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The protein kinase homologue Ste20p is required to link the yeast pheromone response G-protein $\beta\gamma$ subunits to downstream signalling components

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In the yeast *Saccharomyces cerevisiae* the G-protein $\beta\gamma$ subunits have been shown to trigger downstream events of the pheromone response pathway. We have identified a new gene, designated *STE20*, which encodes a protein kinase homologue with sequence similarity to protein kinase C, which is required to transmit the pheromone signal from $G_{\beta\gamma}$ to downstream components of the signalling pathway. Overproduction of the kinase suppresses the mating defect of dominant-negative G_β mutations providing genetic evidence for an interaction with G_β, and epistasis experiments show that this kinase functions after or at the same point as $G_{\beta\gamma}$, but before any of the other currently identified components of the signalling pathway. This points to a potentially new mechanism of G-protein mediated signal transduction, the activation of a protein kinase through $G_{\beta\gamma}$.

Key words: G-protein/mating pheromone/*Saccharomyces cerevisiae*/signal transduction/Ste20p kinase

Introduction

Many eukaryotic signal transduction pathways involve heterotrimeric G-proteins that couple surface receptors with seven transmembrane domains to intracellular effector enzymes (Bourne *et al.*, 1990; Simon *et al.*, 1991). In the yeast *Saccharomyces cerevisiae*, a receptor G-protein system mediates the initial steps of the pheromone response pathway (for review see Marsh *et al.*, 1991). The **a**- and α-factor pheromones are released from haploid *MAT***a** and *MAT*α cells, respectively, and interact with cell type-specific receptors that are exposed on the cell surface of cells of the opposite mating type. Pheromone binding triggers a signalling pathway that is common to cells of both mating types (Bender and Sprague, 1986; Nakayama *et al.*, 1987). Activation of this signalling pathway ultimately leads to arrest of the cells in G_1 of the cell cycle, the induction of gene products that facilitate mating and the subsequent fusion of the haploid cells to form a zygote (Marsh *et al.*, 1991).

The genetic analysis of the G-protein involved in yeast mating has provided evidence that the pheromone signal is transmitted through the β and γ subunits to downstream components of the signalling pathway. Loss of function of the G-protein α subunit, encoded by *GPA1*, leads to constitutive activation of signalling (Dietzel and Kurjan, 1987a; Miyajima *et al.*, 1987). Disruptions of either *STE4*, encoding G_β, or *STE18*, encoding G_γ, cause unresponsiveness of the cells to pheromone (Whiteway *et al.*, 1989). Overproduction of Ste4p (Cole *et al.*, 1990; Nomoto *et al.*, 1990; Whiteway *et al.*, 1990) or a hyperactive mutation in *STE4* (Blinder *et al.*, 1989) stimulate the signalling pathway even in the absence of pheromone. Moreover, overproduction of dominant-negative Ste4p mutants impedes

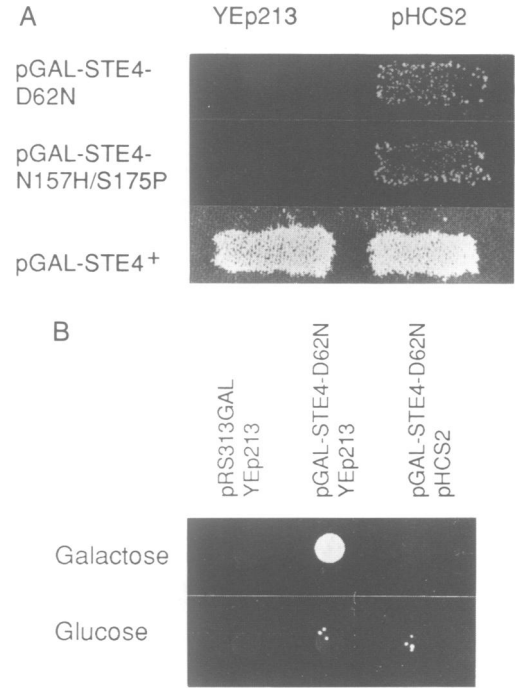
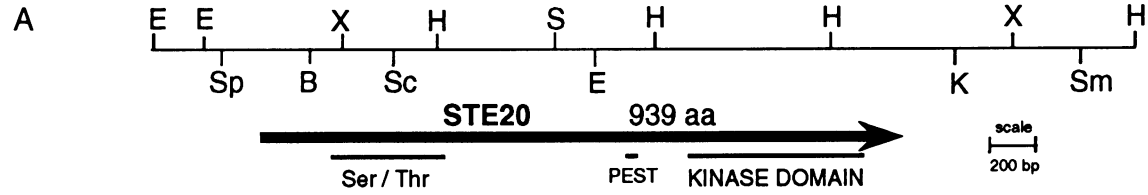


Fig. 1. Isolation of *STE20*. (**A**) Partial complementation of the mating defect of interfering *STE4* mutants by pHCS2. The *ste4*Δ *MAT***a** strain YEL2 was transformed with the indicated plasmids. Mating of patches of these transformants was tested with the *MAT*α strain A232-32D. pHCS2 confers weak mating competence to cells expressing the dominant-negative *STE4* mutants when compared with cells expressing wild type *STE4*. This effect was also observed with the multicopy plasmids pHCS9 and pVTU-STE20 carrying the full-length *STE20* gene (data not shown). The effect was, however, dependent on the galactose-induced expression of the *STE4* mutants indicating that a *ste4* null mutation is not complemented by the multicopy suppressor plasmids (data not shown). (**B**) Suppression of an inhibitory *STE4* mutant by multicopy plasmid pHCS2. The supersensitive (*sst2::URA3*) *MAT***a** strain YEL105 was transformed with the indicated plasmids and grown overnight at 30°C in selective liquid medium. From each transformant, 10^4 cells were then spotted onto selective plates (80 mm) onto which 100 μl of a 0.1 mg/ml solution of α-factor were spread, and which contained either 3% galactose or 2% glucose. Cells were grown for 48 h at 30°C. Galactose-induced expression of the *STE4-D62N* allele interferes with the α-factor-induced growth arrest, and this interference is abrogated by introduction of pHCS2. This effect of pHCS2 was also observed with pHCS9 and a full-length *STE20* multicopy plasmid (data not shown). The appearance of the few pheromone-resistant colonies seen on glucose medium was variable from experiment to experiment, and is most probably the result of random sterile mutations that frequently occur within supersensitive cells.

signalling (Leberer *et al.*, 1992). These observations support the model that the α subunit fulfils a negative regulatory role by coupling the G-protein heterotrimer to the pheromone receptor, and the β and γ subunits function together to activate the signalling pathway.

Known components of the pheromone response pathway include the Ste7p and Ste11p kinases, the Ste12p transcription factor and the Ste5p of unknown function (for review see Marsh *et al.*, 1991). The molecular mechanism by which the information is ferried from the activated G-protein $\beta\gamma$ element to these components is unclear. Here we have employed a genetic approach to identify genes whose products are potential $G_{\beta\gamma}$ targets. We have identified a new gene, designated *STE20*, which encodes a protein kinase



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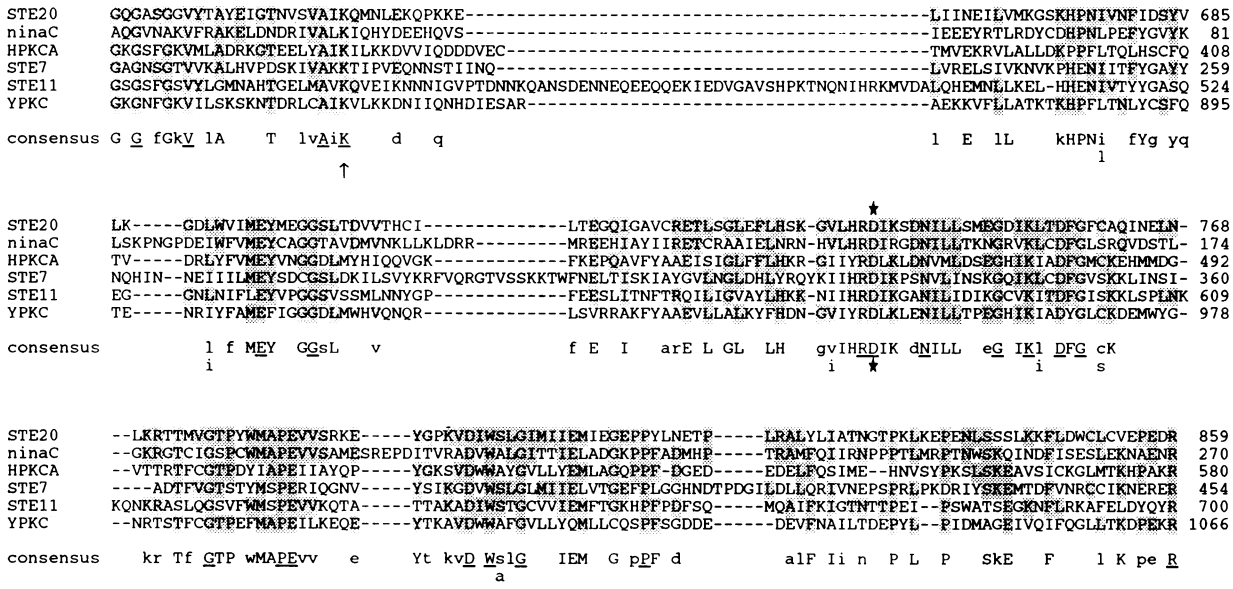

Fig. 2. Map and sequence of *STE20*. (**A**) Restriction endonuclease map, location of the ORF (arrow in bold face) and positions of protein domains. The endonuclease restriction sites are as follows: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; S, *Sal*I; Sc, *Sac*I; Sm, *Sma*I; Sp, *Sph*I; X, *Xba*I. The locations of the 281 residues segment rich in serine and threonine (Ser/Thr), a PEST sequence (PEST), postulated to be involved in protein degradation (Rogers *et al.*, 1986) and the protein kinase domain (Hanks *et al.*, 1988) are shown below. (**B**) Nucleotide and predicted amino acid sequences of *STE20*. Numerals at the left margin indicate nucleotide and amino acid positions. Nucleotide 1 corresponds to the first nucleotide of the initiation codon and amino acid 1 to the first residue of the deduced protein. The PEST sequence motif (Rogers *et al.*, 1986) has been shadowed, the kinase domain has been boxed and a highly charged sequence preceding the kinase domain has been underlined. The GXGXXG motif of the ATP binding domain (Hanks *et al.*, 1988) has been highlighted in bold face, and the invariant lysine (position 649) and aspartate (position 739) residues of the catalytic domain (Hanks *et al.*, 1988) have been indicated by an arrow and a star, respectively. The first amino acid encoded by pHCS2 (isoleucine at position 222) has been indicated by arrow heads (>>>). The aspartate residue at position 70 is the first amino acid encoded by pHCS9. The AATAAA polyadenylation signal at position 2,969 has been depicted by the wavy line (^^^). Another such signal is also found at position 3526 (not shown). The GenBank/EMBL Data Library accession number for the nucleotide sequence is M94719. (**C**) Amino acid alignment of the catalytic kinase domains of Ste20p, the *Drosophila* protein kinase ninaC (ninaC) (Montell and Rubin, 1988), human protein kinase C α (HPKCA) (Finkenzeller *et al.*, 1990), the yeast signal transduction kinases Ste7p (Teague *et al.*, 1986) and Ste11p (Rhodes *et al.*, 1990) and the yeast protein kinase C homologue Pkc1p (YPKC) (Levin *et al.*, 1990). Sequence identities with Ste20p are shadowed. Consensus residues conserved among at least three of the six kinases are shown in lower case letters below the protein sequences. Residues conserved among at least four of the kinases are shown in upper case letters. Residues conserved within all six kinases are underlined in the consensus sequence. The numbers at the end of each line specify the amino acid positions within each protein.

homologue whose function is essential to transmit the pheromone signal from $G_{\beta\gamma}$ to the other components of the response pathway and thus is a candidate for a $G_{\beta\gamma}$ effector.

Results

Isolation of STE20

We have previously identified dominant-negative *STE4* mutants that when overexpressed from the galactose-inducible *GAL1* promoter, interfere with the signalling function of wild type G_{β} (Leberer *et al.*, 1992). We have found that interference with pheromone signalling by these mutants is independent of G_{α} (encoded by *GPA1*) and not eliminated by overexpression of G_{γ} (encoded by *STE18*), suggesting that the interfering G_{β} variants inhibit the function of a component working downstream of the G-protein and interacting with G_{β}.

Here we have made use of one of these dominant-negative mutants, the Ste4p-D62N variant (containing an aspartate to asparagine substitution at position 62 of Ste4p), which fails to restore the mating defect in cells deleted for the endogenous *STE4* gene (Figure 1A). Since interference with signalling implies that the mutant protein is capable of performing some but not all of the functions of the normal protein (Herskowitz, 1987), the Ste4p-D62N mutant protein appears to have lost the ability to interact properly with some downstream components thereby blocking its normal function. We postulated that this improper interaction may cause reduced signalling and hence sterility in *ste4* deleted cells, and we expected, therefore, that overproduction of both the Ste4p-D62N mutant and the putative interacting component should result in partial restoration of the signalling function in *ste4* deleted cells.

Based upon this assumption, we have designed a genetic screen to search for components, which when overproduced, may restore mating capability in *ste4* deleted cells that overproduce the Ste4p-D62N mutant. We introduced a genomic DNA library constructed in the multicopy plasmid YEp213 into *ste4* deleted cells that contained a plasmid carrying the *STE4-D62N* mutant gene under control of the *GAL1* promoter, and screened the transformants for their capability to undergo mating on galactose medium. We isolated plasmids pHCS2 and pHCS9, which were capable of partially complementing the mating defect of *STE4-D62N* cells (Figure 1A). This effect was also observed in cells overexpressing the STE4-N157H/S175P double mutant (with asparagine to histidine and serine to proline substitutions at positions 157 and 175, respectively, of Ste4p) (Figure 1A) and all the other *STE4* mutants (data not shown), which we previously identified as dominant interfering mutant alleles (Leberer *et al.*, 1992). The effect of the multicopy suppressor plasmids was dependent on galactose-induced expression of the dominant-negative *STE4* mutants, demonstrating that a *ste4* null mutation is not suppressed by

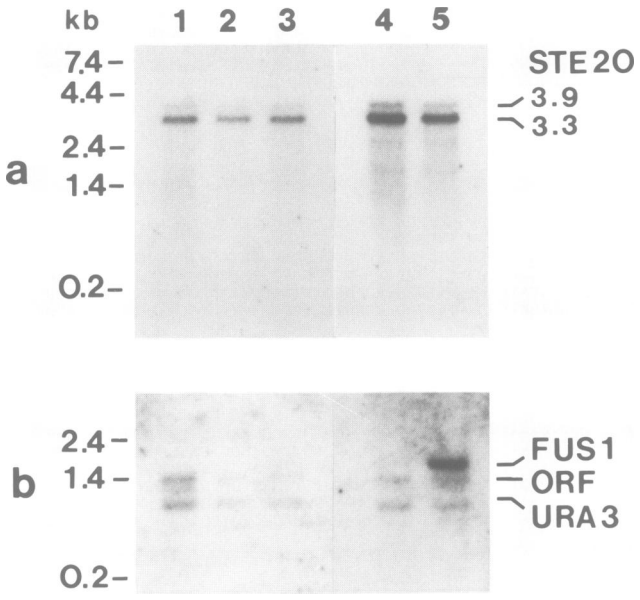

Fig. 3. Northern blot analyses of poly(A)$^+$ RNAs from diploid (lane 1), *MAT***a** (lane 2), *MAT*α (lane 3) and supersensitive (*sst1*Δ::*LEU2*) *MAT***a** cells without (lane 4) and with (lane 5) α-factor treatment (2 μM α-factor for 2 h). The yeast strains were isogenic to strain W303 (Table I). The blots were first hybridized with a *STE20* probe (**a**), and were then, after washing, hybridized with a probe containing sequences of *FUS1*, *URA3* and an ORF of unknown function located upstream of *FUS1* (Trueheart *et al.*, 1987) (**b**). *FUS1* served as a positive control for a pheromone-inducible gene and *URA3* and *ORF* as pheromone-unresponsive standards. The positions of RNA molecular weight standards are indicated on the left side of the panels (kb).

overexpression of the proteins encoded by the plasmids. Moreover, we observed that these plasmids had only slight effects on the low mating efficiency of the previously identified recessive *ste4-25* mutant allele (Whiteway *et al.*, 1989) suggesting that the effects of the multicopy suppressor plasmids are *STE4* allele-specific and not the consequence of a non-specific augmentation of a weak mating signal (data not shown). We also found that introduction of the plasmids into *STE4* wild type cells overexpressing the Ste4p-D62N mutant reverses the inhibitory effect of the mutant protein on signalling (Figure 1B). These results are consistent with a specific interaction of the proteins encoded by the multicopy suppressor plasmids with the dominant-inhibitory Ste4p mutants.

Restriction endonuclease mapping indicated that pHCS2 and pHCS9 contained overlapping DNA inserts. Sequencing revealed that both DNA fragments encompassed an extensive ORF (designated *STE20*) missing an amino-terminus (Figure 2). A chromosomal disruption of this ORF caused sterility of haploid cells (see Figure 4). We subsequently isolated a plasmid (pSTE20-5) that was able to complement the mating defect caused by disruption of *STE20* and carried the full-length *STE20* gene (Figure 2) from a library constructed in the low copy vector pRS316. Similar to the results obtained when the truncated versions of *STE20* encoded by pHCS2 and pHCS9 were overexpressed, overexpression of the full-length *STE20* gene was capable of both partially complementing the mating defect of *STE4-D62N* cells and preventing the interfering effect of overexpression of the Ste4p-D62N mutant in *STE4* wild type cells, when placed under control of the *ADH1* promoter in the multicopy plasmid pVTU-STE20 (data not shown).

Sequence analysis of STE20

The insert in plasmid pSTE20-5 contained a complete ORF encoding a deduced protein of 939 amino acids with a molecular weight of 102 kDa (Figure 2A and B). The carboxyl-terminal half of the deduced protein (Ste20p) has extensive homologies with the catalytic domains of protein serine/threonine kinases (Hanks *et al.*, 1988), with largest identities (30–34%) to kinases related to protein kinase C, including the *Drosophila ninaC* gene product (Montell and Rubin, 1988), believed to be involved in rhodopsin-mediated signal transduction, human protein kinase C α (Finkenzeller *et al.*, 1990), the yeast protein kinases Ste7p (Teague *et al.*, 1986) and Ste11p (Rhodes *et al.*, 1990), which are also components of the pheromone signalling pathway, and the yeast protein kinase C homologue Pkc1p (Levin *et al.*, 1990) (Figure 2C). The putative catalytic domain of Ste20p contains a GXGXXG sequence motif at position 627 and invariant lysine and aspartate residues at positions 649 and 739, respectively, all of which are characteristic of the ATP binding site of protein kinases (Hanks *et al.*, 1988). The sequences DIKSDN (from positions 739–744) and GTPYWMAPE (from positions 776–784) are strong indicators of protein serine/threonine kinases including kinases of the protein kinase C family (Hanks *et al.*, 1988). Based on these structural features, Ste20p is predicted to function as a protein serine/threonine kinase.

The amino-terminal half of Ste20p has no extensive homology to any known protein but is rich in serine and threonine residues (Figure 2B). The sequence from residues 95–375 contains 32% serine or threonine and is also rich in protein kinase recognition sequence motifs (Kemp and Pearson, 1990). This region may represent a regulatory domain involved in pheromone-dependent regulation of the kinase domain. In this context it is noteworthy that the Bck1p protein kinase, which was identified by a mutation bypassing the loss of function of the yeast protein kinase C homologue *PKC1* (Lee and Levin, 1992), has a similar bipartite organization into a carboxyl-terminal kinase domain and a serine/threonine rich amino-terminal domain. Since pHCS2 and pHCS9 encode truncated forms of Ste20p (Figure 2) and are capable of complementing the mating defect of *ste20* disruptant cells (data not shown), the first 221 amino acids, including half of the serine/threonine rich region, are not needed for the function of Ste20p. The kinase domain is preceded by a PEST sequence motif found in many proteins with a high turnover rate (Rogers *et al.*, 1986) and by a highly charged stretch of sequence from residues 590–601 (Figure 2). Hydropathy plots of the predicted amino acid sequence (Kyte and Doolittle, 1982) do not indicate any transmembrane domain suggesting that Ste20p is a cytosolic protein.

Chromosomal localization and cell type distribution

STE20 was localized next to the centromere of chromosome VIII by hybridization to an ordered set of λ clones (kindly provided by L.Riles and M.Olson, Washington University Medical Center, St Louis). The pattern of spore viability shown in Figure 6 provides genetic confirmation of the physical localization of *STE20*. *GPA1* is also centromere-linked on chromosome VIII (Miyajima *et al.*, 1987), therefore two different patterns of spore viability are obtained. If the two disruptants (*gpa1::LEU2* and *ste20::URA3*) are *in trans*, very few of the *gpa1::LEU2* disruptants are also *ste20::URA3*, and only two viable spores

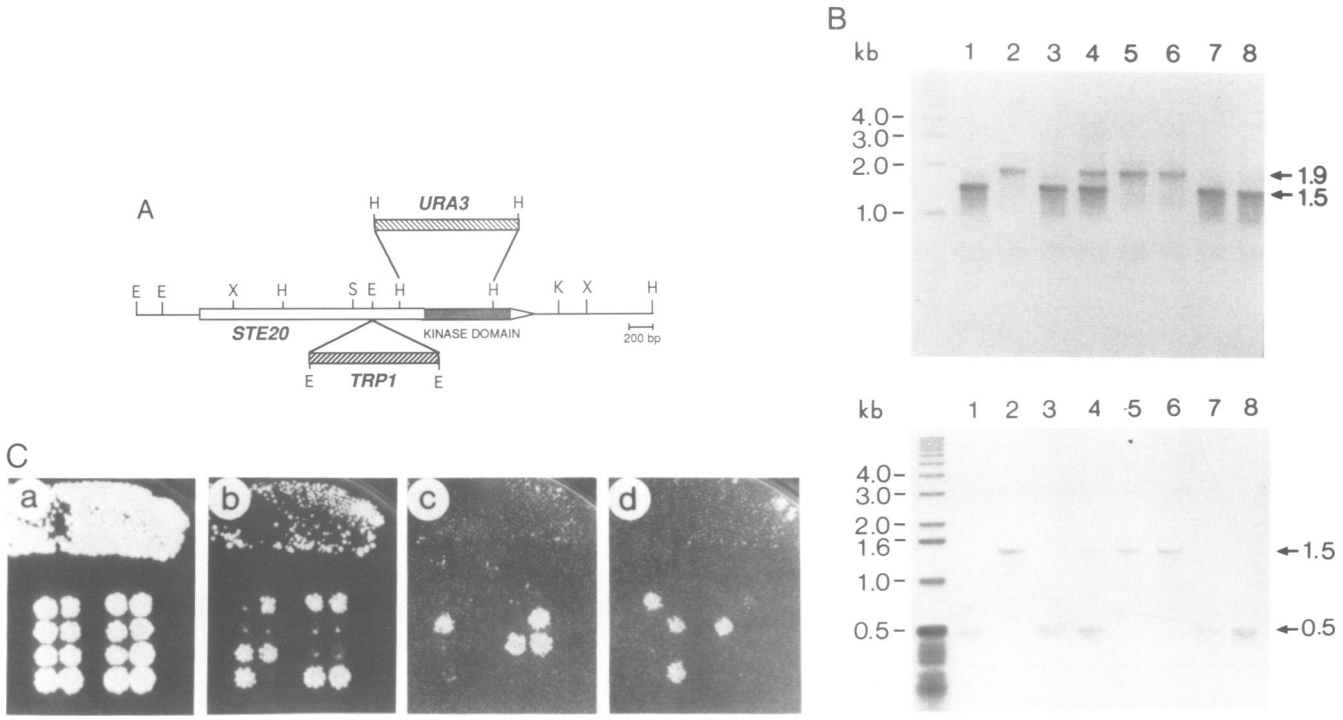

Fig. 4. Chromosomal disruption of *STE20*. (**A**) Schematic representation of the partial replacement of the kinase domain of *STE20* with *URA3* and the *TRP1* insertion. Abbreviations of the restriction sites are as defined in Figure 2. (**B**) PCR data to confirm chromosomal disruptions of *STE20*. Upper panel, partial replacement through *URA3*. DNAs from plasmids pHCS2 carrying wild type *STE20* (lane 1) and pEL45 carrying the *URA3* disrupted *STE20* gene (lane 2) and genomic DNAs from the diploid yeast strains DM225 (lane 3; homozygous for *STE20*) and YEL23 (lane 4; heterozygous for *ste20Δ::URA3*) and the haploid progeny strains YEL37 (lane 5; *ste20Δ::URA3*), YEL38 (lane 6; *ste20Δ::URA3*), YEL26 (lane 7; *STE20*) and YEL28 (lane 8; *STE20*) were amplified by the PCR procedure as described in Materials and methods. Wild type *STE20* gave rise to an amplified fragment of 1.5 kb, whereas a 1.9 kb fragment was amplified from *ste20Δ::URA3*. Lower panel, insertion of *TRP1*. DNAs from plasmids pHCS2 carrying wild type *STE20* (lane 1) and pEL46-2 carrying the *TRP1*-disrupted *STE20* gene (lane 2) and genomic DNAs from the diploid yeast strains W303 (lane 3; homozygous for *STE20*) and YEL33-7 (lane 4; heterozygous for *ste20::TRP1*) and the haploid progeny YEL33-7-3B (lane 5; *ste20::TRP1*), YEL33-7-4A (lane 6; *ste20::TRP1*), YEL33-7-1B (lane 7; *STE20*), and YEL 33-7-1C (lane 8; *STE20*) were amplified by the PCR procedure as described in Materials and methods. In this case, wild type *STE20* gave rise to an amplified fragment of 0.5 kb, whereas a 1.5 kb fragment was obtained from *ste20::TRP1*. The panels show Southern blots that were performed to confirm that the amplified fragments were derived from the *STE20* gene locus. The positions of DNA standards are indicated on the left side of the panels (kb). (**C**) Dissection of haploid progeny derived from *ste20* disrupted diploid cells. The heterozygous *ste20Δ::URA3* strain YEL23 was sporulated and then tetrads were dissected by micromanipulation. The four spores from individual asci are aligned vertically. The spores were allowed to germinate on YPD medium and grown for 3 days at 30°C. The plate was then replica plated to YPD (**a**), −ura (**b**) media, and to lawns of the *MAT***a** (**c**) or *MATα* (**d**) mating tester strains DC14 and DC17, respectively, on permissive YPD plates. Mating was performed overnight at 30°C and cells were then replica plated to selective minimal medium to select for diploid cells and incubated for a further 32 h at 30°C. All spores disrupted for the *STE20* gene (carrying the *URA3* marker) failed to undergo mating.

are obtained per tetrad. If the two disruptants are in *cis* (as shown in Figure 6), almost all the *gpa1::LEU2* spores will also be *ste20::URA3*, and because the *ste20* disruption supresses the inviability of the *gpa1* disruption, four viable (2 *LEU2+*, *URA3+* and 2 *leu2− ura3−*) spores will be found. Because two tetratypes were detected in 25 tetrads, the genetic linkage is ~4 cM.

In Northern blots, two *STE20* transcripts of 3.3 and 3.9 kb in size were recognized (Figure 3). The bands corresponding to these transcripts had similar intensities in haploid and diploid cells (Figure 3) indicating that expression of *STE20* is not regulated transcriptionally by the *MAT* locus. The 3.3 kb band had a higher intensity than the 3.9 kb band in all cell types. These transcripts may result from the differential use of two polyadenylation AATAAA consensus sequences found at nucleotide positions 2969 (Figure 2A) and 3526 (not shown). Alternatively, one of the bands may result from cross-hybridization to a transcript from a homologous gene. However, we have obtained no evidence for a cross-hybridizing gene in Southern blot analyses of genomic DNA using the same DNA probe and hybridization conditions. The intensity of both bands remained unaffected in *MAT***a** cells after treatment with α-factor (Figure 3) indicating that expression of *STE20*, unlike a variety of genes involved in pheromone signalling or mating (Marsh *et al.*, 1991), is unresponsive to pheromone.

Phenotypic consequences of ste20 disruptions

We have used two approaches to perform chromosomal disruptions of the *STE20* gene through homologous recombination. First, we have inserted the *TRP1* gene at a position preceding the kinase domain (Figure 4A). Secondly, we have replaced most of the kinase domain by the *URA3* gene (Figure 4A). The disruptions were confirmed by PCR (Figure 4B) or Southern blotting (data not shown). Both disruptions of *STE20* had no effect on cell viability but conferred sterility to *MAT***a** and *MATα* cells, as judged by the incapability of *ste20* disruptant cells to undergo mating with *STE20* wild type cells of the opposite mating type (Figures 4C and 7A). The gene disruptions also showed that

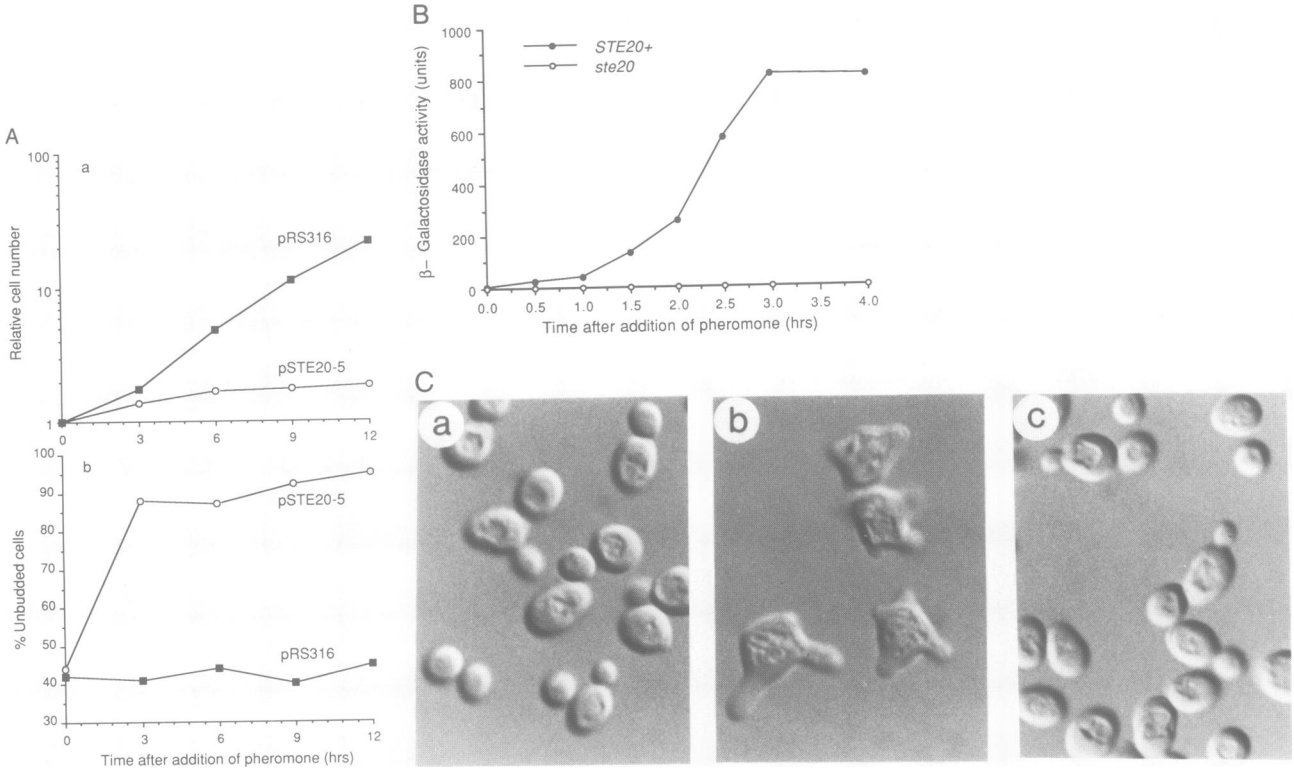

Fig. 5. Phenotypic consequences of disruption of *STE20*. (**A**) Defects in pheromone-induced growth arrest. The *ste20* disruptant and supersensitive (*sst1*) strain YEL120 was transformed with the control plasmid pRS316 or plasmid pSTE20-5 carrying the *STE20* gene and grown in selective glucose medium to mid-exponential phase at 30°C. 1 μM of α-factor was added and aliquots were taken at the indicated time points to count the cell number (a) and to determine the percentage of unbudded cells (b). (**B**) Defects in the induction of a mating-specific gene. The supersensitive (*sst1*) strains YEL106 (*STE20+*) and YEL120 (*ste20*) were transformed with plasmid pSB234 carrying a *FUS1::lacZ* fusion gene (Trueheart *et al.*, 1987) and grown in selective glucose medium to mid-exponential phase at 30°C. 0.1 μM of α-factor was added and aliquots were taken at the indicated time points to measure β-galactosidase activity. The activity is expressed in Miller units (Miller, 1972). (**C**) Defects in the induction of mating-specific morphologies. Strain YEL120 (*sst1 ste20*) was transformed with either plasmid pSTE20-5 carrying *STE20* (a and b) or the control plasmid pRS316 (c) and grown in selective glucose medium to mid-exponential phase at 30°C. Panel a shows untreated cells. Panels b and c show cells that were incubated with 1 μM α-factor for 6 h at 30°C. Photomicrographs were taken by Nomarski optics with a 100× objective.

STE20 is required for pheromone-induced arrest of the cells in G_1 of the cell cycle (Figure 5A), and for the pheromone-induced expression of a mating-specific gene, *FUS1* (Trueheart *et al.*, 1987), as monitored by the induction of a *FUS1–lacZ* fusion gene (Figure 5B). Moreover, the *ste20* disruptant cells were defective for the induction of mating-specific morphological changes, called 'shmoos' (Marsh *et al.*, 1991), in response to pheromone (Figure 5C). Taken together, these results indicate that Ste20p is an essential component of the pheromone signalling pathway and *STE20* depletion results in unresponsiveness of the cells to pheromone.

Point of function of Ste20p within the signalling pathway

Epistasis experiments were performed to define the point of function of Ste20p within the signalling pathway. We found that the constitutive G_1 arrest caused by loss of function of G_α (Dietzel and Kurjan, 1987a; Miyajima *et al.*, 1987) was blocked by the *ste20* disruption (Figure 6). Similarly, we found that the pheromone-independent activation of the growth arrest signal through galactose-induced overexpression of Ste4p (Cole *et al.*, 1990; Nomoto *et al.*, 1990; Whiteway *et al.*, 1990) was interrupted by *ste20* disruption (Figure 7D). These results are consistent with a function of Ste20p downstream of or at the same level as the G-protein β and γ subunits.

To establish further the position of Ste20p function, we made use of hyperactive *STE5* and *STE11* mutants. These gain of function mutants were identified by their capability to constitutively activate the signalling pathway in *ste4* disruptant cells, and were used to demonstrate that Ste5p acts before Ste11p and Ste7p (M.S.Hasson, D.Blinder, J.Thorner and D.D.Jenness, manuscript submitted; Stevenson *et al.*, 1992), and Ste11p prior to Ste7p (Stevenson *et al.*, 1992). We found that these mutants conferred mating competence to *ste20* disruptant cells (Figure 7B and C) suggesting that Ste20p functions prior to Ste5p and Ste11p.

Discussion

The mating response in the yeast *S.cerevisiae* is mediated through the action of a pheromone receptor-coupled heterotrimeric G-protein to downstream target enzymes (for review see Marsh *et al.*, 1991). Strong evidence has been accumulated that the β and γ subunits of the G-protein act to transmit the pheromone signal to downstream components. Intracellular processing of the pheromone signal involves several known components including protein kinases (Ste7p, Ste11p, Fus3p and Kss1p), a transcription factor (Ste12p) and a protein with unknown function (Ste5p) (Marsh *et al.*, 1991). Based upon epistasis experiments, a pathway with the order of Ste5p, Ste11p, Ste7p and Ste12p has recently been proposed (Cairns *et al.*, 1992; Stevenson *et al.*, 1992;

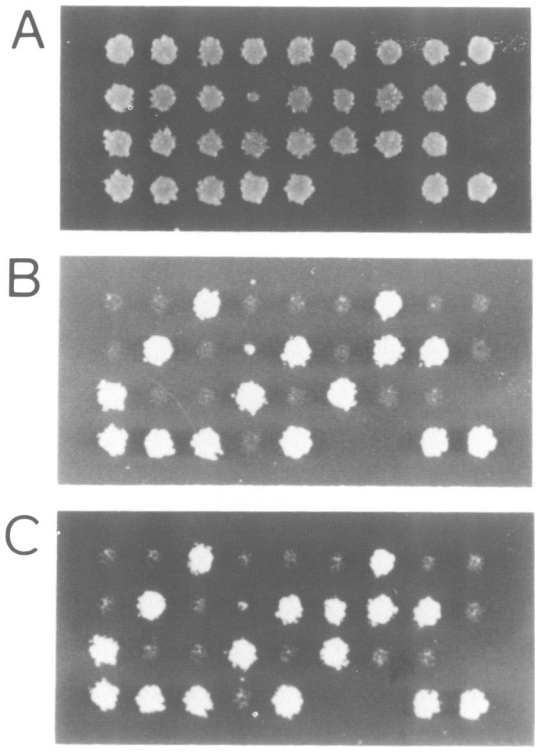

Fig. 6. Disruption of *STE20* prevents growth arrest caused by loss of *GPA1* function. The diploid strain YEL103-3 (*GPA1/gpa1::LEU2 STE20/ste20Δ::URA3*) was sporulated and tetrads were dissected by micromanipulation. The four spores from individual asci were aligned vertically. The spores were allowed to germinate on YPD medium and grown for 3 days at 30°C. The plate was then replica plated to YPD medium (**A**), −leu (**B**) and −ura minimal media (**C**) and grown for a further day at 30°C. All *LEU2*$^+$ spores (disrupted in *GPA1*) were also *URA3*$^+$ (disrupted in *STE20*) indicating that disruption of *STE20* prevents lethality caused by loss of *GPA1* function.

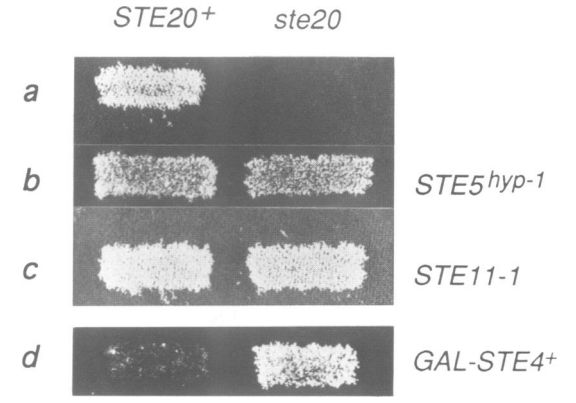

Fig. 7. Epistatic relationship of *STE20* with other components in the mating pheromone signalling pathway. (**a**) Mating of the *STE20* wild type strain W303-1A (*STE20*$^+$) and the isogenic *ste20* disruptant strain YEL33-7-3B (*ste20*). The sterile phenotype seen here in *ste20 MAT*a cells was also observed in isogenic *ste20 MAT*α cells (data not shown). (**b**) Mating of the same strains after transformation with plasmid pDJ174 carrying a gain of function (hyp-1) mutant of *STE5* (M.S.Hasson, D.Blinder, J.Thorner and D.D.Jenness, manuscript submitted). The *STE5*$^{hyp-1}$ mutant complements the mating defect of the *ste20* disruptant strain. (**c**) Mating of the *STE20* wild type strain SY1865 (*STE20*$^+$) and the isogenic *ste20* disruptant strain YEL67 (*ste20*). Both strains contain the gain of function mutation *STE11-1* (Stevenson *et al.*, 1992). A *ste20* disruption in the isogenic *STE11* wild type strain YEL64 engendered a sterile phenotype (data not shown). The *STE11-1* mutation complements the mating defect of the *ste20* disruptant cells. (**d**) Galactose-induced overexpression of *STE4* from plasmid pGAL-STE4$^+$ in the *STE4* wild type strain W303-1A (*STE20*$^+$) and the *ste20* disruptant strain YEL33-7-3B (*ste20*). The constitutive growth arrest caused by Ste4p overproduction is blocked by the *ste20* deletion.

M.S.Hasson, D.Blinder, J.Thorner and D.D.Jenness, manuscript submitted). The primary target of the $G_{\beta\gamma}$ element, however, has not been determined.

In this study we have identified a new gene, *STE20*, which encodes a protein kinase homologue that is an essential component of the signalling pathway. Disruption of *STE20* in haploid cells causes defects in mating (Figures 4 and 7), in pheromone-induced proliferation arrest (Figure 5A), in the induction of a mating-specific gene, *FUS1* (Figure 5B) and in the induction of mating-specific morphologies (Figure 5C). Epistasis experiments (Figures 6 and 7) suggest that Ste20p functions between the $G_{\beta\gamma}$ element and Ste5p, or at the same level as $G_{\beta\gamma}$. This places the Ste20p kinase among all the other currently known components of the signalling pathway closest to the point of function of the G-protein β and γ subunits. Thus the Ste20p kinase is a strong candidate for a primary target of $G_{\beta\gamma}$.

The *STE20* gene was isolated in a genetic screen that was designed to identify components potentially interacting with G_β. We made use of a dominant-negative Ste4p mutant protein whose phenotype suggested that it may have lost the function to properly activate the putative G-protein effector (Leberer *et al.*, 1992). Partial complementation of the signalling defect of this mutant by overexpression of the Ste20p kinase (Figure 1) provides genetic evidence for an interaction of Ste20p with G_β and supports the hypothesis that the Ste20p kinase may be a primary target of $G_{\beta\gamma}$. This hypothesis has to be proven in future biochemical experiments.

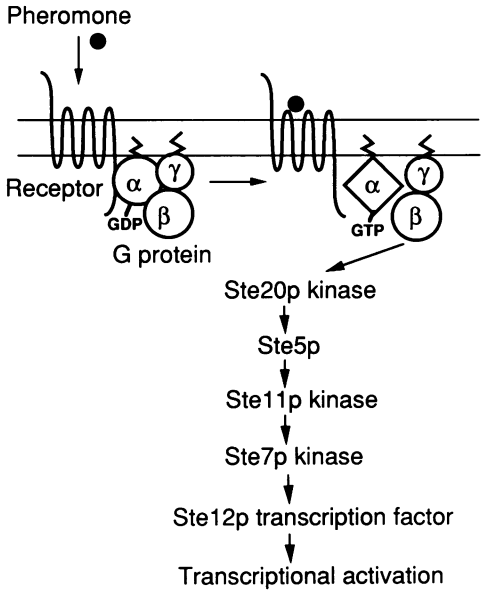

Fig. 8. Proposed model for the function of Ste20p. Our model for the order of function of the components of the signalling pathway incorporates data presented here and elsewhere (Stevenson *et al.*, 1992; Cairns *et al.*, 1992; M.S.Hasson, D.Blinder, J.Thorner and D.D.Jenness, manuscript submitted). We propose that pheromone binding to the receptor causes the activation of $G_{\beta\gamma}$, which transmits the signal through the Ste20p kinase, Ste5p and the Ste11p and Ste7p kinases to the Ste12p transcription factor, which is modified to stimulate transcriptional activation of mating-specific genes. Our model is based on genetic interactions and does not imply direct interactions between the various components, nor does it exclude the involvement of not yet identified components. Lipid modifications of G_α and G_γ that are thought to serve as membrane anchors for the G-protein (Bourne *et al.*, 1990; Simon *et al.*, 1991) are indicated.

From our epistasis experiments and those of others (Cairns *et al.*, 1992; Stevenson *et al.*, 1992; M.S.Hasson, D.Blinder, J.Thorner and D.D.Jenness, manuscript submitted) we propose the model (Figure 8) that Ste20p acts to link the activated $G_{\beta\gamma}$ subunits to the downstream components of the signalling pathway. Since Ste5p has been demonstrated to be phosphorylated in a pheromone-dependent manner (D.Jenness, personal communication), we speculate that Ste20p phosphorylates Ste5p, which, in turn, carries the signal to Ste11p. The Ste11p kinase may then phosphorylate the Ste7p kinase, which has been shown to be phosphorylated in response to pheromone (B.Errede, personal communication). The Ste7p kinase may then phosphorylate the Ste12p transcription factor to stimulate transcriptional activation of mating-specific genes.

It should be emphasized that this model of Ste20p function is solely based on genetic evidence and does not exclude interactions by as yet unidentified components. Whether indeed a physical interaction exists between $G_{\beta\gamma}$ and Ste20p, and Ste5p acts as the Ste20p kinase substrate, has to be substantiated in further genetic and biochemical studies. In this regard, however, it is noteworthy that the muscarinic acetylcholine receptor kinase has been recently demonstrated to be activated by $G_{\beta\gamma}$ in a reconstituted system (Haga and Haga, 1992). This gives reason to speculate that there may exist $G_{\beta\gamma}$ regulated kinases, and Ste20p may represent a yeast homologue of such kinases.

An alternative model for the mode of function of Ste20p is that the Ste20p kinase acts to modulate G_{β}. This interpretation is also consistent with our finding that loss of function of *STE20* blocks constitutive signalling caused by overproduction of G_{β} or ablation of *GPA1* (Figures 6 and 7). Further experiments, e.g. the analysis of hyperactive mutations of *STE20* that bypass the requirement of G_{β} for signalling, will be needed to unequivocally distinguish these models. It should be noted, however, that phosphorylation of Ste4p has been shown to be unessential for the ability of $G_{\beta\gamma}$ to transmit the mating signal (Cole and Reed, 1991). On the contrary, phosphorylation has been implicated in inactivation of Ste4p and to be part of the desensitization process (Cole and Reed, 1991). In considering all these results we are inclined to suggest that the Ste20p kinase is rather an actively signal transmitting component of the response pathway than a G_{β} modulating enzyme.

Unlike a variety of genes involved in pheromone signalling, such as the G-protein subunits and the pheromone receptors (for review see Marsh *et al.*, 1991), *STE20* is expressed in both haploid and diploid cells (Figure 3). This feature is shared by the *STE11* and *STE7* genes (Marsh *et al.*, 1991) suggesting that these kinases and Ste20p may also play a role in other functions than in pheromone signalling. This possibility has to be tested in future analyses of homozygous gene disruptions in diploid cells.

In summary, we have identified a new gene from the yeast *S.cerevisiae*, *STE20*, which encodes a protein kinase homologue that is needed to transmit the mating pheromone signal from the activated G-protein β and γ subunits to all the other currently known signalling components of the pheromone response pathway. Our genetic experiments suggest that Ste20p is a strong candidate for a $G_{\beta\gamma}$ effector. Thus the identification of *STE20* may point to a potentially new mechanism of G-protein mediated signal transduction, the initiation of a protein kinase cascade by activated $G_{\beta\gamma}$ subunits. The high degree of conservation of G-protein subunits from yeast to man (Dietzel and Kurjan, 1987a; Miyajima *et al.*, 1987; Whiteway *et al.*, 1989) suggests that a similar mechanism may also play a role in signal transduction pathways in mammalian cells.

Materials and methods

Materials

Restriction endonucleases and DNA-modifying enzymes were obtained from Boehringer Mannheim, Bethesda Research Laboratories, Pharmacia LKB Biotechnology Inc. and New England Biolabs. [α-^{32}P]dATP was obtained from Amersham International. Synthetic α-factor was purchased from Sigma, dissolved in 90% methanol at a concentration of 1 mg/ml and stored at −20°C. All other reagents were of the highest purity grade commercially available.

Strains, plasmids and DNA libraries

Escherichia coli strain MC1061 was used for the propagation of plasmids. Bacterial media, culture conditions and calcium phosphate transformation procedures were as described in Maniatis *et al.* (1982).

Plasmid pRS313GAL was constructed by introducing an *Eco*RI−*Bam*HI fragment of the *GAL1/10* promoter (Whiteway *et al.*, 1990) into the centromere plasmid pRS313 carrying *HIS3* as a selective marker (Sikorski and Hieter, 1989). Plasmids pGAL-STE4-D62N, pGAL-STE4-N157H/S175P and pGAL-STE4^{+} are pRS313 plasmids carrying dominant-negative *STE4* alleles or wild type *STE4*, respectively, under control of the *GAL1* promoter (Leberer *et al.*, 1992). These plasmids allowed the conditional expression of the Ste4p mutants or wild type protein in galactose medium. Plasmid YEp213 is a multicopy plasmid with *LEU2* as a selective marker (Broach, 1983) and pRS316 is a centromere plasmid carrying *URA3* as a selective marker (Sikorski and Hieter, 1989). Plasmid pSB234 is a high copy plasmid carrying a *FUS1*−*lacZ* fusion gene (Trueheart *et al.*, 1987) and *URA3* as a selective marker, and was kindly provided by J.L.Pinkham. Plasmid pDJ174 is a multicopy YEp352 plasmid carrying a hyperactive *STE5* mutant and *URA3* as a selective marker (M.S.Hasson, D.Blinder, J.Thorner and D.D.Jenness, manuscript submitted) and was kindly provided by M.S.Hasson and D.D.Jenness.

Genomic DNA libraries of *S.cerevisiae* were constructed by ligating partial *Sau*3A digests of genomic DNA into the *Bam*HI sites of either YEp213 (the library was kindly provided by Dr Terry Chow, University of Sherbrooke, Sherbrooke) or pRS316 (the library was kindly provided by Dr Charles Boone, University of Oregon, Eugene).

The yeast strains used in this study are listed in Table I. Strain SY1865 contains *HIS3* under control of the *FUS1* promoter and the hyperactive mutation *STE11-1* (Stevenson *et al.*, 1992). Yeast media, culture conditions and manipulations of yeast strains were as described by Rose *et al.* (1990). Yeast transformations with circular or linearized plasmid DNA were carried out after treatment of yeast cells with lithium acetate according to Ito *et al.* (1983). Plasmid DNA was isolated from yeast cells as described by Rose *et al.* (1990).

Gene disruptions were performed by the one-step gene replacement method of Rothstein (1983). The *ste4* deletion in strain YEL2 was constructed by replacing the complete coding region of *STE4* with *URA3* (Leberer *et al.*, 1992). The *sst2* disruption in strain YEL105 was conducted as described (Dietzel and Kurjan, 1987b). The *sst1* disruptions in strains YEL106 and YEL120 were performed by using a fragment of pZV77 (kindly provided by V.L.MacKay, ZymoGenetics Inc., Seattle) containing *LEU2* and flanking sequences of *SST1* (MacKay *et al.*, 1988).

STE20 was disrupted by either insertion of *TRP1* or partial replacement with *URA3* (Figure 4). The *Sal*I−*Xba*I fragment from nucleotides 1302−3324 of *STE20* was subcloned into the Bluescript KS(+) vector (Stratagene) and a 1.0 kb *Eco*RI fragment of *TRP1* derived from pJJ246 (Jones and Prakash, 1990) was then inserted into the *Eco*RI site at position 1485 of the *STE20* coding region to constitute plasmid pEL46-2. Partial deletion of the coding region for the kinase domain of *STE20* was achieved by replacing the *Hin*dIII−*Hin*dIII fragment from nucleotide positions 1746 to 2526 of the *STE20* gene with a 1.2 kb *Hin*dIII fragment of *URA3* derived from pJJ244 (Jones and Prakash, 1990) to constitute plasmid pEL45. Linear fragments of these plasmids were then used to disrupt the chromosomal *STE20* gene by homologous recombination (Rothstein, 1983). The *STE20* gene disruptions were confirmed by either Southern blotting (data not shown) or PCR (Figure 4B). Amplification of genomic DNA isolated from control and disruptant strains by PCR (Saiki *et al.*, 1988) was carried out with *Taq* thermostable DNA polymerase (Cetus). The oligodeoxynucleotide primers

Table I. Yeast strains

Strain	Genotype	Source
W303	*MAT***a**/α *ade2/ade2 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 can1/can1*	R.Rothstein
W303-1A	*MAT***a** *ade2 his3 leu2 trp1 ura3 can1*	R.Rothstein
W303-1B	*MAT*α *ade2 his3 leu2 trp1 ura3 can1*	R.Rothstein
YEL2	W303-1A *ste4Δ::URA3*	This work
DM225	*MAT***a**/α *leu2/leu2 trp1/TRP1 his3/HIS3 ura3/ura3*	This work
YEL23	DM225 *STE20/ste20Δ::URA3*	This work
YEL37	*MAT*α *leu2 ura3 his3 ste20Δ::URA3* (segregant from YEL23)	This work
YEL38	*MAT***a** *leu2 ura3 his3 ste20Δ::URA3* (segregant from YEL23)	This work
YEL26	*MAT*α *leu2 ura3 his3* (segregant from YEL23)	This work
YEL28	*MAT***a** *leu2 ura3 his3* (segregant from YEL23)	This work
YEL33-7	W303 *STE20/ste20::TRP1*	This work
YEL33-7-3B	W303-1A *ste20::TRP1* (segregant from YEL33-7)	This work
YEL33-7-4A	W303-1B *ste20::TRP1* (segregant from YEL33-7)	This work
YEL33-7-1B	W303-1A (segregant from YEL33-7)	This work
YEL33-7-1C	W303-1B (segregant from YEL33-7)	This work
D111	W303 *MAT***a**/α *GPA1/gpa1::LEU2*	J.Kurjan
YEL103-3	D111 *STE20/ste20Δ::URA3*	This work
YEL105	W303-1A *sst2::URA3*	This work
YEL106	W303-1A *sst1::LEU2*	This work
YEL120	YEL106 *ste20::TRP1*	This work
SY1865	*MAT*α *his3 leu2 ura3 trp1 FUS1::HIS3 STE11-1*	B.J.Stevenson
YEL67	SY1865 *ste20::TRP1*	This work
YEL64	YEL67 *STE11*$^+$	This work
A232-32D	*MAT*α *his3 ura3 can1*	A.Murray
DC14	*MAT***a** *his1*	J.Hicks
DC17	*MAT*α *his1*	J.Hicks

corresponded to the nucleotide sequences from positions 1357−1377 for both disruptions and from positions 1831−1854 for the *TRP1* insertion and from positions 2821−2844 for the replacement by *URA3*. This gave rise to amplified fragments of 0.5 or 1.5 kb for the wild type *STE20* gene and fragments of 1.5 and 1.9 kb for the *TRP1* and *URA3* disrupted *STE20* genes, respectively. The amplified fragments were then analysed by Southern blotting using an *Eco*RI−*Kpn*I fragment from nucleotide positions 1485−3075 of the *STE20* DNA as a hybridization probe to confirm that the solely amplified fragments were derived from the *STE20* gene locus. Hybridizations were done in 50% formamide at 42°C as described by Maniatis *et al.* (1982).

Isolation and sequencing of STE20

The genomic DNA library constructed in the multicopy plasmid YEp213 was transformed into the *ste4* deleted *MAT***a** yeast strain YEL2 carrying plasmid pGAL-STE4-D62N and ~6000 independent transformants were patched onto selective glucose (2%) plates. Plasmids pHCS2 and pHCS9 were then selected by their ability to partially complement the mating defect of the dominant-negative *STE4* mutant allele on galactose plates. Mating tests were performed by replica plating the patches to a lawn of the *MAT*α tester strain A232-32D on permissive YEP-galactose (3%) plates at 30°C overnight. These plates were then replica plated to selective minimal medium to select for diploid cells and incubated for a further 32 h at 30°C.

The full-length *STE20* clone pSTE20-5 was isolated from the genomic DNA library constructed in the low copy plasmid pRS316 by complementation of the mating defect of the *ste20* disruptant *MAT***a** strain YEL33-7-3B. Mating of transformants was performed with the *MAT*α tester strain DC17 on glucose medium as described above. The full-length *STE20* multicopy plasmid pVTU-STE20 was constructed as follows. The *STE20* gene from plasmid pSTE20-5 was amplified by PCR (Saiki *et al.*, 1988) with *Taq* thermostable DNA polymerase and the oligodeoxynucleotide primers 5′-GATCCTCGACTAAAGATCTTAATGAGCAATGATCC-3′ and 5′-CCGCTCGAGTTTACTTTTGTTTATCATC-3′. A 2.8 kb fragment, the sole PCR product produced, was digested with *Bgl*II and *Xho*I, and then cloned into the *Bam*HI−*Xho*I site of pVT102-U carrying *URA3* as selective marker (Vernet *et al.*, 1987) to constitute pVTU-STE20. This places the complete coding region of *STE20* under control of the *ADH1* promoter. This plasmid was found to complement fully the mating defect of *ste20* disruptant cells and to stimulate mating in *STE4-D62N* and *STE4-N157H/S175P* cells (data not shown).

Both DNA strands of *STE20* were sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) with the Klenow fragment of DNA polymerase I and [α-^{32}P]dATP after subcloning of fragments into M13 using restriction sites. The predicted protein sequence was analysed using the University of Wisconsin Genetics Computer Group package (Devereux *et al.*, 1984) and compared with databases using the FASTA and TFASTA programs (Pearson and Lipman, 1988); significant homologies were found only for the kinase domain.

Northern blot analyses

Total RNA was isolated from logarithmically growing yeast cells using the hot phenol method as described by Koehrer and Domdey (1991). Poly(A)$^+$ RNA was prepared by chromatography on oligo(dT)-cellulose (Maniatis *et al.*, 1982). 4 μg RNA were separated electrophoretically in denaturing formaldehyde−1.2% agarose gels (Maniatis *et al.*, 1982) and transferred to Zeta Probe nylon membrane (Bio-Rad). Hybridizations were performed at 42°C in 50% formamide (Maniatis *et al.*, 1982). The *STE20* hybridization probe was an *Eco*RI−*Kpn*I fragment from nucleotide positions 1485−3075 of the *STE20* DNA. The *FUS1* probe was a *Bam*HI−*Hin*dIII fragment of pSB234 comprising *URA3* and partial sequences of *ORF* and *FUS1* (Trueheart *et al.*, 1987). In order to test pheromone-inducibility of *STE20* expression, logarithmically growing cells were treated with 2 μM α-factor for 2 h at 30°C prior to RNA isolation.

Miscellaneous

All other recombinant techniques were carried out essentially according to standard protocols (Maniatis *et al.*, 1982). β-galactosidase activity was measured as described by Slater and Craig (1987) and units were defined as $(OD_{420}\times1000)/(OD_{600}\times t\times v)$ (Miller, 1972).

Determination of cell number was conducted in a cell counting chamber after fixation of the cells in 3.7% formaldehyde and 75 mM NaCl (fixation solution) for 10 min and a brief sonication. For microscopic analyses, yeast cells were fixed for 10 min in fixation solution, briefly sonicated and then embedded in 90% glycerol. Photomicrographs were taken by using a 100× objective with a Leica Aristoplan microscope with differential interference contrast (Nomarski) optics.

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