Pheromone-dependent phosphorylation of the yeast STE12 protein correlates with transcriptional activation

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Haploid a and α cells of yeast respond to the pheromones α- and α-factor, respectively, by increasing the transcription of many genes whose products are essential for mating. The STE12 protein acts in this process by binding to the DNA sequence that mediates the increased transcription of pheromone-responsive genes. We show here that a hybrid protein containing STE12 fused to the DNA-binding domain of GAL4 can activate transcription of a reporter gene containing GAL4-binding sites but only after treatment of cells with pheromone. Thus, STE12 alone, when bound to DNA, is sufficient to mediate pheromone-induced transcription. By constructing hybrids of different STE12 regions with the GAL4 domain, we map the domain of STE12 necessary for this activation to the central third of the protein. Upon α-factor treatment, the hybrid of GAL4 with the complete STE12 sequence is rapidly phosphorylated, with kinetics consistent with the observed transcriptional induction of pheromone-responsive genes. The domain of STE12 necessary for this phosphorylation correlates with that involved in transcriptional activation. We propose that induction of pheromone-responsive genes is mediated by phosphorylation of STE12 to alter its activation function but not its DNA-binding ability.

[Key Words: Yeast; STE12 protein; pheromone response; transcription; DNA-binding protein; phosphorylation]

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omone. We find that STE12 is rapidly phosphorylated in response to the binding of pheromone and that this phosphorylation correlates with the enhanced ability of STE12 to act in transcription. These data are consistent with a model that pheromone treatment leads to transcriptional induction by modifying the STE12 protein, making it a more potent transcriptional activator.

Results

Pheromone-dependent transcriptional activation by GAL4–STE12 fusion proteins

In regulating cell-type-specific transcription, the STE12 protein acts cooperatively with several proteins that bind to other DNA sequences; in addition, STE12 is not the only protein capable of binding to the PRE [F. Gimble and J. Thorner, pers. comm.]. We wished to analyze the transcriptional role of STE12 in the absence of these other activities and, therefore, made use of a strategy employing hybrid proteins. In this approach, a heterologous DNA-binding domain that is unable to activate transcription is fused to a test protein. The hybrid thus generated is then assayed for its ability to activate transcription of a reporter gene containing a binding site for the heterologous DNA-binding domain. For our purposes, we used the amino-terminal 147 amino acids of the GAL4 protein, which bind to the upstream activation sequence (UASG) present in the regulatory region of genes for galactose metabolism [Keegan et al. 1986]. This domain alone produces no transcription of a GAL1–lacZ gene activated by UAS~ (Keegan et al. 1986).

A hybrid gene was generated that encodes the entire STE12 sequence (688 amino acids) fused to the GAL4 DNA-binding domain and whose expression is under the control of the strong ADH1 promoter. This hybrid gene, carried on a multicopy plasmid, was introduced into a yeast a strain deleted for GAL4 and containing a GAL1–lacZ gene. The presence of this hybrid protein resulted in approximately the same very low level of GAL1–lacZ transcription as did the GAL4 DNA-binding domain itself [Fig. 1, lines a and b]. However, addition of α-factor to cells carrying this hybrid led to a sixfold increase in β-galactosidase activity [Fig. 1, line b]. This result indicates, first, that the full-length STE12 protein can function as a transcriptional activator, but only after pheromone treatment. Thus, we have effectively converted the UASc into a PRE, demonstrating that STE12 is sufficient for pheromone-responsive transcription, independent of other DNA-binding proteins. Second, this result indicates that even after pheromone treatment, the full-length GAL4 protein produces >200-fold more GAL1–lacZ transcription [Fig. 1, line h].

To define the region of the STE12 protein responsible for this transcriptional induction in response to pheromone, we constructed two carboxy-terminal and two amino-terminal deletions of the STE12-coding sequence fused to the GAL4 DNA-binding domain [Fig. 1]. GAL4–STE121–473 [line c] and GAL4–STE12214–688 [line e] both led to pheromone-dependent transcriptional activity. However, GAL4–STE121–215 [line d] and GAL4–STE12472–688 [line f] failed to activate: β-Galactosidase activity of cells carrying these fusion proteins was low and not stimulated by α-factor treatment. These results demonstrate that neither the amino-terminal 213 nor the carboxy-terminal 215 amino acids of STE12 are necessary for pheromone-dependent transcriptional activity. To test whether the sequence between these two deletion endpoints is sufficient for this activity, we constructed GAL4–STE12214–473: This fusion protein caused a high level of transcription in untreated cells, and this level was only minimally pheromone responsive [line g]. One explanation for this result is that the central region of STE12 contains a transcriptional activating sequence that lies exposed to the transcriptional machinery in this fusion but not in the larger fusions. The presence of pheromone would then be required for

Figure 1. Transcriptional activation by GAL4–STE12 hybrid proteins. The GAL4 domain is indicated by the shaded region, and the STE12 domain by the open region. The hybrids were transformed into strain YM709::171, and β-galactosidase activity was assayed either without treatment or after 2 hr in α-factor. Activity is expressed in Miller units [Miller 1972].
the exposure of this activating domain in the larger fusions.

Another factor that may contribute to the low levels of GAL1–lacZ transcription produced by the fusions carrying large regions of STE12 is that these proteins have additional functions. The amino-terminal 215 amino acids of STE12 are sufficient for specific DNA binding [Y.O. Yuan and S. Fields, unpubl.], and reduced transcriptional activation by fusion proteins containing more than one DNA-binding domain has been observed previously (Weston and Bishop 1989). Thus, any fusion proteins carrying the first 215 amino acids of STE12 may not only bind to UASG by virtue of the GAL4 DNA-binding domain but also to PRE sites by virtue of the STE12 domain. As a test of this possibility, we assayed whether the fusion proteins could complement a ste12 mutation (Fig. 2). Both GAL4–STE12\(^{1-688}\) and GAL4–STE12\(^{1-473}\) restored mating ability, indicating that these two fusion proteins could also act in cell-type-specific transcription. Comparison of the activity of GAL4–STE12\(^{214-688}\) and GAL4–STE12\(^{214-473}\) shows that the carboxy-terminal region of STE12 also has a negative effect on GAL1–lacZ transcription. The region from amino acids 470 to 688 appears to interact with the MCM1 protein (Errede and Ammerer 1989), and this interaction may affect the ability of the GAL4–STE12\(^{214-688}\) hybrid to act at UASG. Alternatively, the amino- or carboxy-terminal region may somehow mask an internal transcriptional activating region.

Apart from the pheromones and receptors, the pheromone-responsive transduction pathway appears to use the same components in both \(a\) and \(\alpha\) cells (Bender and Sprague 1986; Nakayama et al. 1987). \(a\)-Factor treatment of \(a\) cells carrying the GAL4–STE12 fusion proteins led to the same pattern of transcriptional induction as observed with \(\alpha\)-factor treatment of \(a\) cells [data not shown].

The STE12 protein is phosphorylated in response to pheromone

The rapid pheromone induction of cell-type-specific transcription occurs independently of new protein synthesis (Hagen and Sprague 1984). This result, combined with the demonstration that STE12 binds to the PRE (Dolan et al. 1989; Errede and Ammerer 1989) and is a pheromone-dependent transcriptional activator, suggests that the STE12 protein itself may be a target for a pheromone-induced post-translational modification. To test this idea, we generated polyclonal antiserum to the amino-terminal 215 amino acids of STE12 and used it to immunoprecipitate STE12 from \(^{35}\)S-labeled yeast extracts (Fig. 3). The anti-STE12 serum did not detect any specific proteins in an extract from cells containing the control vector encoding the GAL4 DNA-binding domain [lane 1], indicating that the level of chromosomally encoded wild-type STE12 protein is too low to be detected with this assay. However, the antibody detected a protein with an apparent molecular mass of 133 kD in an extract of cells containing GAL4–STE12\(^{1-688}\) (lane 3), indicating that the level of chromosomally encoded STE12 protein is too low to be detected with this assay. However, the antibody detected a protein with an apparent molecular mass of 133 kD in an extract of cells containing GAL4–STE12\(^{1-688}\) (lane 3). Chromosomally encoded STE12 can be detected by immunoprecipitation of an extract from vector-containing cells treated with \(\alpha\)-factor (lane 2). This protein migrates at the approximate position of in vitro-translated STE12 [not shown]. STE12 transcription is induced severalfold by pheromone (J.W. Dolan and S. Fields, unpubl.), and this induction may at least partially account for the ability to detect the endogenous protein from pheromone-treated cells. Extracts of cells carrying GAL4–STE12\(^{1-688}\) treated with \(\alpha\)-factor also showed this level of endogenous STE12 protein (lane 4). The GAL4–STE12\(^{1-688}\) fusion protein from \(\alpha\)-factor-treated cells migrated slightly more slowly and more diffusely than the fusion protein from untreated cells (lane 4).

To determine whether the altered mobility of the fusion protein from \(\alpha\)-factor-treated cells is due to phosphorylation, we treated \(^{35}\)S-labeled immunoprecipitates with potato acid phosphatase. As shown in Figure 4, pheromone treatment led to a characteristic shift in migration of the fusion protein [cf. lanes 1 and 2]; but after incubation with phosphatase [lane 3], the protein migrated with a mobility characteristic of that from cells not treated with \(\alpha\)-factor. Incubation with phosphatase in the presence of phosphatase inhibitors (Verjee 1969) did not affect the rate of migration, and the fusion protein continued to exhibit the slower electrophoretic mo-

Figure 2. Mating assay of strains producing GAL4–STE12 hybrid proteins. Patches of an \(a\) ste12 strain [430] containing different plasmids were replica-plated onto an SD–minimal plate spread with an \(a\) strain, such that ability to mate is indicated by the growth of a prototrophic diploid patch. Numbers in parentheses represent the STE12 residues present in the GAL4–STE12 hybrids. The vector is the plasmid carrying the GAL4 DNA-binding domain alone, and the STE\(^{+}\) strain is isogenic to strain 430 but with the wild-type STE12 allele.
Figure 3. The immunoprecipitation of STE12 and GAL4-STE12(l_688) from cells grown in the absence or presence of α-factor. The cells contained either the vector [GAL4 DNA-binding domain alone] or the GAL4-STE12 fusion. Equivalent amounts of [35S]-labeled protein were immunoprecipitated with an antibody to STE12, and the precipitates were fractionated by SDS-polyacrylamide gel. Molecular masses of standards are indicated in kilodaltons. The upper arrow indicates GAL4-STE12; the lower arrow indicates the native STE12.

Figure 4. α-Factor leads to phosphorylation of GAL4-STE12. 3SS-Labeled cells carrying GAL4-STE12(l_688) were untreated or treated with α-factor as indicated. Cell extracts were immunoprecipitated as in Fig. 3, and the precipitates were treated with potato acid phosphatase or phosphatase plus inhibitors [PO4 and MoO4] as indicated. (Lanes 1–4) The upper arrow indicates GAL4-STE12; the lower arrow indicates the native STE12. (Lanes 5 and 6) The arrow indicates GAL4-STE12.
Phosphorylation of the yeast STE12 protein

following pheromone treatment (Fig. 1). To examine whether the induced transcriptional activity correlated with phosphorylation following α-factor treatment, we immunoprecipitated extracts from cells containing certain GAL4–STE12 fusion proteins [Fig. 7]. Like GAL4–STE121/1–688 [lanes 1 and 2], GAL4–STE121/1–473 also showed pheromone-dependent phosphorylation [lanes 3 and 4]. However, GAL4–STE121/1–215 [lanes 7 and 8] and the GAL4 DNA-binding domain alone [lanes 9 and 10] migrated the same after pheromone treatment as without treatment. There is thus a good correlation between the ability of these fusion proteins to activate transcription following pheromone treatment and their ability to be phosphorylated by the response pathway. The GAL4–STE121/214–473 fusion, after treatment of cells with pheromone, showed a small increase in the amount of protein present as a slightly slower migrating species [lanes 5 and 6]. The relatively minor biochemical change in this fusion protein in response to pheromone correlates with the ability of this protein to activate transcription only 1.6-fold better after pheromone treatment (Fig. 1). The lack of a pronounced pheromone-dependent shift observed with GAL4–STE121/214–473 may be due to its high level of phosphorylation in the absence of pheromone, as incubation with phosphatase indicated that all of the GAL4–STE121/214–473 protein present in cells not treated with pheromone is phosphorylated [data not shown]. Alternatively, there may be phosphorylation that requires, but occurs outside of, the region between amino acids 214 and 473.

Pheromone treatment does not significantly alter the DNA-binding activity of STE12

The previous experiments examined the properties of fusion proteins containing the DNA-binding domain of a heterologous protein that is not responsive to pheromone treatment. We also tested directly whether pheromone treatment affects the DNA-binding ability of the native STE12 protein [Fig. 8]. Cells overproducing STE12 were treated with α-factor, followed by preparation of extracts for gel-mobility shift assays. Treatment of cells with α-factor [lane 3] failed to increase significantly [or decrease] the ability of STE12 to bind to DNA in vitro. This result is therefore consistent with pheromone treatment affecting some aspect of STE12 function other than DNA binding. We cannot rule out the possibility, however, that in vivo, the DNA-binding ability is altered, for example, by a pheromone-dependent alteration in a DNA-binding factor with which STE12 must interact. Such a pheromone-dependent effect might be detectable only with DNA probes containing certain STE12-binding sites.

Discussion

The STE12 protein is required for both constitutive and pheromone-induced levels of cell-type-specific transcription and binds to the DNA sequence that mediates transcriptional induction. We show here that a fusion pro-

Correlation between transcriptional activation and phosphorylation

In addition to GAL4–STE121/1–688, other fusion proteins were also capable of enhanced GAL1–lacZ transcription following pheromone treatment [Fig. 1]. To examine whether the induced transcriptional activity correlated with phosphorylation following α-factor treatment, we immunoprecipitated extracts from cells containing certain GAL4–STE12 fusion proteins [Fig. 7]. Like GAL4–STE121/1–688 [lanes 1 and 2], GAL4–STE121/1–473 also showed pheromone-dependent phosphorylation [lanes 3 and 4]. However, GAL4–STE121/1–215 [lanes 7 and 8] and the GAL4 DNA-binding domain alone [lanes 9 and 10] migrated the same after pheromone treatment as without treatment. There is thus a good correlation between the ability of these fusion proteins to activate transcription following pheromone treatment and their ability to be phosphorylated by the response pathway. The GAL4–STE121/214–473 fusion, after treatment of cells with pheromone, showed a small increase in the amount of protein present as a slightly slower migrating species [lanes 5 and 6]. The relatively minor biochemical change in this fusion protein in response to pheromone correlates with the ability of this protein to activate transcription only 1.6-fold better after pheromone treatment (Fig. 1). The lack of a pronounced pheromone-dependent shift observed with GAL4–STE121/214–473 may be due to its high level of phosphorylation in the absence of pheromone, as incubation with phosphatase indicated that all of the GAL4–STE121/214–473 protein present in cells not treated with pheromone is phosphorylated [data not shown]. Alternatively, there may be phosphorylation that requires, but occurs outside of, the region between amino acids 214 and 473.

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tein of the GAL4 DNA-binding domain and STE12 is capable of activating transcription of a gene that carries GAL4-binding sites but only after the cells carrying the fusion have been treated with pheromone. This result establishes that STE12 alone, when bound to DNA, is sufficient to mediate pheromone-induced transcription. STE12 itself could activate this induced transcription or it may bring other transcription factors to the DNA via protein–protein contacts. The GAL4–STE12 fusion protein also becomes phosphorylated after pheromone treatment. The pheromone-induced phosphorylation of STE12 is rapid and independent of protein synthesis, with kinetics that are similar to the transcriptional induction of cell-type-specific genes by pheromone. Fusion proteins capable of pheromone-induced transcription also show pheromone-induced phosphorylation. Our results are therefore consistent with a model that pheromone treatment leads to transcriptional induction by increasing the phosphorylation and consequent activity of STE12. In addition, pheromone treatment leads to increased transcription of the STE12 gene itself, and this autoregulation may amplify the pheromone response.

STE12 resembles other transcriptional activators that respond to an environmental signal by phosphorylation. For example, the GAL4 protein has highly phosphorylated forms correlating with growth in media that induce

Figure 6. Kinetics of GAL4–STE12 phosphorylation and evidence that protein synthesis is not required for the phosphorylation. (A) Cells carrying GAL4–STE12(1–688) were labeled with 35S-labeled amino acids and treated with α-factor. Aliquots were removed at the times indicated and immunoprecipitated with antibody to STE12. (B) Cells carrying GAL4–STE12(1–688) were labeled with 35S-labeled amino acids for 5 min and, where indicated, treated with cycloheximide for 5 min. α-Factor was then added [lanes 2 and 4] for an additional 5 min, after which all cultures were lysed and immunoprecipitated with antibody to STE12.

Figure 7. Pheromone-dependent phosphorylation of hybrids carrying different regions of STE12. Numbers in parentheses indicate the region of STE12 fused to the GAL4 DNA-binding domain; vector is the GAL4 domain only. Cultures were 35S-labeled, and half of each culture was treated with α-factor as indicated. Extracts were treated with antibody to STE12 [lanes 1–4, 7, and 8] or to GAL4 [lanes 5, 6, 9, and 10]. Immunoprecipitates were electrophoresed on 6% [lanes 1–4] or 8% [lanes 5–10] SDS–polyacrylamide gels. Molecular weight markers for lanes 1–4 are to the left of lane 1, and for lanes 7–10 to the left of lane 7. Arrows indicate the positions of the hybrid proteins.
transcription of the GAL genes [Mylin et al. 1989, 1990]. Several different mechanisms may operate to alter the activity of transcription factors following phosphorylation. [1] The transcriptional activation domains of certain factors, for example, the yeast GAL4 and GCN4 proteins [Hope and Struhl 1986; Ma and Ptashne 1987], are acidic, and mutations that increase the acidity of the domain frequently enhance the ability of the factor to activate transcription [Gill and Ptashne 1987]. Thus, phosphorylation of the activation domain may represent a controllable means of increasing the acidity of an activation domain in response to changes in conditions. Such a mechanism has been proposed for the yeast heat-shock factor [HSF], which is more active in transcription at higher temperatures and shows heat-induced phosphorylation [Sorger et al. 1987; Sorger and Pelham 1988]. [2] In some cases, the DNA-binding ability of a protein is altered by phosphorylation. For example, the DNA-binding activity of serum response factor [SRF] is dramatically increased by casein kinase II phosphorylation [Manak et al. 1990]. The affinity of SV40 large T antigen for the viral origin of replication is increased by phosphorylation of a single threonine residue [McVey et al. 1989], although this modification has been shown to affect replication and not transcription. [3] Phosphorylation may cause conformational changes in the structure of the activator. For the cAMP response element-binding protein [CREB], phosphorylation by protein kinase A appears to result in an allosteric change that allows a distal site to interact with the transcription apparatus [Yamamoto et al. 1990]. [4] Transcriptional activators may be bound to inhibitory components, and these interactions may be regulated by phosphorylation. Phosphorylation on the inhibitory factor IκB appears to dissociate it from the transcription factor NFκB, which can then translocate to the nucleus and activate transcription [Ghosh and Baltimore 1990]. [5] Phosphorylation of the transcription factor itself may influence its entry into the nucleus. The yeast SWI5 protein enters the nucleus in early G1, and this phenomenon may be mediated by cell-cycle-regulated phosphorylation [Nasmyth et al. 1990].

Our results suggest that the efficacy of the STE12 activation domain is increased following pheromone-induced phosphorylation. Nuclear localization, dimerization, and specific DNA binding of the GAL4–STE12 fusion proteins can all be accomplished by the GAL4 domain, which is highly unlikely to vary in response to pheromone. In this regard, the GAL4 DNA-binding domain does not exhibit altered electrophoretic mobility following pheromone treatment, nor does the full-length GAL4 activate more transcription after pheromone treatment. Our results also indicate that the native STE12 protein present in extracts from both untreated and pheromone-treated cells binds to DNA with similar affinity. Phosphorylation of STE12 may make it a better transcriptional activator because of its increased acidity. Alternatively, phosphorylation may change the conformation of the protein such that an activating region becomes exposed to the transcription machinery. The presence of such a region is suggested by the results with GAL4–STE12(214–473), which is a potent activator even in the absence of pheromone. In the full-length protein this activation domain may be masked by the amino or carboxyl terminus until pheromone-induced phosphorylation occurs. The sequence of STE12 indicates that the region from residue 214 to 473 is highly acidic (net charge of −13) and relatively rich in serine, threonine, and tyrosine residues [20%], as well as proline residues [13%]. Abundance of these four amino acids has been noted in other transcriptional activation domains [Mermod et al. 1989; Theill et al. 1989].

On the basis of our results with the GAL4–STE12 fusions, the STE12 moiety may be either unphosphorylated, phosphorylated in a pheromone-independent manner, or phosphorylated in a pheromone-dependent manner. In the absence of pheromone, the GAL4–STE12(1–68) fusion is almost completely inactive for transcription of GAL1–lacZ. This observation is consistent with the low level of constitutive expression of genes such as FUS1 [McCaffrey et al. 1987; Trueheart et al. 1987], whose regulatory regions contain multiple copies of the PRE and no strong UAS elements. Although approximately half of the population of the STE12 fusion protein is phosphorylated in the absence of pheromone [on the basis of 32P labeling], this modification does not appear to provide significant transcriptional activity. It may be that the forms that are apparently more highly phosphorylated (and in very low abundance), seen only by 32P-labeling, are active in transcription and allow the fusion protein to complement a ste12 deletion. Pheromone treatment appears to lead to at least two modified
forms of STE12, suggesting multiple phosphorylations. Modulation of the extent of pheromone-induced phosphorylation may allow the cell to respond to different levels of pheromone with different increases in transcription. The pheromone response is not an all-or-nothing phenomenon, as different aspects of the response require different concentrations of pheromone (Moore 1983). It is possible that different protein kinases are responsible for the phosphorylations observed in the absence or presence of pheromone, or that the same kinase(s) become more active after pheromone treatment. Some of the phosphorylations occurring in the absence of pheromone may be dependent on a functional pheromone response pathway, such that mutations in this pathway decrease constitutive transcription through their effect on STE12 phosphorylation.

The pheromone-responsive transcriptional activator STE12 is another member of the growing set of transcription factors whose activities are modulated by phosphorylation. Phosphorylation represents a rapid and reversible means of altering the activity of a protein in response to changing conditions, such as an encounter with a cell of the opposite mating type. For STE12 it should be possible to determine which component(s) of the pheromone response pathway catalyze these modifications and, ultimately, to assay the various forms of the protein in an in vitro transcription system. It will also be of interest to characterize the changes STE12 undergoes later in the pheromone response, when transcriptional activity returns to uninduced levels, and as cells adapt for growth in the continuous presence of pheromone.

Materials and methods

Yeast strains and growth media

Yeast strains used were YM709::171 [MATα ura3-52 his3-200 met trp1 can1 gal4a gal80a GAL1-lacZ(URA3)]; Ma and Ptashne 1987], W303-1a [MATα ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1], from R. Rothstein, 430 (isogenic to W303-1a except ste21Δ::URA3), and EG123 [MATα trp1 leu2 ura3 his4 can1], (Siliciano and Tatchell 1984). Media used were YEP, synthetic [S] (Sherman et al. 1986), and modified Wickerham’s (Wickerham 1946), in which the sulfates and phosphates were replaced by chlorides. Carbon sources were 2% glucose (YEPD and SD), 4% raffinose (SRaf), or 2% galactose/2% glycercol/2% ethanol (SGGE). Synthetic media were supplemented with amino acids and nucleosides (Sherman et al. 1986). To maintain plasmids with selectable markers, the media were supplemented with amino acids and nucleosides (Sherman et al. 1986). To construct pGAL4-STE121_688, an EcoRI-BamHII subfragment of the EcoRI cassette of pY08 was cloned into pUC18 and then transferred into pMA424. pGAL4-STE12Δ1_215 was constructed by first digesting pY06 with XbaI and religating, removing a 0.6-kb XbaI fragment and inserting a XbaI-EcoRI linker into the XbaI site, generating pYY3. The resultant EcoRI fragment was then cloned into pMA424. pGAL4-STE12Δ2_473 was constructed by cloning an EcoRI-BamHII subfragment of pYY3 into pMA424. To construct pGAL4-STE12Δ1_215, the EcoRI-HindIII fragment from pYY08 was cloned into pUC19 digested with EcoRI and HindIII and a HindIII-BglII linker was inserted to yield pYR1. The BamHI-BgllII fragment of pYR1 was cloned into the BamHII site of pUC9, and a linker (5’-GATCGGATCC-3’) was inserted at the BamHII site to adjust the reading frame of the final construction. Finally, a BamHI-SalI fragment was cloned into pMA424. The junctions of all constructions were verified by sequence analysis. The fusion proteins stopped at the end of the STE12 sequence except in the following constructions: pGAL4-STE12Δ1_215 [16 additional amino acids], pGAL4-STE12Δ1_215 [8 additional amino acids], and pGAL4-STE12Δ2_473 [16 additional amino acids]. Plasmids were transformed into yeast by the lithium chloride method (Ito et al. 1983).

Production of anti-STE12 antiserum

The plasmid pYE contains a bacteriophage T7 promoter with the STE12-coding region under its control. The STE12-coding region was truncated by removing an XbaI–HindIII fragment, resulting in the expression of just the first 215 amino acids of STE12. This plasmid was transformed into Escherichia coli strain BL21 (DE3), and the STE12 fragment was expressed after IPTG induction [Studier and Moffatt 1986]. Induced cells were lysed by French press, and the lysates were fractionated on 12.5% SDS–polyacrylamide gels. The gels were stained with Coomassie blue, and the slices containing the STE12 fragment were cut out and the gel slice was crushed in 0.13 M NaCl, 10 mM sodium phosphate [pH 7.2], to elute the fragment. This fragment was used to immunize rabbits at Cocalico, Inc. (Reamstown, PA).

Labeling and immunoprecipitation

Procedures used for labeling and immunoprecipitation were modified from Hochstrasser and Varshavsky (1990). For 35S-labeling, cells were grown in 5 ml of SRA–histidine to an OD600 of ~0.5. The cells were harvested by centrifugation, washed three times with 1 ml of SD (no supplements), and resuspended in 300 μl of 50 mM NaPO4 [pH 7.5]/0.5% glucose. The cells were incubated at 30°C for 15 min, 150 μCi of 35S-labeled amino acids [either ExpressS35S [NEN] or Trans35SLabel [ICN]] was added, and cells were incubated for an additional 10 min. For labeling in the presence of α-factor, cells were resuspended in 600 μl of NaPO4/glucose and incubated at 30°C for 5 min, α-factor was added to a final concentration of 0.7 μM, incubation was continued for an additional 10 min, and label was added as above. Following incubation with label, cells were pelleted and resuspended in 0.5 ml of buffer A complete, 0.3 gram of glass beads was added, and the cells were lysed by vigorously vortexing for 3 min, followed by boiling for 3 min. Cells and cell debris were removed by centrifugation, and the supernatants were used for immunoprecipitations. Buffer A consisted of 50 mM Tris-Cl [pH
8.0), 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100. Buffer A complete contained the following additions: 20 µg/ml of pepstatin, 2 µg/ml of chymostatin, 20 µg/ml of leupeptin, 0.5 mM PMSF, 50 mM 2-mercaptoethanol, and 0.5% SDS.

For 32P-labelling, cells were grown overnight in modified Wickerham’s media with raffinose and amino acids, supplemented with 100 µM sodium phosphate (pH 7.5). The culture was diluted into 5 ml of the above media without phosphate and grown to an OD600 of ~0.5. Cells were harvested by centrifugation, washed three times with 10 mM Tris-Cl (pH 8.0)/1 mM EDTA, and resuspended in 300 µl of Wickerham salts/0.5% glucose. Resuspended cells were incubated at 30°C for 15 min, 250 µCi of H32PO4 was added, and the cells were incubated for 60 min. For labeling in the presence of pheromone, α-factor was added to a final concentration of 0.7 µM for the last 15 min of labeling. The cells were pelleted and lysed as described above.

For immunoprecipitations, equal amounts of TCA-precipitable counts (generally 1 x 10^6 to 2 x 10^6 cpm) were added to buffer A in a total volume of 400 µl. Rabbit antiserum against STE12 amino acids 1–215) or GAL4 amino acids 1–147 or 1–881; gifts of P. Silver and L. Mylin) was added, and the reactions were incubated at 4°C for 2 hr. Protein A-Sepharose (Sigma) was added, and the reactions were incubated at 30°C for an additional 30 min. The immunoprecipitates were collected by centrifugation and washed three times with buffer A containing 0.1% SDS. The washed precipitates were resuspended in 25 µl of 2x sample buffer [Laemmli 1970], boiled for 3 min, and separated on a 6% SDS–polyacrylamide gel. Cells were fixed, washed with 1 M sodium salicylate for 30 min, dried, and exposed to Kodak X-AR5 film at ~70°C.

**Phosphatase treatment of immunoprecipitates**

Samples to be treated with phosphatase were labeled and immunoprecipitated as described above. The washed immunoprecipitates were resuspended in 50 µl of 50 mM Na acetate (pH 5.4) and 0.5 units of potato acid phosphatase [Boehringer Mannheim Biochemicals] and incubated at 37°C for 30 min. Where indicated, 25 mM NaPO4 (pH 7.5) and 100 mM Na2MoO4 were added to specifically inhibit the phosphatase [Verjee 1969].

**Time course of α-factor treatment**

Cells were grown in 10 ml of SRaf-histidine to an OD600 of ~0.5, harvested by centrifugation, and washed three times with SD [no supplements]. The cells were resuspended in 1 ml of NaPO4/glucose and incubated at 30°C for 15 min. Then, 750 µCi of 35S-labeled amino acids were added, and cells were incubated for 5 min. α-Factor was added to a final concentration of 0.7 µM, and 0.2-ml aliquots were removed after 0, 1, 2.5, and 5 min. The aliquots were added to an equal volume of buffer A complete and 0.3 grams of glass beads, immediately boiled for 3 min, and vortexed vigorously for 3 min. The lysate was clarified by centrifugation, and the supernatant was immunoprecipitated as described above.

**Cycloheximide treatment**

Cells were grown and labeled with 35S as described above with the following changes. The cells were labeled for 5 min, and, where indicated, cycloheximide was added to a final concentration of 100 µg/ml and incubation continued for 5 min. α-Factor was added where indicated, and incubation was continued for an additional 5 min. The cells were then lysed and immunoprecipitated as described. The inhibition of translation by cycloheximide was confirmed by removing 2-µl aliquots immediately before the addition of cycloheximide and after 5 min and adding the aliquots to 200 µl of 10% TCA and measuring the precipitable radioactivity. The amount of TCA-precipitable radioactivity continued to increase in cultures that did not receive cycloheximide but remained essentially unchanged in cultures that received cycloheximide.

**β-Galactosidase assays**

β-Galactosidase activity was assayed as described previously [Dolan and Fields 1990]. The cells were grown in SSG6–histidine, and, where indicated, α-factor was added to a final concentration of 0.7 µM and cells were incubated at 30°C for 2 hr prior to being assayed.

**Gel mobility-shift assay**

Preparation of extracts for gel shifts and the gel-shift procedure was as described previously, using as probe a 209-bp fragment of the MFA2 gene [Dolan et al. 1989]. The strain was EG123 carrying the plasmid pSY2.

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