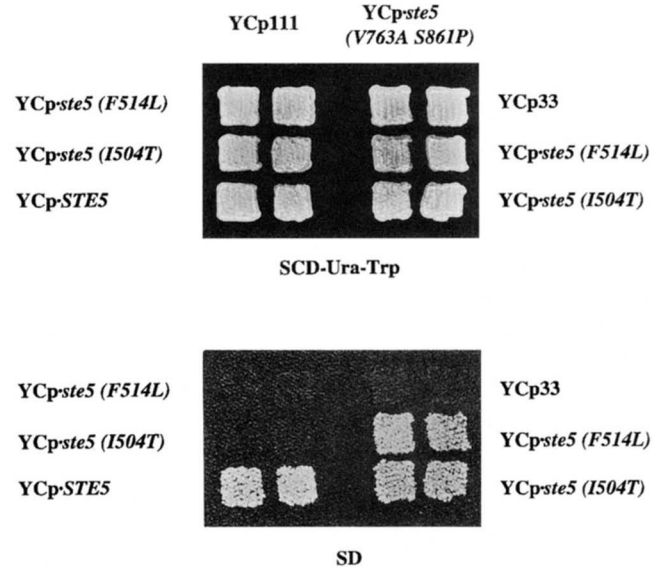

FIGURE 6.—Class I and Class II *ste5* mutants display interallelic complementation. Strain BYB69 was transformed with either vector alone (YCp111) or the same vector expressing the indicated Class II mutant, Ste5(V763A S861P). These two derivatives were then co-transformed with either a vector control (YCp33), with the same vector expressing either wild-type Ste5, or each of the two different Class I mutants indicated, Ste5(I504T) and Ste5(F514L). The resulting plasmid-bearing strains were patched onto selective plates (top panel), mated to an appropriate tester strain (DC17), and replica-plated onto a medium selective for diploids (bottom panel). Patches from two independent transformants containing each combination of plasmids are shown.

The association of Ste5 with the MAPKs themselves (Fus3 and Kss1) is presumably also required for signaling, and a segment of Ste5 (residues 241–336) that is nonoverlapping with the Ste11 and Ste7 binding regions defined in our study appears to be both necessary and sufficient for the binding of either Fus3 or Kss1 to Ste5 (CHOI *et al.* 1994). The interaction of Ste11 with Ste5 appears to be a tight association because Ste5-Ste11 complexes can be detected by co-sedimentation (CHOI *et al.* 1994). In marked contrast, high-affinity binding of Ste7 to Ste5 does not appear to be a prerequisite for efficient signaling because one Class II allele, Ste5(D746G), which caused a severely decreased affinity for Ste7 (as judged by three independent tests), nonetheless mated with near normal efficiency. These findings suggest that for signaling to occur only a minor fraction of the Ste5 molecules need to be associated with Ste7, or that this interaction need only be transient. In this regard, it has been observed previously that little, if any, Ste7 co-sediments with Ste5 (CHOI *et al.* 1994). In fact, Ste7 binds to its cognate MAPKs with very high affinity, even in the absence of both Ste5 and Ste11 (BARDWELL *et al.* 1996). Thus, Ste7-Fus3 and Ste7-Kss1 complexes may associate with Ste5 as a unit; however, since Fus3 associates with Ste5(D746G) and Ste5(V763A S861P) at wild-type levels, MAPK interaction with Ste5 cannot obligatorily require simultaneous interaction of Ste5 with Ste7. Furthermore, Ste11, Ste7, Fus3, and Kss1 can each interact with Ste5, even when the other components of the MAPK cascade are absent, at least as judged by the two-hybrid method (CHOI *et al.* 1994; MARCUS *et al.* 1994; PRINTEN and SPRAGUE 1994).

Another opportunity provided by the isolation and characterization of *ste5* point mutants was the ability to assess intragenic interactions between alleles that affect only Ste11 binding and alleles that affect only Ste7 binding. We found that combining two such completely mating-defective alleles in the same cell restored mating ability. Such positive interallelic complementation strongly suggests that Ste5 must oligomerize *in vivo* to perform its function. Indeed, while our work was in progress, it was reported that Ste5 appears to be an oligomer in cell extracts, and that distinct regions of the protein (residues 138–239 and 335–586) are required for this interaction (YABLONSKI *et al.* 1996). This self-association was also shown to be necessary for Ste5 function *in vivo* because, similar to our findings, certain deletion mutants that lack different interaction domains were able to complement one another when co-expressed (YABLONSKI *et al.* 1996). The apparent requirement for oligomerization raises the possibility that one (or more) of the Ste5-associated enzymes may be activated via the ensuing conformational change (and/or by cross-phosphorylation) in a manner analogous to receptor-tyrosine kinases that have been dimerized by ligand binding (LEMMON and SCHLESSINGER 1994). If so, the ability of rather large Ste5 deletions to complement each other suggests that the sequences remaining may provide rather extensive contacts between Ste5 monomers that properly position the various binding domains, consistent with the fact that at least two extended regions of Ste5 appeared to be necessary for oligomerization, at least under the assay conditions used by YABLONSKI *et al.* (1996). A simpler model in which Ste5 oligomers merely act to concentrate the ap-

propriate kinases, which then promotes signaling through mass action, cannot be ruled out at the present time.

Association of one or more of the kinases with the Ste5 oligomer may also be necessary to induce structural changes in these enzymes that stimulate their catalytic activity. In fact, the association of the MEKK, Ste11, with Ste5 is mediated by its N-terminal extension (and not the catalytic domain). This segment of Ste11 appears to act as a negative regulatory domain because alterations of this region result in a hyperactive kinase that can partially restore mating in a *ste5*Δ strain (CAIRNS *et al.* 1992; STEVENSON *et al.* 1992). Oligomerization of Ste5 may alter its structure and concomitantly the structure of the bound Ste11 regulatory domain, thereby relieving its inhibitory effect. Given the tight association observed between Ste5 and Ste11, once the MEKK is activated, the system is "primed," so that only transient association with Ste7, the MEK, would be needed for its phosphorylation and activation by Ste11, consistent with the behavior of the Ste5(D746G) mutant. Of course, association of Ste7 (or Ste7-Fus3 and Ste7-Kss1 complexes) with Ste5 may also induce conformational changes in Ste7 (and/or in Fus3 and Kss1) that favor MEK-dependent phosphorylation of the MAPKs, or that may position Ste7 appropriately to favor its efficient phosphorylation by Ste11. Additional analysis of the regions required for oligomerization and how the resulting structure promotes signaling by the MAPK module will be required to understand the precise molecular basis of signaling and the role of Ste5 in this process.

We thank past and present members of the THORNER laboratory, who generated some of the information and reagents that made this study possible. We especially acknowledge JANET SCHULTZ, MIRIAM S. HASSON, RICHARD L. FREEDMAN, and ELISABETH T. BARFOD. We also thank LEE BARDWELL, JEANETTE GOWEN COOK, PAMELA TORRANCE, JOANNE ADAMKEWICZ and JUDITH ROE for critical reading of the manuscript, and BRAD CAIRNS, JULIE BRILL, JODI HIRSCHMAN and DUANE JENNESS for generously providing specific antisera. This work was supported by Postdoctoral Fellowship PF-3785 from the American Cancer Society (C.I.), by National Cancer Institute Postdoctoral Traineeship CA09041 and by National Research Service Award Postdoctoral Fellowship GM-17573 from the National Institutes of Health (NIH) (to N.D.), by National Research Service Award Postdoctoral Fellowship GM-16915 from the NIH (T.D.), by Department of Energy Research Grant 88ER13882 (P.C.Z.), by Research Grant GM-21841 from the National Institute of General Medical Sciences (J.T.), and by resources provided by the Berkeley campus Cancer Research Laboratory.

LITERATURE CITED

AKADA, R., L. KALLAL, D. I. JOHNSON and J. KURJAN, 1996 Genetic relationships between the G protein $\beta\gamma$ complex, Ste5p, Ste20p and Cdc42p: investigation of effector roles in the yeast pheromone response pathway. Genetics **143:** 103–117.

ALLEN, J. B., M. W. WALBERG, M. C. EDWARDS and S. J. ELLEDGE, 1995 Finding prospective partners in the library: the two-hybrid system and phage display find a match. Trends Biochem. Sci. **20:** 511–516.

BAI, C., and S. J. ELLEDGE, 1996 Gene identification using the yeast two-hybrid system. Methods Enzymol. **273:** 331–347.

BARDWELL, L., J. G. COOK, C. J. INOUYE and J. THORNER, 1994 Signal propagation and regulation in the mating pheromone response pathway of the yeast *Saccharomyces cerevisiae*. Dev. Biol. **166:** 363–379.

BARDWELL, L., J. G. COOK, E. C. CHANG, B. R. CAIRNS and J. THORNER, 1996 Signaling in the yeast pheromone response pathway: specific and high-affinity interaction of the mitogen-activated protein (MAP) kinases Kss1 and Fus3 with the upstream MAP kinase kinase Ste7. Mol. Cell. Biol. **16:** 3637–3650.

BARNES, D. A., and J. THORNER, 1986 Genetic manipulation of *Saccharomyces cerevisiae* by use of the *LYS2* gene. Mol. Cell. Biol. **6:** 2828–2838.

BOEKE, J. D., F. LACROUTE and G. R. FINK, 1984 A positive selection for mutants lacking orotidine 5-phosphate decarboxylase activity in yeast: 5′-fluoro-orotic acid resistance. Mol. Gen. Genet. **197:** 345–346.

BREEDEN, L., and K. NASMYTH, 1985 Regulation of the yeast *HO* gene. Cold Spring Harbor Symp. Quant. Biol. **50:** 643–650.

BRILL, J. A., E. A. ELION and G. R. FINK, 1994 A role for autophosphorylation revealed by activated alleles of FUS3, the yeast MAP kinase homolog. Mol. Biol. Cell. **5:** 297–312.

CAIRNS, B. R., S. W. RAMER and R. D. KORNBERG, 1992 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. Genes Dev. **6:** 1305–1318.

CHENEVERT, J., N. VALTZ and I. HERSKOWITZ, 1994 Identification of genes required for normal pheromone-induced cell polarization in *Saccharomyces cerevisiae*. Genetics **136:** 1287–1297.

CHOI, K. Y., B. SATTERBERG, D. M. LYONS and E. A. ELION, 1994 Ste5 tethers multiple protein kinases in the MAP kinase cascade required for mating in *S. cerevisiae*. Cell. **78:** 499–512.

CID, V. J., A. DURAN, F. DEL REY, M. P. SNYDER, C. NOMBELA *et al.*, 1995 Molecular basis of cell integrity and morphogenesis in *Saccharomyces cerevisiae*. Microbiol. Rev. **59:** 345–386.

COLE, G. M., and S. I. REED, 1991 Pheromone-induced phosphorylation of a G protein *beta* subunit in *S. cerevisiae* is associated with an adaptive response to mating pheromone. Cell **64:** 703–716.

DURFEE, T., K. BECHERER, P. L. CHEN, S. H. YEH, Y. YANG *et al.*, 1993 The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. Genes Dev. **7:** 555–569.

ELION, E., 1995 Ste5: A meeting place for MAP kinases and their associates. Trends Cell Biol. **5:** 322–327.

ELION, E. A., B. SATTERBERG and J. E. KRANZ, 1993 FUS3 phosphorylates multiple components of the mating signal transduction cascade: evidence for STE12 and FAR1. Mol. Biol. Cell. **4:** 495–510.

ESTOJAK, J., R. BRENT and E. A. GOLEMIS, 1995 Correlation of two-hybrid affinity data with *in vitro* measurements. Mol. Cell. Biol. **15:** 5820–5829.

EVAN, G. I., G. K. LEWIS, G. RAMSEY and J. M. BISHOP, 1985 Isolation of monoclonal antibodies specific for human *c-myc* proto-oncogene product. Mol. Cell. Biol. **5:** 3610–3616.

FIELDS, S., and I. HERSKOWITZ, 1985 The yeast STE12 product is required for expression of two sets of cell-type specific genes. Cell **42:** 923–930.

FIELDS, S., and O. SONG, 1989 A novel genetic system to detect protein-protein interactions. Nature **340:** 245–246.

GARTNER, A., K. NASMYTH and G. AMMERER, 1992 Signal transduction in Saccharomyces cerevisiae requires tyrosine and threonine phosphorylation of FUS3 and KSS1. Genes Dev. **6:** 1280–1292.

GIETZ, R. D., and A. SUGINO, 1988 New yeast-*Escherichia coli* shuttle vectors constructed with *in vitro* mutagenized yeast genes lacking six-base pair restriction sites. Gene **74:** 527–534.

HAGEN, D. C., G. MCCAFFREY and G. F. J. SPRAGUE, 1991 Pheromone response elements are necessary and sufficient for basal and pheromone-induced transcription of the *FUS1* gene of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **11:** 2952–2961.

HASSON, M. S., 1992 Analysis of *Saccharomyces cerevisiae* pheromone response: biochemical and genetic characterization of the Ste5 protein. Ph.D. Thesis, University of California, Berkeley.

HASSON, M. S., D. BLINDER, J. THORNER and D. D. JENNESS, 1994 Mutational activation of the *STE5* gene product bypasses the requirement for G protein β and γ subunits in the yeast pheromone response pathway. Mol. Cell. Biol. **14:** 1054–1065.

HERSKOWITZ, I., 1995 MAP kinase pathways in yeast: for mating and more. Cell **80:** 187–197.

HIRSCHMAN, J. E., G. S. D. ZUTTER, W. F. SIMONDS and D. J. JENNESS, 1996 The G$\beta\gamma$ complex of the yeast pheromone response pathway: subcellular fractionation and protein-protein interactions. J. Biol. Chem. **272:** 240–248.

INOUYE, C., N. DHILLON and J. THORNER, 1997 Ste5 RING-H2 domain: Role in Ste4-promoted oligomerization for yeast pheromone signaling. Science (in press).

JENNESS, D. D., and H. K. SCHACHMAN, 1983 Genetic characterization of the folding domains of the catalytic chains in aspartate transcarbamoylase. J. Biol. Chem. **258:** 3266–3279.

JONES, E. W., 1991 Tackling the protease problem in *Saccharomyces cerevisiae.* Methods Enzymol. **194:** 428–453.

KRANZ, J. E., B. SATTERBERG and E. A. ELION, 1994 The MAP kinase Fus3 associates with and phosphorylates the upstream signaling component Ste5. Genes Dev. **8:** 313–327.

LEBERER, E., D. DIGNARD, D. HARCUS, D. Y. THOMAS and M. WHITEWAY, 1992 The protein kinase homologue Ste20p is required to link the yeast pheromone response G-protein $\beta\gamma$ subunits to downstream signalling components. EMBO J. **11:** 4815–4824.

LEBERER, E., D. DIGNARD, D. HARCUS, L. HOUGAN, M. WHITEWAY *et al.,* 1993 Cloning of *Saccharomyces cerevisiae STE5* as a suppressor of a Ste20 protein kinase mutant: structural and functional similarity of Ste5 to Far1. Mol. Gen. Genet. **241:** 241–254.

LEBERER, E., J. CHENEVERT, T. LEEUW, D. HARCUS, I. HERSKOWITZ *et al.,* 1996 Genetic interactions indicate a role for Mdg1p and the SH3 domain protein Bem1p in linking the G-protein mediated yeast pheromone signalling pathway to regulators of cell polarity. Mol. Gen. Genet. **252:** 608–621.

LEMMON, M. A., and J. SCHLESSINGER, 1994 Regulation of signal transduction and signal diversity by receptor oligomerization. Trends Biochem. Sci. **19:** 459–463.

LYONS, D. M., S. K. MAHANTY, K. Y. CHOI, M. MANANDHAR and E. A. ELION, 1996 The SH3-domain protein Bem1 coordinates mitogen-activated protein kinase cascade activation with cell cycle control in Saccharomyces cerevisiae. Mol. Cell. Biol. **16:** 4095–106.

MA, D., J. G. COOK and J. THORNER, 1995 Phosphorylation and localization of Kss1, a MAP kinase of the Saccharomyces cerevisiae pheromone response pathway. Mol. Biol. Cell **6:** 889–909.

MACKAY, V. L., 1983 Cloning of yeast *STE* genes in 2 microns vectors. Methods Enzymol. **101:** 325–343.

MANSER, E., T. LEUNG, H. SALIHUDDIN, Z. S. ZHAO and L. LIM, 1994 A brain serine/threonine protein kinase activated by Cdc42 and Rac1. Nature **367:** 40–46.

MARCUS, S., A. POLVERINO, M. BARR and M. WIGLER, 1994 Complexes between STE5 and components of the pheromone-responsive mitogen-activated protein kinase module. Proc. Natl. Acad. Sci. USA **91:** 7762–7766.

MENDELSOHN, A. R., and R. BRENT, 1994 Applications of interaction traps/two-hybrid systems to biotechnology research. Curr. Opin. Biotechnol. **5:** 482–486.

MILLER, J. H., 1972 *Experiments in Molecular Genetics.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

MUHLRAD, D., R. HUNTER and R. PARKER, 1992 A rapid method for localized mutagenesis of yeast genes. Yeast **8:** 79–82.

MUKAI, Y., S. HARASHIMA and Y. OSHIMA, 1993 Function of the Ste signal transduction pathway for mating pheromones sustains *MAT*α1 transcription in *Saccharomyces cerevisiae.* Mol. Cell. Biol. **13:** 2050–2060.

NEIMAN, A. M., and I. HERSKOWITZ, 1994 Reconstitution of a yeast protein kinase cascade *in vitro*: activation of the yeast MEK homologue STE7 by STE11. Proc. Natl. Acad. Sci. USA **91:** 3398–3402.

PERLMAN, R., D. YABLONSKI, G. SIMCHEN and A. LEVITZKI, 1993 Cloning of the *STE5* gene of *Saccharomyces cerevisiae* as a suppressor of the mating defect of *cdc25* temperature-sensitive mutants. Proc. Natl. Acad. Sci. USA **90:** 5474–5478.

PETER, M., and I. HERSKOWITZ, 1994 Direct inhibition of the yeast cyclin-dependent kinase Cdc28-Cln by Far1. Science **265:** 1228–1231.

PHIZICKY, E. M., and S. FIELDS, 1995 Protein-protein interactions: methods for detection and analysis. Microbiol. Rev. **59:** 94–123.

PRINTEN, J. A., and G. F. SPRAGUE, 1994 Protein-protein interactions in the yeast pheromone response pathway: Ste5p interacts with members of the MAP kinase cascade. Genetics **138:** 609–619.

PRYCIAK, P. M., and L. H. HARTWELL, 1996 AKR1 encodes a candidate effector of the G$\beta\gamma$ complex in the *Saccharomyces cerevisiae* pheromone response pathway and contributes to control of both cell shape and signal transduction. Mol. Cell. Biol. **16:** 2614–2626.

RAMER, S. W., and R. W. DAVIS, 1993 A dominant truncation allele identifies a gene, *STE20,* that encodes a putative protein kinase necessary for mating in *Saccharomyces cerevisiae.* Proc. Natl. Acad. Sci. USA **90:** 452–456.

RHODES, N., L. CONNELL and B. ERREDE, 1990 STE11 is a protein kinase required for cell-type-specific transcription and signal transduction in yeast. Genes Dev. **4:** 1862–1874.

SAMBROOK, J., E. F. FRITSCH and T. MANIATIS, 1989 *Molecular Cloning: A Laboratory Manual,* Ed. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

SCHULTZ, J., B. FERGUSON and G. F. J. SPRAGUE, 1995 Signal transduction and growth control in yeast. Curr. Opin. Genet. Dev. **5:** 31–37.

SIMON, M. N., C. DE VIRGILIO, B. SOUZA, J. R. PRINGLE, A. ABO *et al.,* 1995 Role for the Rho-family GTPase Cdc42 in yeast mating-pheromone signal pathway. Nature **376:** 702–705.

SONG, J., J. HIRSCHMAN, K. GUNN and H. G. DOHLMAN, 1996 Regulation of membrane and subunit interactions by N-myristoylation of a G protein α subunit in yeast. J. Biol. Chem. **271:** 20273–20283.

SPRAGUE, G. F., JR., 1991 Assay of yeast mating reaction. Methods Enzymol. **194:** 77–93.

SPRAGUE, G. F., and J. W. THORNER, 1992 Pheromone response and signal transduction during the mating process of *Saccharomyces cerevisiae,* pp. 657–744 in *The Molecular and Cellular Biology of the Yeast Saccharomyces cerevisiae: Gene Expression.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

STEVENSON, B. J., N. RHODES, B. ERREDE and G. F. SPRAGUE, JR., 1992 Constitutive mutants of the protein kinase STE11 activate the yeast pheromone response pathway in the absence of the G protein. Genes Dev. **6:** 1293–1304.

TYERS, M., and B. FUTCHER, 1993 Far1 and Fus3 link the mating pheromone signal transduction pathway to three G1-phase Cdc28 kinase complexes. Mol. Cell. Biol. **13:** 5659–5669.

WHITE, M. A., 1996 The yeast two-hybrid system: forward and reverse. Proc. Natl. Acad. Sci. USA **93:** 10001–10003.

WHITE, M. A., C. NICOLETTE, A. MINDEN, A. POLVERINO, L. VAN AELST *et al.,* 1995 Multiple Ras functions can contribute to mammalian cell transformation. Cell **80:** 533–541.

WHITEWAY, M. S., C. WU, T. LEEUW, K. CLARK, A. FOUREST-LIEUVIN *et al.,* 1995 Association of the yeast pheromone response G protein $\beta\gamma$ subunits with the MAP kinase scaffold Ste5p. Science **269:** 1572–1575.

WU, C., M. WHITEWAY, D. Y. THOMAS and E. LEBERER, 1995 Molecular characterization of Ste20p, a potential mitogen-activated protein or extracellular signal-related kinase kinase (MEK) kinase kinase from *Saccharomyces cerevisiae.* J. Biol. Chem. **270:** 15984–15992.

YABLONSKI, D., I. MARBACH and A. LEVITZKI, 1996 Dimerization of Ste5, a mitogen-activated protein kinase cascade scaffold protein, is required for signal transduction. Proc. Natl. Acad. Sci. USA **93:** 13864–13869.

YASHAR, B., K. IRIE, J. A. PRINTEN, B. J. STEVENSON, G. F. SPRAGUE, JR. *et al.,* 1995 Yeast MEK-dependent signal transduction: response thresholds and parameters affecting fidelity. Mol. Cell. Biol. **15:** 6545–6553.

ZABIN, I., and M. R. VILLAREJO, 1975 Protein complementation. Annu. Rev. Biochem. **44:** 295–313.

ZHAO, Z. S., T. LEUNG, E. MANSER and L. LIM, 1995 Pheromone signalling in *Saccharomyces cerevisiae* requires the small GTP-binding protein Cdc42p and its activator CDC24. Mol. Cell. Biol. **15:** 5246–5257.

Communicating editor: M. JOHNSTON