Identification of Novel Pheromone-response Regulators through Systematic Overexpression of 120 Protein Kinases in Yeast*

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Protein kinases are well known to transmit and regulate signaling pathways. To identify additional regulators of the pheromone signaling apparatus in yeast, we evaluated an array of 120 likely protein kinases encoded by the yeast genome. Each kinase was fused to glutathione S-transferase, overexpressed, and tested for changes in pheromone responsiveness *in vivo*. As expected, several known components of the pathway (*YCK1*, *STE7*, *STE11*, *FUS3*, and *KSS1*) impaired the growth arrest response. Seven other kinases also interfered with pheromone-induced growth arrest; in rank order they are as follows: YKL116c (renamed *PRR1*) = YDL214c (renamed *PRR2*) > YJL141c (*YAK1*, *SRA1*) > YNR047w = YCR091w (*KIN82*) = YIL095w (*PRK1*) > YCL024w (*KCC4*). Inhibition of pheromone signaling by *PRR1*, but not *PRR2*, required the glutathione S-transferase moiety. Both kinases inhibited gene transcription after stimulation with pheromone, a constitutively active kinase mutant *STE11*-4, or overexpression of the transcription factor *STE12*. Neither protein altered the ability of the mitogen-activated protein kinase (MAPK) Fus3 to feedback phosphorylate a known substrate, the MAPK kinase Ste7. These results reveal two new components of the pheromone-signaling cascade in yeast, each acting at a point downstream of the MAPK.

Many cell-signaling processes require the action of receptors, G proteins, and protein kinases that propagate the signal. In humans, G protein-coupled receptors respond to hormones, neurotransmitters, odors, taste, and light. In the yeast *Saccharomyces cerevisiae*, G protein-linked pheromone receptors trigger events required for mating and cell fusion. Upon activation of the receptor (Ste2 or Ste3 in yeast), GTP binds to the G protein α subunit (Gpa1), which triggers its dissociation from the G protein $\beta\gamma$ subunits (Ste4/Ste18). G$\beta\gamma$ in turn activates a protein kinase cascade comprised of Ste20, Ste11, Ste7, and Fus3 (1). Ste5 promotes signaling, through the assembly and recruitment of Ste11, Ste7, and Fus3, to the activating G protein, as well as to effector targets within the nucleus (2). Sst2 promotes signal inactivation by accelerating Gpa1 GTPase activity and promoting subunit reassembly (3). The casein kinase I isoforms (Yck1, Yck2) promote signal inactivation by phosphorylating receptors, triggering their uptake and degradation (4–6). Notably, most (if not all) of the signaling components must exist as dimers to function. Prominent examples include the receptor (7, 8) Ste5 and its associated kinases (9–11), as well as the nuclear transcription factor Ste12 (12–15).

A central player in the kinase cascade is Fus3, a member of the MAPK[1] family. In mammals, MAPKs can respond to a variety of signaling molecules and chemical or physical stresses (temperature, salt, etc.) (16). MAPKs in turn regulate almost all aspects of cell growth and homeostasis. In yeast, the MAPK Fus3 phosphorylates and regulates proteins required for pheromone detection (Ste3), kinase scaffolding and activation (Ste5) (17), morphological and cytoskeletal changes (Far1) (18–20), cell division arrest (Far1) (19–21), transcriptional activation (Ste12) (18, 22, 23), transcription inhibition (Dig1, Dig2) (24–26), and feedback inhibition (Sst2) (27). In most cases, phosphorylation by Fus3 leads to enhanced signal transduction, but in a few instances phosphorylation promotes signal attenuation or desensitization. For instance, phosphorylation of the receptor Ste3 leads to its endocytosis and degradation (28). Phosphorylation of Sst2 leads to its stabilization and to further inactivation of the G protein (27). Thus Fus3 allows a coordinated response to pheromone in preparation for cell fusion, with both positive and negative outcomes for signaling.

The completion of the yeast genome sequence has yielded a complete inventory of 6144 open reading frames. Of these, 123 are likely to encode protein kinases, many of which have not been characterized. Given the prominent role that kinases have in G protein signaling and desensitization, we undertook a comprehensive screen of these proteins for those affecting the pheromone response in yeast. Here we describe two novel regulators of the G protein/MAPK cascade, which we have designated Prr1 and Prr2.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Mutagenesis—Standard methods for the growth, maintenance, and transformation of yeast and bacteria, and for the manipulation of DNA, were used throughout (29). The yeast *S. cerevisiae* strains used in this study were EJ 758 (MAT**a** *his3*-200, *leu2*-3, 112, *ura3*-52, *pep4*::URA3) (30), C699–32 (*MAT**a** bar1*Δ::HisG *FUS1-lacZ* ::LEU2 ade2 ste7::ADE2 his3 leu2 trp1 ura3 ssd1 can1), BY4741 (*MAT**a** leu2*Δ *met15*Δ *ura3*Δ), and BY4741-derived mutants lacking *FUS3* (YBL016w), *PRR1* (YKL116c), and *PRR2* (YDL214c; all from Research Genetics, Huntsville, AL).

The following steps were used for construction by gene replacement of strain C699–32 from strain C699 (identical to strain W303–1A)

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; GST, glutathione S-transferase; PIPES, 1,4-piperazinediethanesulfonic acid; NLS, nuclear localization signals.

(from K. Nasmyth). The *BAR1* locus was replaced with the *bar1Δ::HisG::URA3::HisG* allele from the plasmid C1329 (from K. Nasmyth). Loss of the *URA3* marker was carried out by selection on 5-fluoroorotic acid-containing medium (31, 32). *Eco*RI-linearized pNC276[2] was used for integration of the *FUS1-lacZ* reporter gene at the *leu2* locus. Finally, the *STE7* locus was replaced with the *ste7Δ::ADE2* allele from pSL2270 (33).

For expression of kinase-GST fusions, plasmid pYEX 4T-2 (CLON-TECH, Palo Alto, CA) (2 μ, *CUP1* promoter, *URA3* and *leu2*-d, amp^R, GST) was modified by the addition of a 140-nucleotide recombination domain, 3′ of its *Eco*RI site, linearized within the recombination domain by restriction digestion, and cotransformed with polymerase chain reaction-amplified open reading frames that had the same ends as the linearized plasmid, as described previously (34). For overexpression without GST, each open reading frame was polymerase chain reaction-amplified using Genepair primers (Research Genetics, Huntsville, AL), which include start and stop codons and a universal flanking sequence, and subcloned into the yeast expression vector pYES2.1/V5-His-TOPO (2 μ, *URA3*, *GAL1* promoter, *CYC1* terminator) (Invitrogen, Carlsbad, CA). *STE7* was expressed as a Myc epitope-tagged protein (Ste7M) using pNC318 (*CEN3-ARS1*, *CYC1* promoter, *TRP1*, amp^R), as described previously (35). *STE11*-4 was overexpressed using YCp50-*STE11*-4 (2 μ, *URA3*, amp^R) (from George Sprague, University of Oregon). *STE12* was overexpressed using PFILZ-112.1 (*TRP1*, 2 μ, amp^R, *GAL1* promoter) (from Joseph Dolan, Medical University of South Carolina).

The Lys to Arg mutations were constructed using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). The mutagenic oligonucleotides (plus complementary strands; not shown) are 5′-CAT CGT GAT TTA AAG CCC TCC AAC-3′ (*FUS3*), 5′-GGT CAC CGG CAA TTT GCT GCT GAG TTT ATA GGG-3′ (*PRR1*), and 5′-CAA AAA CGT AAT TTC CTT ATG TCC AGT TTC-3′ (*PRR2*).

Pheromone-response Bioassays—For the pheromone-dependent growth inhibition assay (halo assay), overnight cultures were grown in selective media, and 100 μl was diluted with 2 ml of sterile water, followed by the addition of an equal volume of 1% (w/v) dissolved agar (55 °C), and poured onto an agar plate containing the same medium. Sterile filter discs were spotted with synthetic α-factor pheromone and placed onto the nascent lawn to induce growth arrest. The resulting zone of growth-arrested cells was documented after 2 days.

For pheromone-dependent reporter transcription assays (36), strains were grown for 36 h in dextrose selective medium and then diluted in galactose selective medium to induce expression of the kinases. Mid-log phase cells were then aliquoted (90 μl) to a 96-well plate and mixed with 10 μl of α-factor for 90 min, in triplicate. β-Galactosidase activity was measured by adding 20 μl of a freshly prepared solution of 83 μM fluorescein di-β-D-galactopyranoside (10 mM stock in Me₂SO; Molecular Probes), 137.5 mM PIPES, pH 7.2, 2.5% Triton X-100, and incubating for 90 min at 37 °C. The reaction was stopped by the addition of 20 μl of 1 M Na₂CO₃, and the resulting fluorescence activity was measured at 485-nm excitation, 530-nm emission.

Ste7 phosphorylation was monitored by immunoblotting. Cells were grown in galactose selective medium to induce *PRR1*, *PRR1*-KR, *PRR2*, *PRR2*-KR, *FUS3*, and *FUS3*-KR to a density of 1×10^7 cells per ml. Protein extracts (150 μg) were prepared as described previously (37, 38), fractionated by SDS-polyacrylamide (10%) gel electrophoresis, and transferred to nitrocellulose using a mini trans-blot electrophoretic transfer cell (Bio-Rad) for 1 h at 110 V. 9E10 monoclonal antibodies (1.4 μg/ml) (39) were used to detect the Myc epitope-tagged form of Ste7 (Ste7M). The Promega Protoblot immunoblot system with anti-mouse IgG alkaline phosphatase conjugate (0.13 μg/ml) was used to detect the primary antibody by calorimetric methods. Protein concentrations were determined using the Bio-Rad protein assay kit.

Bioinformatics—Prr2 homologues were identified with the advanced BLAST algorithm using the Prr2 protein kinase domain as the query (40). Multiple alignments of the Prr2 homologues were performed using CLUSTAL W (41) with Phylip set as output. For the visualization of the degree of relatedness among the Prr2 homologues in *S. cerevisiae*, the CLUSTAL W alignment was imported into TREEVIEW (42). Nuclear localization signals (NLS) were identified using the Internet version of PSORT II (43). For the detection of classical NLS patterns, PSORT uses the following two rules: four-residue pattern (pat4) composed of four basic amino acids (Lys or Arg) or composed of three basic amino acids (Lys or Arg) and either His or Pro; the other (pat7) is a pattern starting with Pro and followed within three residues by a basic segment con-

taining Lys/Arg at three of four positions. Another type of NLS pattern (bipartite) is two basic residues, a ten-residue spacer, and another basic region consisting of at least three positions of five basic residues.

RESULTS

To identify novel proteins that regulate the mating pathway, we screened an expression array of 120 (of 123 total) protein kinases encoded by the yeast genome. Each open reading frame was fused to GST and overexpressed in a high copy plasmid, using the copper-inducible promoter from *CUP1*. Most of the 6144 open reading frames are available in this format and are typically tested in arrayed pools containing a defined set of gene products (30). Because we were primarily interested in finding novel kinase regulators of the pathway, we elected to test this subset of genes individually. Our approach has significant advantages over conventional genetic screening strategies. First, it is quite rapid; each round of screening can be conducted in about 2 weeks. Second, it is highly systematic, allowing us to test nearly all of the likely kinases encoded by the yeast genome, and provides immediate identification of the gene responsible for a particular activity. Third, it is sensitive, because each kinase is strongly overexpressed. Fourth, each open reading frame is fused to GST, which will facilitate eventual purification and biochemical analysis of the encoded kinase.

The following strategy was used to identify kinases that promote or inhibit pheromone signaling. Transformed cells were grown to saturation and plated on solid medium containing copper sulfate to induce kinase expression. α-Factor pheromone was spotted onto the nascent lawn, and the resulting zone of growth inhibition was documented after 2–3 days (halo assay). After screening all 120 clones in duplicate, the vast majority yielded no difference in growth. None caused an increase in halo size, whereas a few produced halos that were smaller and/or more turbid than normal. Of these, 12 were selected for further analysis and validation.

The following five of the 12 clones encode known kinases of the pathway: *YCK1*, *STE7*, *STE11*, *FUS3*, and *KSS1* (a MAPK that can partially complement *FUS3*). The only other known kinase in the pathway, *STE20*, was not included in the screen, because it is one of three genes that could not be cloned into the expression vector. Our ability to identify essentially all of the known kinase components in the pathway indicates that our screening strategy produced few false negatives. Further, recovery of all the expected kinases reinforces the idea that this set is functionally relevant and that the seven other clones identified in the screen are likely to have some role in pheromone signaling. These are as follows, in rank order of inhibitory potency: YKL116c = YDL214c > YJL141c (*YAK1*, *SRA1*) > YNR047w = YCR091w (*KIN82*) = YIL095w (*PRK1*) > YCL024w (*KCC4*). Notably, the screen does not reveal whether the other clones recovered here might have a positive or negative role in pheromone signaling. For instance, Ste11 and Ste7 are primarily needed to transmit the pheromone signal, yet each inhibits the response in this overexpression method (see below).

Pheromone-induced growth inhibition experiments with representative kinases from the above set are shown in Fig. 1. In this and all subsequent figures, *FUS3* is used as a positive control, whereas an irrelevant kinase or empty vector is used as a negative control. The two most potent inhibitors were renamed *PRR1* (YKL116c) and *PRR2* (YDL214c), for pheromone response regulators 1 and 2. Because they had not previously been implicated in the pheromone-response pathway, we focused our attention on their further characterization.

The halo assay is a convenient but fairly qualitative measure of pheromone sensitivity. A more quantitative measure of pher-

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[2] B. Errede, unpublished data.

FIG. 1. **Overexpression of kinase-GST fusions leads to diminished pheromone-dependent growth arrest response.** Cells were transformed with *FUS3*, *PRR1*, *PRR2*, YNR047w, *KIN82*, or *PTK2*, all in the vector pYEX-4T-2, plated, and exposed to sterile filter discs spotted with α-factor (*clockwise from bottom*: 0, 15, 25, 50, 60, and 75 μg) for 48 h and then photographed. Depicted are the four unknown protein kinases recovered in the screen, Prr1, Prr2, Ynr047w, and Kin82. Fus3 is the positive control. Ptk2 is the negative control. Rare colonies within the zone of growth inhibition are spontaneous sterile mutants.
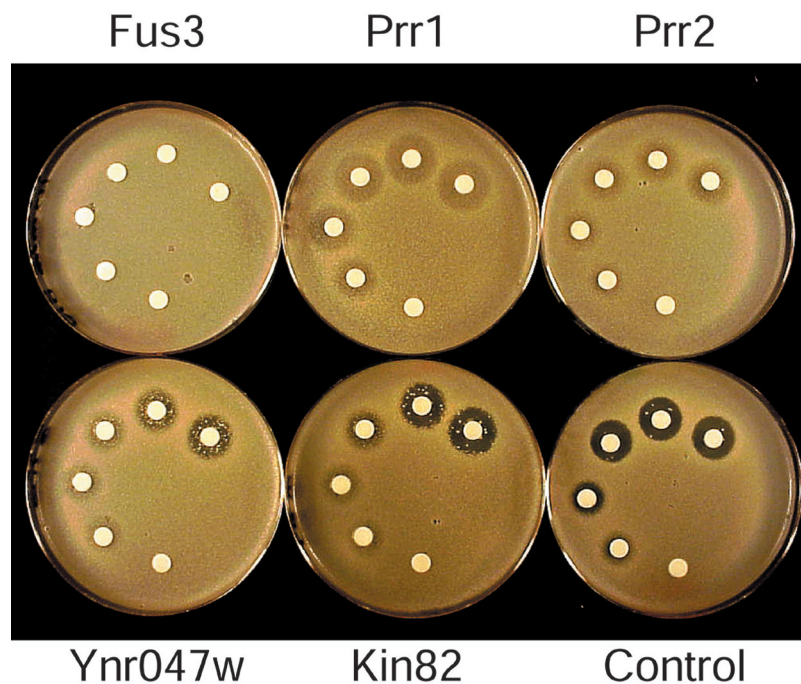
omone response can be obtained from analysis of transcriptional induction, using a reporter comprised of the *FUS1* promoter and *lacZ* (β-galactosidase) gene. As shown in Fig. 2, overexpression of either *PRR1* or *PRR2* results in a marked inhibition of pheromone-dependent transcription. Compared with the vector control, overexpression of Prr1 or Prr2 reduced the maximum response at 30 μM α-factor by 1.24- and 2.77-fold, respectively, with no change in EC_{50}. Overexpression of our positive control kinase Fus3 yielded an even larger (11.7-fold) decrease in activity, as shown previously (44).

As noted above, the overexpression assays employed here do not distinguish whether a given kinase has a positive or negative role in pheromone-induced signaling. It was shown previously through systematic gene disruption analysis that neither *PRR1* nor *PRR2* is essential for cell viability (45). Therefore, the transcription response of deletion mutant strains could be evaluated over a range of α-factor concentrations. As shown in Fig. 3, the pheromone response in either the *prr1*Δ or *prr2*Δ single mutant is identical to that of the wild-type strain. By contrast, the pheromone response is diminished with the control *fus3*Δ mutant. Although there is substantial precedent and several reasons for single deletion mutants failing to give a phenotype, this outcome meant that different strategies would be needed to establish whether *PRR1* and *PRR2* act as positive or negative regulators (see below).

To determine where in the pathway Prr1 and Prr2 operate, we examined their ability to inhibit signaling at different points downstream of the pheromone receptor. For this analysis, the pathway was activated in two alternative ways. In the first case, we used an allele of the MAPK kinase kinase *STE11* (*STE11*-4), containing a single amino acid substitution (T596I) within the catalytic domain. Cells expressing *STE11*-4 exhibit a significant increase in basal signal pathway activity (46). Constitutive activation by *STE11*-4 is unaffected by mutations in the receptor (Ste2) or Gβ (Ste4) but is blocked by mutations in the downstream MAPK kinase (Ste7), MAPK (Fus3, Kss1), or transcription factor (Ste12) (46). Thus *STE11*-4 activates signaling at a well defined step in the pathway, between Ste20 and Ste7. In the second case, we activated the pathway through expression of *STE12* under the control of the inducible *GAL1* promoter. *STE12* overexpression leads to transcriptional in-
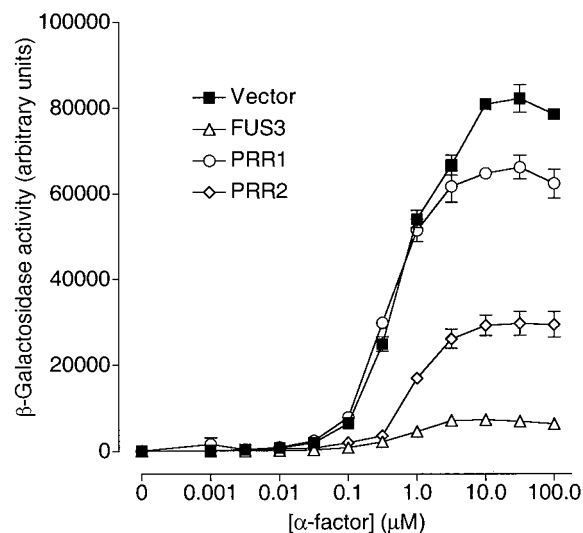
FIG. 2. **Overexpression of *PRR1*-GST or *PRR2*-GST leads to diminished pheromone-dependent transcriptional induction.** Cells were cotransformed with a plasmid containing the pheromone-responsive *FUS1* promoter-*lacZ* reporter and a plasmid containing *FUS3*-GST, *PRR1*-GST, *PRR2*-GST, or no insert (*Vector*; pYEX 4T-2). Cells were treated with the indicated concentrations of α-factor, and β-galactosidase activity was determined as detailed under "Experimental Procedures." Data shown are typical of three independent experiments performed in triplicate. *Error bars*, $\pm$ S.E.

duction in the absence of any upstream components or added pheromone (47).

As shown in Fig. 4, Prr1 and Prr2 are both potent inhibitors of transcriptional induction, in response to either *STE11*-4 (top panel) or *STE12* overexpression (bottom panel). In agreement with previous reports, our positive control Fus3 is also a potent inhibitor of transcriptional induction (48–50). When overexpressed, a portion of Fus3 is presumably in the unphosphorylated and catalytically inactive state. This pool may interfere with the ability of any activate Fus3 to transmit a signal to Ste12 (48). These data show that Prr1 and Prr2 (like Fus3) regulate signaling downstream of Ste11, most likely at the level of Ste12.

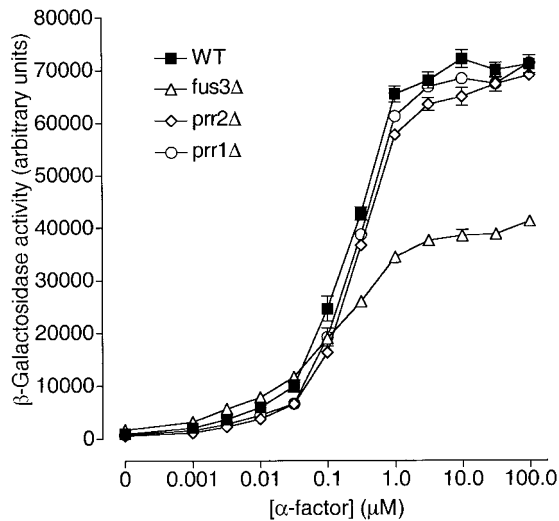To determine whether signal inhibition requires the GST

FIG. 3. **Disruption of *PRR1* or *PRR2* does not alter pheromone-dependent transcriptional induction.** Gene disruption mutants lacking *FUS3* (*fus3Δ*), *PRR1* (*prr1Δ*), or *PRR2* (*prr2Δ*) were transformed with the *FUS1-lacZ* reporter plasmid. Cells were treated with α-factor, and β-galactosidase activity was determined as described above. Data shown are typical of three independent experiments performed in triplicate. *Error bars*, ± S.E.

moiety, each open reading frame was polymerase chain reaction-amplified and cloned into another vector lacking any fusion protein. As shown in Fig. 5, overexpression of Prr2 or Fus3 reduced the maximum response at 30 μM α-factor by 3.1- and 29.7-fold, respectively, compared with the vector control. This level of inhibition is comparable with that observed for the GST fusions (Fig. 2). In contrast, overexpression of Prr1 provided almost no reduction, considerably less than the 1.24-fold reduction observed with Prr1-GST (Fig. 5 *versus* Fig. 2). Because inhibition by Prr1 is largely dependent on the presence of GST, we cannot rule out the possibility that Prr1 is only active as a GST fusion protein. In contrast, the inhibition by Prr2, like that of Fus3, is independent of the GST moiety.

Overexpression of Prr1 or Prr2 could inhibit signaling by at least two different mechanisms. First, overexpression could lead to an enhanced (perhaps unregulated) phosphorylation and inhibition of a pathway component. Second, overexpression of the kinase could lead to reduced phosphorylation of a substrate protein, through competitive binding and inhibition of a needed regulatory subunit or of a substrate protein (see below). To distinguish between these possibilities, we prepared a mutant version of Prr1 and Prr2 that lacks a conserved Lys essential for kinase activity. If the competitive inhibition mechanism applies, wild-type and kinase-dead versions should be equally inhibitory. For example, a kinase-dead form of Fus3 (Fus3^{K42R}) has been reported to inhibit signaling when overexpressed, evidently through its ability to bind and inhibit its substrates in competition with wild-type Fus3 (48–50). Alternatively, if the enhanced phosphorylation mechanism applies, the kinase-dead enzyme should fail to inhibit. As shown in Fig. 6, Fus3^{K42R} inhibits in this assay. However, the kinase-dead version is slightly less potent than the wild-type counterpart. This outcome suggests that inhibition by Fus3 overexpression involves both competitive and enhanced phosphorylation mechanisms. Likewise, the kinase-dead version of Prr2 is a less potent inhibitor than its wild-type counterpart. This suggests that Prr2, like Fus3, acts through competitive binding, as well as enhanced phosphorylation. By contrast, inhibition by Prr1^{K225R} was equal to that of wild-type Prr1. This result supports the view that Prr1 inhibits the pheromone response primarily through a competitive binding mechanism.
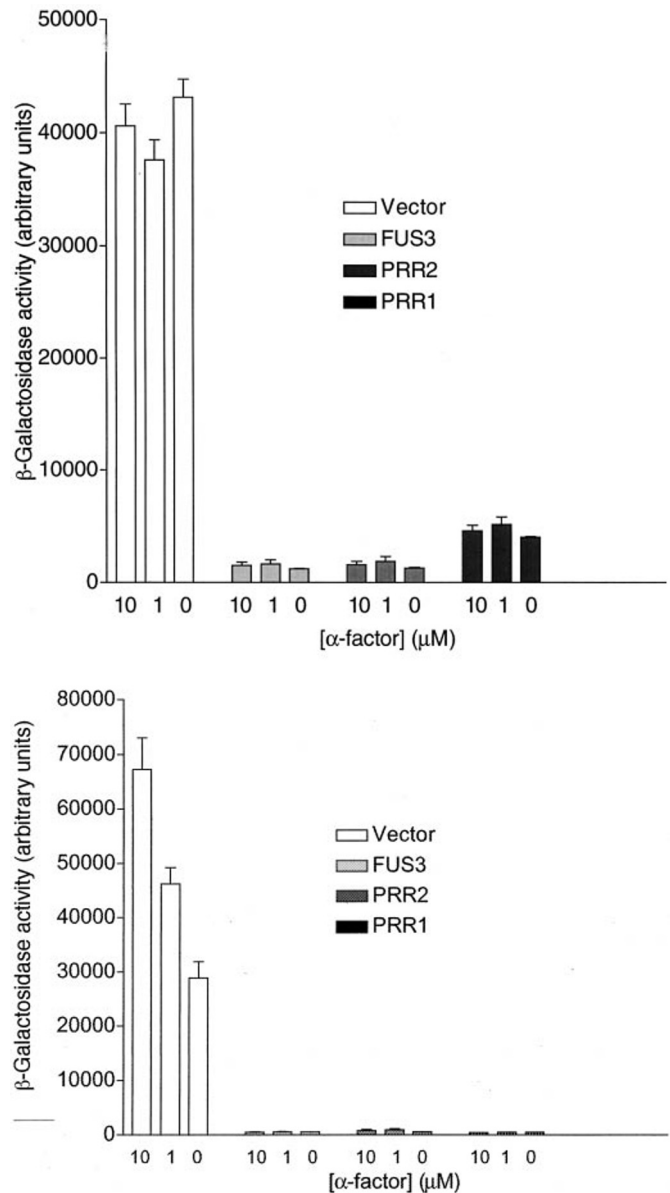

FIG. 4. **Overexpression of *PRR1*-GST or *PRR2*-GST leads to diminished transcriptional induction through activation by *STE11*-4 or *STE12*.** Cells were transformed with a plasmid containing the *FUS1* promoter-*lacZ* reporter, a plasmid that overexpresses either a constitutively active allele *STE11*-4 (*top panel*) or wild-type *STE12* (*bottom panel*), and a plasmid containing *FUS3*-GST, *PRR1*-GST, *PRR2*-GST, or no insert (*Vector*; pYEX 4T-2). Cells were treated with the indicated concentrations of α-factor or water, and β-galactosidase activity was determined as detailed under "Experimental Procedures." Data shown are typical of three independent experiments performed in triplicate. *Error bars*, ± S.E.

The ability to suppress both *STE11*-4 and *GAL1-STE12* indicates that Prr2 operates at the level of the transcription factor Ste12. Ste12 activity depends in part on Fus3 binding and phosphorylation. We therefore examined whether Prr2 inhibits Ste12 indirectly, through inhibition of Fus3. It has been shown previously that pheromone activation results in feedback phosphorylation of Ste7. This reaction provides a particularly convenient indicator of Fus3 activity *in vivo*, because the resulting hyperphosphorylated species of Ste7 exhibits a markedly reduced mobility (~10 kDa) on SDS-polyacrylamide gel electrophoresis (35). Thus we used the Ste7 mobility shift assay to determine whether Prr2 or Prr2^{K390R} can inhibit Fus3 activity. As shown in Fig. 7, pheromone induction caused a typical shift in Ste7 mobility (Fig. 7, *lane 1 versus lane 6*). As
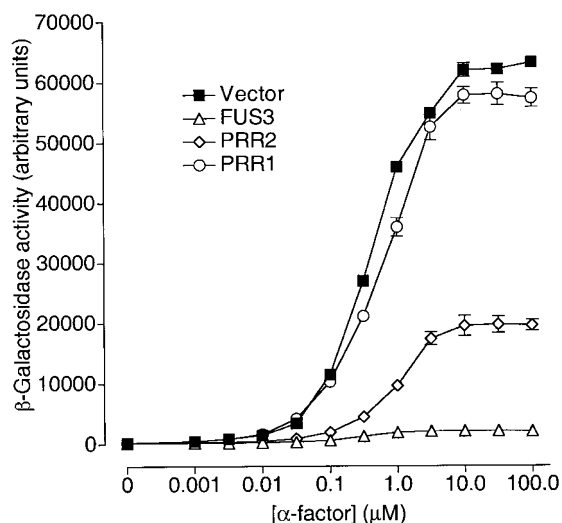
FIG. 5. **PRR1 but not PRR2 requires GST for inhibition of pheromone-dependent transcriptional induction.** Cells were co-transformed with a plasmid containing the FUS1 promoter-*lacZ* reporter and a plasmid containing *FUS3*, *PRR1*, *PRR2*, or no insert (*Vector*; pYES2.1 TOPO). Cells were treated with the indicated concentrations of α-factor, and β-galactosidase activity was determined as described above. Data shown are typical of three independent experiments performed in triplicate. *Error bars*, ± S.E.
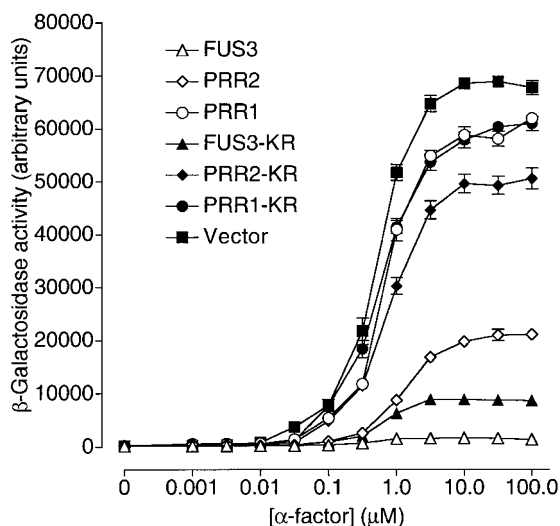

FIG. 6. **PRR2 kinase activity is required for full inhibition of pheromone-dependent transcriptional induction.** Cells were co-transformed with the *FUS1* promoter-*lacZ* reporter and a plasmid containing either wild-type or kinase-dead (*KR*) forms of *FUS3*, *PRR1*, *PRR2*, or no insert (*Vector*; pYES2.1/V5). Cells were treated with α-factor, and β-galactosidase activity was determined as described above. Data shown are typical of three independent experiments performed in triplicate. *Error bars*, ± S.E.

expected, overexpression of either Fus3 or Fus3^{K42R} completely inhibited pheromone-induced Ste7 phosphorylation. In contrast, overexpression of Prr2 or the catalytically inactive derivative Prr2^{K390R} had no effect on Ste7 phosphorylation (Fig. 7, *lanes 9* and *10*). Overexpression of Prr1 or Prr1^{K225R} similarly had no effect on pheromone-induced feedback phosphorylation of Ste7 (data not shown). These results confirm that inhibition of the pheromone-response pathway by Prr2 (and Prr1) occurs downstream of the MAPK.

DISCUSSION

In recent years a number of eukaryotic genomes have been sequenced, including that of humans, *Drosophila melanogaster*, *Caenorhabditis elegans*, and the yeast *S. cerevisiae*. The
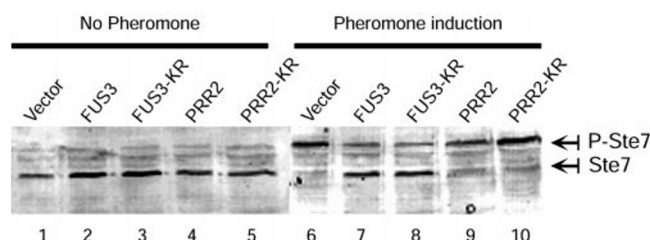

FIG. 7. **PRR2 does not alter Fus3/Kss1 phosphorylation of Ste7.** Immunoblots comparing phosphorylated (*P-Ste7*; *upper arrow*) and non-phosphorylated (*lower arrow*) forms of Ste7 are shown. Strain C699–32 (*bar1*::HisG *ste7*::*ADE2*) expressing Myc-tagged Ste7 (Ste7M) from the *CYC1* promoter (pNC318) and one of the following from the *GAL1,10* promoter is shown: *lane 1*, empty vector (pYES2.1/V5); *lane 2*, *Fus3*; *lane 3*, *Fus3*-KR; *lane 4*, *Prr2*; *lane 5*, *Prr2*-KR; *lane 6*, empty vector (pYES2.1/V5); *lane 7*, FUS3; *lane 8*, FUS3-KR; *lane 9*, PRR2; *lane 10*, PRR2-KR. *KR*, kinase-dead. Extracts were prepared from each culture either before (*right*) or after (*left*) pheromone induction (50 nM α-factor; 90 min). 150 μg of each extract was fractionated by SDS-polyacrylamide gel electrophoresis (10%) and transferred to nitrocellulose filters for immune detection of Ste7M using anti-Myc monoclonal antibody and anti-mouse IgG conjugated to alkaline phosphatase.

wealth of information provided by these efforts will require the development of more systematic approaches to the analysis of gene function. Here we have tested a subset of the *Saccharomyces* genome, specifically the genes encoding likely protein kinases. Our immediate goal was to identify novel kinase regulators of the pheromone-response pathway. A broader goal was to assess the feasibility of such a systematic screening method for functional genomic analysis in yeast and eventually in mammals.

By screening a protein kinase-GST overexpression library, we identified 12 kinase regulators of the pheromone response. Five of the genes represent previously known components and a regulator of the signaling pathway (*STE7*, *STE11*, *FUS3*, *YCK1*, and *KSS1*). The remaining seven have not been shown previously to act in this manner. The two most active regulators, designated *PRR1* and *PRR2*, were characterized further.

Overexpression of either *PRR1* or *PRR2* is able to diminish signaling, as assessed by two standard bioassays of pheromone sensitivity (Figs. 1 and 2). Gene disruption mutants have no effect on signaling (Fig. 3). Both kinases appear to act late in the pathway, as demonstrated by their ability to diminish signaling by activated forms of *STE11* and *STE12* (Fig. 4), without affecting the activity of the MAPK (Fig. 7). Inhibition of signaling by *PRR1*, but not *PRR2*, is largely dependent upon GST fusion (Fig. 5). Inhibition by *PRR2*, but not *PRR1*, is dependent upon its kinase catalytic activity (Fig. 6). Thus our genetic analysis indicates that the pheromone-response regulators identified here act downstream of the MAPK and most likely in conjunction with a terminal-signaling component Ste12.

Future experiments will be aimed at finding the target of the most potent regulator Prr2. One obvious candidate substrate is Ste12. Ste12 is known to be heavily phosphorylated through the action of Fus3 and Kss1 and almost certainly by other kinases (18, 22, 23). Demonstrating that Prr2 phosphorylates Ste12 may be difficult, however. The presence of multiple independent phosphorylation sites has frustrated efforts in the past to identify kinases in addition to the MAPKs that act on Ste12 (23).

Other candidate substrates include a number of DNA-binding proteins that also interact with Ste12, including Mcm1, Matα1, Tec1, Kar4, Dig1, Dig2, and Ste12 itself. Ste12 homodimers are needed for the expression of pheromone-inducible genes in both **a**- and α-haploid cell types (12). Ste12-Tec1 heterodimers are needed for expression at promoters contain-
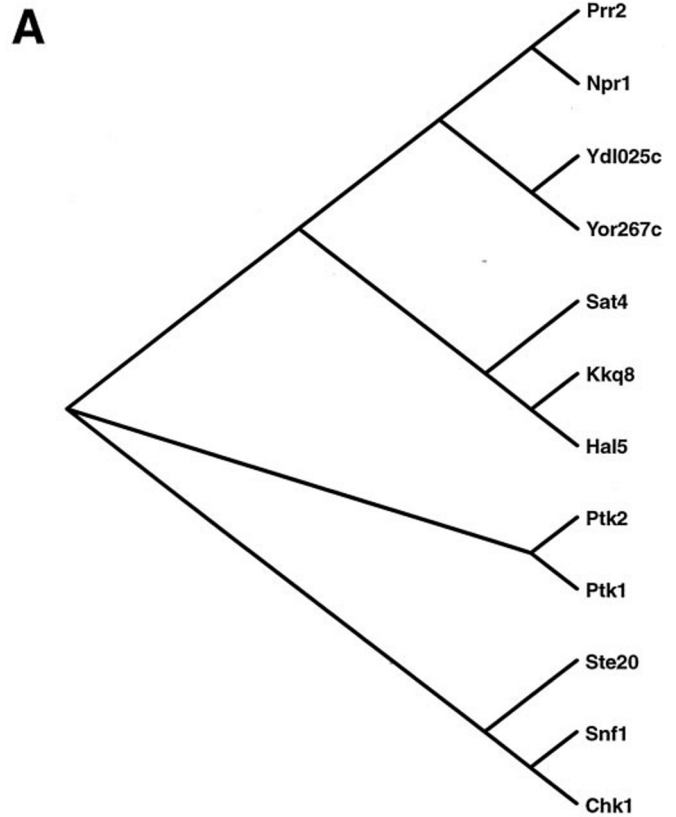
ing a filamentous growth response element (13, 15, 51, 52). Ste12-Mcm1 heterodimers are needed for expression of **a**-cell-specific genes in response to pheromone (13, 14). A ternary complex involving Ste12, Mcm1, and Matα1 is needed for expression of α-cell-specific genes in response to pheromone (*e.g.* *MFα2*). Conversely, a ternary complex involving Ste12, Dig1, or Dig2 and either Fus3 or Kss1 (24, 25) can inhibit transcription at pheromone-response elements (48, 50) or filamentous growth response elements (49, 53), respectively. In principle, Prr1/Prr2 could modulate Ste12 activity directly or indirectly through phosphorylation of a Ste12-binding protein.

Prr1, and to a certain extent Prr2, may regulate signaling through a mechanism that does not require kinase activity. We showed that wild-type and kinase-dead forms of Prr1 were equally active in their ability to inhibit the pheromone response. The kinase-dead form of Prr2 was less active than its wild-type counterpart (Fig. 6). In this regard, Prr2 behaves very much like Fus3; a kinase-dead form of Fus3 inhibits pheromone signaling, though less potently than the wild-type version (Fig. 6). Inhibition of signaling by catalytically inactive Fus3 has been attributed, at least in part, to its direct binding and inhibition of Ste12 (48–50). In the absence of a sustained signal, a dephosphorylated form of Fus3 accumulates, blocks Ste12-mediated gene transcription, and permits cell cycle progression. Under pheromone-inducing conditions Ste7 phosphorylates and activates Fus3, and this leads to both removal of inhibition (through dissociation) and activation (through phosphorylation) of Ste12. Thus, Fus3 can act as both an inhibitor and an activator of Ste12, depending on its state of phosphorylation. A similar phenomenon has also been observed for Kss1 (49, 50, 54). Taken together, these results suggest that Prr2 behaves similarly to Fus3, able to function as both an inhibitor and activator of Ste12.

Once the target of Prr2 kinase activity is found, we will attempt to identify upstream activators of Prr2. One likely candidate is the MAPK Fus3. Although the most intensively studied substrates of MAPKs are transcription factors (55), some substrates are themselves protein kinases (16, 66). Although some MAPK kinase substrates act upstream in the same pathway (Ste7, Ste11), it is unlikely that Prr2 acts upstream of Fus3, because Fus3 activity is not altered by *PRR2* overexpression (Fig. 7).

There are other reasons to believe that Prr2 acts within the nucleus. We identified three NLS motifs within Prr2 (Fig. 8*B*). NLS are utilized for the import of proteins into the nucleus from the cytoplasm through the nuclear pore complex (56). Translocation of Prr2 to the nucleus from the cytosol and/or activation of Prr2 within the nucleus may be involved in mediating the inhibition of pheromone signaling. Exploring these areas may lead to new insights into the molecular mechanism of the regulation of nuclear signal transduction.

Another issue is whether Prr1 or Prr2 exists as a dimer, either as a homodimer or as a heterodimer with another regulatory protein. We have shown that the pheromone-response regulation by Prr1 requires the GST moiety. Because the GST moiety exists as a dimer (57), GST could activate Prr1 by imposing homodimerization. Alternatively, GST might sterically exclude binding by another subunit or a regulatory protein. Given the position at which Prr1 and Prr2 act in the pathway (downstream of the MAPK) it seems likely that these proteins do form some sort of dimer. Many MAPK substrates exist as dimers, most notably transcription factors (55). Moreover, many MAPKs appear to undergo dimerization upon activation, which likely facilitates the phosphorylation of dimeric substrates (58). Finally, biochemical (9, 59) and genetic (11, 60) methods indicate that the MAPK scaffold protein Ste5 also

FIG. 8. **Prr2 and its homologues in *S. cerevisiae*. A**, Cladogram depicting the Npr1/Hal5 family of protein kinases in *S. cerevisiae*. A tree depicting the entire kinase family was described previously (65). The closest Prr2 homologues are Npr1, YDL025c, and YOR267c. Ste20, Snf1, and Chk1 are not members of the Npr1/Hal5 family but are closely related. *B*, Prr2 protein sequence. Prr2 contains all three of the classical nuclear localization signals (*black highlight*) found in animal proteins, and they are as follows: a four-residue pattern within the protein kinase domain (KKKR), a seven-residue pattern within the N terminus (PKGRLRK), and a bipartite element also located within the N terminus (RKHSYTTSLSSIKRLFK). Also indicated is the critical lysine mutated to arginine (K390R) creating the dead kinase version of Prr2 (*clear box*). The protein kinase domain is also indicated (*gray highlight*).

self-associates and exists as an oligomer in cell extracts. Ste5 mutants that cannot oligomerize (11) are unable to transmit the pheromone signal (9, 11).

It is not certain why the *prr1Δ* and *prr2Δ* mutants lack any signaling phenotype. The absence of an observable phenotype for the *prr1Δ* mutant might be expected, because only the GST

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fusion protein inhibits the response. However, this explanation does not apply to the *prr2Δ* mutant. One possibility to consider in this case is that a closely related gene may compensate for the loss of Prr2. For example, loss of *STE20* is compensated in part by the closely related gene, *CLA4* (14, 61–63). In fact, at least three other kinases appear closely related to *PRR2*, including *NPR1*, YDL025c, and YOR267c (Fig. 8*A*). Analysis of double, triple, and quadruple gene disruption mutants are in progress and may reveal some defect in signaling (if indeed these mutants are viable). Alternatively Prr2 activity might regulate both positive and negative components of the pathway. If this were the case, the deletion mutation could be without a phenotype. For example, it has been established that the MAPK Kss1 is a component of the pseudohyphal response pathway, yet the *kss1Δ* deletion mutant has no phenotype, because Kss1 has both positive and negative regulatory roles in the pathway (49, 50).

In conclusion, Prr2 is one of a growing list of proteins that modulate pheromone signaling and which act downstream of the MAPK. Clearly, much remains to be learned about its mechanism of action, where it is located, and how it is regulated. The availability of the kinase-GST fusions will facilitate the identification of proteins that regulate, or are regulated by, this novel kinase. Recent advances in the use of mass spectrometry for proteomics should make it feasible to perform a global analysis on *S. cerevisiae* cells to determine all proteins that are phosphorylated in a Prr2- or Prr1-specific manner (64). More generally speaking, the approach used here serves as a model for identification and characterization of unknown gene products, as they are identified through genome sequencing programs.

REFERENCES

1. Gustin, M. C., Albertyn, J., Alexander, M., and Davenport, K. (1998) *Microbiol. Mol. Biol. Rev.* **62,** 1264–1300
2. Elion, E. A. (2000) *Curr. Opin. Microbiol.* **3,** 573–581
3. Dohlman, H. G., Song, J., Apanovitch, D. M., DiBello, P. R., and Gillen, K. M. (1998) *Semin. Cell Dev. Biol.* **9,** 135–141
4. Hicke, L., Zanolari, B., and Riezman, H. (1998) *J. Cell Biol.* **141,** 349–358
5. Feng, Y., and Davis, N. G. (2000) *Mol. Cell. Biol.* **20,** 5350–5359
6. Hicke, L. (1999) *Trends Cell Biol.* **9,** 107–112
7. Yesilaltay, A., and Jenness, D. D. (2000) *Mol. Biol. Cell* **11,** 2873–2884
8. Overton, M. C., and Blumer, K. J. (2000) *Curr. Biol.* **10,** 341–344
9. Feng, Y., Song, L. Y., Kincaid, E., Mahanty, S. K., and Elion, E. A. (1998) *Curr. Biol.* **8,** 267–278
10. Sette, C., Inouye, C. J., Stroschein, S. L., Iaquinta, P. J., and Thorner, J. (2000) *Mol. Biol. Cell* **11,** 4033–4049
11. Inouye, C., Dhillon, N., and Thorner, J. (1997) *Science* **278,** 103–106
12. Dolan, J. W., and Fields, S. (1991) *Biochim. Biophys. Acta* **1088,** 155–169
13. Sengupta, P., and Cochran, B. H. (1990) *Mol. Cell. Biol.* **10,** 6809–6812
14. Mueller, C. G., and Nordheim, A. (1991) *EMBO J.* **10,** 4219–4229
15. Yuan, Y. O., Stroke, I. L., and Fields, S. (1993) *Genes Dev.* **7,** 1584–1597
16. Chang, L., and Karin, M. (2001) *Nature* **410,** 37–40
17. Kranz, J. E., Satterberg, B., and Elion, E. A. (1994) *Genes Dev.* **8,** 313–327
18. Elion, E. A., Satterberg, B., and Kranz, J. E. (1993) *Mol. Biol. Cell* **4,** 495–510
19. Peter, M., Gartner, A., Horecka, J., Ammerer, G., and Herskowitz, I. (1993) *Cell* **73,** 747–760
20. Tyers, M., and Futcher, B. (1993) *Mol. Cell. Biol.* **13,** 5659–5669
21. Gartner, A., Jovanovic, A., Jeoung, D. I., Bourlat, S., Cross, F. R., and Ammerer, G. (1998) *Mol. Cell. Biol.* **18,** 3681–3691
22. Song, D., Dolan, J. W., Yuan, Y. L., and Fields, S. (1991) *Genes Dev.* **5,** 741–750
23. Hung, W., Olson, K. A., Breitkreutz, A., and Sadowski, I. (1997) *Eur. J. Biochem.* **245,** 241–251
24. Cook, J. G., Bardwell, L., Kron, S. J., and Thorner, J. (1996) *Genes Dev.* **10,** 2831–2848
25. Tedford, K., Kim, S., Sa, D., Stevens, K., and Tyers, M. (1997) *Curr. Biol.* **7,** 228–238
26. Olson, K. A., Nelson, C., Tai, G., Hung, W., Yong, C., Astell, C., and Sadowski, I. (2000) *Mol. Cell. Biol.* **20,** 4199–4209
27. Garrison, T. R., Zhang, Y., Pausch, M., Apanovitch, D., Aebersold, R., and Dohlman, H. G. (1999) *J. Biol. Chem.* **274,** 36387–36391
28. Feng, Y., and Davis, N. G. (2000) *Mol. Cell. Biol.* **20,** 563–574
29. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds) (1987) *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc., New York
30. Martzen, M. R., McCraith, S. M., Spinelli, S. L., Torres, F. M., Fields, S., Grayhack, E. J., and Phizicky, E. M. (1999) *Science* **286,** 1153–1155
31. Boeke, J. D., Trueheart, J., Natsoulis, G., and Fink, G. R. (1987) *Methods Enzymol.* **154,** 164–175
32. Alani, E., Cao, L., and Kleckner, N. (1987) *Genetics* **116,** 541–545
33. Printen, J. A., and Sprague, G. F., Jr. (1994) *Genetics* **138,** 609–619
34. Hudson, A. Jr., Dawson, E. P., Rushing, K. L., Jackson, C. H., Lockshon, D., Conover, D., Lanciault, C., Harris, J. R., Simmons, S. J., Rothstein, R., and Fields, S. (1997) *Genome Res* **7,** 1169–1173
35. Zhou, Z., Gartner, A., Cade, R., Ammerer, G., and Errede, B. (1993) *Mol. Cell. Biol.* **13,** 2069–2080
36. Trueheart, J., Boeke, J. D., and Fink, G. R. (1987) *Mol. Cell. Biol.* **7,** 2316–2328
37. Pfeifer, K., Prezant, T., and Guarente, L. (1987) *Cell* **49,** 19–27
38. Pfeifer, K., Arcangioli, B., and Guarente, L. (1987) *Cell* **49,** 9–18
39. Evan, G. I., Lewis, G. K., Ramsay, G., and Bishop, J. M. (1985) *Mol. Cell. Biol.* **5,** 3610–3616
40. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *J. Mol. Biol.* **215,** 403–410
41. Jeanmougin, F., Thompson, J. D., Gouy, M., Higgins, D. G., and Gibson, T. J. (1998) *Trends Biochem. Sci* **23,** 403–405
42. Page, R. D. (1996) *Comput. Appl. Biosci.* **12,** 357–358
43. Nakai, K., and Kanehisa, M. (1992) *Genomics* **14,** 897–911
44. Bardwell, L., Cook, J. G., Inouye, C. J., and Thorner, J. (1994) *Dev. Biol.* **166,** 363–379
45. Winzeler, E. A., Shoemaker, D. D., Astromoff, A., Liang, H., Anderson, K., Andre, B., Bangham, R., Benito, R., Boeke, J. D., Bussey, H., Chu, A. M., Connelly, C., Davis, K., Dietrich, F., Dow, S. W., El Bakkoury, M., Foury, F., Friend, S. H., Gentalen, E., Giaever, G., Hegemann, J. H., Jones, T., Laub, M., Liao, H., Davis, R. W., *et al.* (1999) *Science* **285,** 901–906
46. Stevenson, B. J., Rhodes, N., Errede, B., and Sprague, G. F., Jr. (1992) *Genes Dev.* **6,** 1293–1304
47. Dolan, J. W., and Fields, S. (1990) *Genes Dev.* **4,** 492–502
48. Farley, F. W., Satterberg, B., Goldsmith, E. J., and Elion, E. A. (1999) *Genetics* **151,** 1425–1444
49. Bardwell, L., Cook, J. G., Voora, D., Baggott, D. M., Martinez, A. R., and Thorner, J. (1998) *Genes Dev.* **12,** 2887–2898
50. Madhani, H. D., Styles, C. A., and Fink, G. R. (1997) *Cell* **91,** 673–684
51. Madhani, H. D., and Fink, G. R. (1997) *Science* **275,** 1314–1317
52. Baur, M., Esch, R. K., and Errede, B. (1997) *Mol. Cell. Biol.* **17,** 4330–4337
53. Bardwell, L., Cook, J. G., Zhu-Shimoni, J. X., Voora, D., and Thorner, J. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 15400–15405
54. Cook, J. G., Bardwell, L., and Thorner, J. (1997) *Nature* **390,** 85–88
55. Treisman, R. (1996) *Curr. Opin. Cell Biol.* **8,** 205–215
56. Hicks, G. R., and Raikhel, N. V. (1995) *Annu. Rev. Cell Dev. Biol.* **11,** 155–188
57. Parker, W., Lo Bello, M., and Federici, G. (1990) *J. Mol. Biol.* **213,** 221–222
58. English, J., Pearson, G., Wilsbacher, J., Swantek, J., Karandikar, M., Xu, S., and Cobb, M. H. (1999) *Exp. Cell Res.* **253,** 255–270
59. Yablonski, D., Marbach, I., and Levitzki, A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 13864–13869
60. Inouye, C., Dhillon, N., Durfee, T., Zambryski, P. C., and Thorner, J. (1997) *Genetics* **147,** 479–492
61. Mitchell, D. A., and Sprague, G. F., Jr. (2001) *Mol. Cell. Biol.* **21,** 488–500
62. Cvrckova, F., De Virgilio, C., Manser, E., Pringle, J. R., and Nasmyth, K. (1995) *Genes Dev.* **9,** 1817–1830
63. Leberer, E., Dignard, D., Harcus, D., Thomas, D. Y., and Whiteway, M. (1992) *EMBO J.* **11,** 4815–4824
64. Haynes, P. A., and Yates, J. R., 3rd. (2000) *Yeast* **17,** 81–87
65. Hunter, T., and Plowman, G. D. (1997) *Trends Biochem. Sci.* **22,** 18–22
66. Frodin, M., and Gammeltoft, S. (1999) *Mol. Cell. Endocrinol.* **151,** 65–77