STE12, a protein involved in cell-type-specific transcription and signal transduction in yeast, is part of protein–DNA complexes

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The STE12 gene of Saccharomyces cerevisiae is essential for the expression of genes required for mating, such as those involved in pheromone response, and for genes unrelated to mating but regulated by the presence of an adjacent copy of the transposable element Ty1. We show that the STE12 protein is a component of specific DNA–protein complexes that form with transcriptional control elements from Ty1 and the α-pheromone receptor gene STE2. Although a sequence involved in pheromone-dependent transcriptional activation is protected in both complexes, competition experiments indicate that the complexes are intrinsically different from each other. We show that another factor involved in cell-type-specific transcription, PRTF/GRM, is a component of the complex with the STE2 fragment but not the Ty1 fragment. We propose that the STE12 product interacts with different transcription factors in different sequence contexts and that PRTF/GRM is one of these factors.

[Key Words: DNA–protein interactions; yeast mating response pathway; gene expression; epitope-tagging]

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The yeast Saccharomyces cerevisiae provides a model system for the study of events controlling cell-type determination and cell communication. The α and α haploid cells of this yeast are able to mate with each other to form an αα diploid cell that is mating incompetent. Recognition between the α and α cell types initiates and coordinates events that are a precondition for cell fusion. This recognition is mediated by peptide pheromones α- and α-factor, which are characteristic of each cell type and their respective receptors [for review, see Cross et al. 1988]. It appears that all components necessary for the response elicited by pheromone receptor binding are the same in both cell types [Bender and Sprague 1986; Nakayama et al. 1987]. Both pheromone receptors are predicted to be structurally similar to vertebrate receptors that are coupled to classical G proteins such as the β-adrenergic receptor [Nakayama et al. 1985; Dixon et al. 1986]. Moreover, three genes in S. cerevisiae (GPA1/SCG1, STE4, and STE18) were found to encode proteins homologous to the α-, β-, and γ-subunits of mammalian G proteins. Genetic evidence has established a role for these three gene products in the mating response pathway [Dietzel and Kurjan 1987; Miyajima et al. 1987; Jahng et al. 1988; Whiteway et al. 1989].

The signal generated by the interaction between receptor and mating pheromone causes arrest of the mitotic cell cycle in the G1 phase, synthesis of cell-surface agglutinins, and morphological changes associated with mating. It also leads to transcriptional induction of several genes, including the receptor and pheromone structural genes [Strazdis and MacKay 1983; Nakayama et al. 1985; Hartig et al. 1986; Jarvis et al. 1988]. One target sequence for the signal causing pheromone-inducible transcription has been identified. The sequence element TGAAACA appears in the promoter region of all pheromone-responsive genes and has been shown to be necessary for induction by pheromone [Kronstad et al. 1987; Van Arsdell and Thorner 1987]. We refer to this sequence as a pheromone response element [PRE]. A trans-acting factor that binds to the PRE has not been identified yet.

Genetic approaches to dissect the signal transduction pathway have led to the identification of several genes (STE4, STE5, STE7, STE11, STE12, and STE18) that are necessary for mating competence in both cell types and may specify, directly or indirectly, components of the signal pathway [Hartwell 1980; Nakayama et al. 1988; Whiteway et al. 1989]. Mutations of these genes prevent mating and response to pheromone, including inducible gene expression [Hartwell 1980]. In addition, these mutations reduce basal transcription of genes required for mating, suggesting that the integrity of the signal
pathway is important for transcription even under non-inducing conditions (Hartig et al. 1986; McCaffrey et al. 1987; Fields et al. 1988). The transcriptional defects caused by these mutations also can be observed for transcription associated with transposable elements such as Ty1 and σ (Errede et al. 1981; Van Arsdell et al. 1987). Although these STE gene products are necessary for full expression of genes required for mating, not all of the components identified by this group are likely to be transcription factors. For example, the predicted STE4 and STE18 proteins are related to the β- and γ-subunits of mammalian G proteins, respectively, and the predicted STE7 and STE11 proteins are related to protein kinases (Teague et al. 1986; Whiteway et al. 1989; B. Errede, unpubl.).

The aim of studies presented here was to determine the function of the STE12-encoded protein. A cis-acting regulatory element within Ty1 that requires the STE12 product for activation of the CYC7 promoter was described recently (Company et al. 1988). For comparison, we wished to identify a STE12-dependent regulatory element from the promoter region of another gene whose expression is decreased in a ste12 mutant background. One such gene is the α-pheromone receptor gene STE2 (Hartig et al. 1986; Fields et al. 1988).

One of the STE2 upstream activating sequences (UAS) that confers cell-type-specific gene expression was shown previously to be within a 97-bp AvaI–HindIII fragment (Miller et al. 1985). We tested this STE2 UAS for the ability to cause STE12-dependent expression of

Figure 1. STE12 dependent regulatory elements. [A] Nucleotide sequence of the Ty1 and STE2 factor binding regions as determined by DNase I protection assays. Fragments used to demonstrate STE12-dependent regulatory and factor binding activity are the 94-bp Ty1 fragment A5 (PvuI–Sau3A fragment described by Company et al. 1988), the 97-bp STE2 UAS (AvaI–HindIII fragment, nucleotide positions 266–362 according to Nakayama et al. 1985) and the synthetic 47-bp STE2 UAS (nucleotide positions 305–351, according to Nakayama et al. 1985). Matches to the P box (solid underline) and PRE sequences (hatched underline) are indicated. Base pair differences for a nonfunctional Ty1 or STE2 regulatory element are given above the corresponding sequences. Methylation interference sites for the Ty1 fragment are indicated (†). [B] Structure and STE12-dependent expression for regulatory fragments inserted upstream of the CYC1-lacZ fusion gene. CYC1 sequences (stippled) and the lacZ-coding region (open) of the fusion gene are indicated. Base pair differences for a nonfunctional Ty1 or STE2 regulatory element are given above the corresponding sequences. Methylation interference sites for the Ty1 fragment are indicated (†).
the CYCI-lacZ fusion. The 97-bp STE2 fragment and a 47-bp subfragment were each inserted upstream of the fusion gene in the vector pLGΔ178 to give pGA1680 and pGA1702, respectively (Fig. 1). Each of these plasmids was used to transform a wild-type STE12 strain (G2, STE12) and an isogenic ste12 deletion strain (G2,Δ12, ste12Δ). The CYCI-lacZ plasmid with a functional CYCI promoter (pLG669-Z) and the CYCI-lacZ plasmid with the disabled promoter (pLGΔ178) were introduced into the two yeast strains for reference. Expression of the fusion genes was quantified as the amount of β-galactosidase produced by each transformed strain (Fig. 1B). Both STE2 UAS fragments allowed significant expression of the reporter gene in the STE12 strain. In the ste12Δ strain, expression was reduced 21-fold for the 97-bp STE2 UAS plasmid (pGA1680) and 96-fold for the 47-bp STE2 UAS plasmid (pGA1702).

We attribute the residual activity of the STE2 UAS fragments in the ste12Δ strain to the presence of a palindromic P-box element (Fig. 1). The P-box element is the binding site for a factor known as the pheromone/receptor gene transcription factor (PRTF) or general regulator of mating (GRM) [Bender and Sprague 1987; Keleher et al. 1988; Tan et al. 1988; Hayes et al. 1988]. PRTF and GRM are the product of the same gene (MCM1) and are therefore the same factor [Passmore et al. 1988; G. Ammerer, in prep.]. We will refer to this factor as PRTF. P-box elements from the promoters of the BAR1, STE6, and STE2 genes have been shown to be sufficient for UAS activity [Kronstad et al. 1987; Jarvis et al. 1988; Keleher et al. 1988; G. Ammerer, in prep.]. It is assumed that this activity is due to the ability of PRTF to bind to the P-box and function as a transcription activator. [Evidence that PRTF binds to the STE2 UAS is presented below.]

We expected that comparison of the Ty1 and STE2 fragments would reveal a common sequence that would identify a recognition element for the STE12-dependent binding factor (Fig. 1A). The most noteworthy similarity between the two sequences is that both contain a match to the PRE (TGAAACA), which is important for pheromone-inducible transcription [Kronstad et al. 1987; Van Arsdell and Thorner 1987]. It has been shown that α-pheromone increases steady-state amounts of STE2 mRNA [Nakayama et al. 1985; Hartig et al. 1986]. Under conditions of α-pheromone induction that cause an approximate fourfold increase in STE2 steady-state mRNA, we found an approximate twofold increase in the steady-state amount of mRNA for a gene whose transcription is controlled by an adjacent Ty1 element (C. Adler and B. Errede, unpubl.). A P-box sequence, which is another feature of the STE2 UAS, is not present in the Ty1 regulatory element.

STE12 dependent protein–DNA complexes

It was shown previously that the STE12-dependent regulatory sequence from Ty1 forms a specific protein–DNA complex and that its formation requires the presence of the STE12 gene product [Company et al. 1988]. To determine whether the same dependence is associated with the STE2 UAS, we used a gel mobility shift assay with whole-cell extracts prepared from isogenic STE12 and ste12Δ strains. When the 97-bp STE2 fragment was used as a probe, two protein–DNA complexes (C1 and C2) were observed with extracts prepared from the STE12 strain (Fig. 2A). Only complex C1 was STE12 dependent, as evidenced by its absence from binding reactions using extract from a ste12 deletion strain (ste12Δ).

Factor binding site(s) within the STE2 regulatory element were mapped by a DNase I protection assay. Two different 3'-end-labeled probes were prepared for the top and bottom strands of the STE2 fragment. Factor binding sites for both complexes C1 and C2 were identified (Fig. 2B). A summary of the protected sites on both strands is given (Fig. 2C). Both complexes C1 and C2 show an identical footprint located at the P-box. The STE12-dependent complex C1 shows an additional footprint at the PRE. These results suggest that the P-box binding factor, PRTF, is present in both complexes C1 and C2 and that an additional factor or factors is present in complex C1.

STE12 nucleotide and predicted amino acid sequence

To begin investigations into the role of the STE12 product, we determined the nucleotide sequence of the STE12 gene that had been cloned in the plasmid pLB1214 (kindly provided by L. Bell, Zymogenetics, Inc.). The sequence of 3519 nucleotides that encompass the STE12 locus is shown (Fig. 3). No DNA sequences that encode the consensus for RNA splicing in yeast were found. An ATG at position 1127 is followed by an open reading frame terminating with a TGA codon at position 3191. A protein of 688 amino acids with a net negative charge and a molecular mass of 78,000 daltons is predicted by the open reading frame.

We looked for features in the predicted STE12 protein that are characteristic of known DNA-binding proteins because the STE12 product is required for protein–DNA complex formation. We found no regions similar to known DNA-binding domains [Pabo and Sauer 1984; Shephard et al. 1984; Berger 1986]. The predicted STE12 protein was compared to the NBRF Protein Sequence Data Base (release 17, January 1989), using the Word-Search program from the Wisconsin Genetics Computer Group Sequence Analysis Software Package. No striking similarities with other proteins were identified.

Tagging the STE12 gene product

To facilitate detection of the STE12 product, we fused the coding sequence of a known epitope to the STE12 gene. The chosen epitope consisted of 10 amino acids (EQKLISEEDL) from human c-myc for which a specific monoclonal antibody (Myc1-9E10) was available [Evan et al. 1985; Munro and Pelham 1987]. Two different in-frame fusions of the STE12 gene were constructed (Fig. 4). One fusion, designated STE12-469M, was made at STE12 codon 469, using the Ncol site at nucleotide
Figure 2. Factor binding to the STE2 regulatory element. (A) Protein–DNA complexes detected by gel mobility shift assays. Binding reactions contained extract prepared from yeast strains with STE12 or ste12A genetic backgrounds, as specified. The positions of complex C1 (C1), complex C2 (C2), and free probe (F) are indicated at left. (B) DNase I protection analysis of factor binding sites in complexes C1 and C2. The top or bottom strand of the 97-bp STE2 UAS was 3'-end-labeled and used as probe DNA. Extract used for factor binding was prepared from yeast with either STE12 or STE12-668M genetic background, as indicated. Lanes display cleavage products of DNase-I treated binding reactions that were fractionated by gel electrophoresis into free (F), complex 2 (C2), and complex 1 (C1) probe DNA. Also shown are the cleavage products from purine-specific reactions with probe DNA (A - G). Regions showing DNase I-protected sites for complexes C1 and C2 are indicated by brackets at right. (C) Summary of footprint analysis. The nucleotide sequence of the footprint sites is given. The locations of hypersensitive (\(|\)) and weakly protected (o) sites corresponding to complex C1 and C2 are indicated. The locations of P-box and PRE elements in the sequence are indicated by brackets (top).

position 2529. This fusion replaces the 219 carboxy-terminal amino acids of the normal STE12 product with an 11-amino-acid peptide tag. The other fusion, designated STE12-668M, was made at STE12 codon 668, using the SacI site at nucleotide position 3129. This fusion replaces the 20 carboxy-terminal amino acids of the normal STE12 product with a 20-amino-acid peptide tag. We established that the monoclonal antibody Mycl-9E10 specifically detected the tagged STE12 proteins produced in yeast by Western blot analysis (data not shown).

In vivo function of the tagged STE12 products

We tested the ability of the STE12-469M and STE12-668M gene products to complement the mating defects of a ste12 deletion mutation. A yeast strain with a ste12 deletion (E929-6C-6, ste12A) was transformed with either a low-copy centromeric yeast plasmid [pNC204] or a high-copy 2-μm yeast plasmid [pGA1635]. The ste12 deletion strain was also transformed with the STE12-668M gene carried on a low-copy centromeric yeast plasmid [pNC228]. As a control, the same recipient strain was transformed with a plasmid carrying the normal STE12 gene on a centromeric plasmid [pSC4]. The STE12-469M, STE12-668M, and STE12-transformed strains were mating competent when assayed by a nonquantitative patch test (Table 1).

The nonquantitative mating assay does not indicate whether the STE12-M products are functionally identical to the normal product. (Strains can differ by an order of magnitude in mating ability and still show equivalent mating by the patch test.) For this reason we tested the STE12-M products for their ability to promote STE12-dependent gene expression. The CYC7-H2 gene is a convenient reporter for these tests. The CYC7-H2 mutation was caused by insertion of a Ty1 element in the 5'-noncoding region of CYC7, which is the structural gene for iso-2-cytochrome c. The inserted Ty1 element causes iso-2-cytochrome c expression to come under mating type control and to be dependent on a functional STE12 product (Errede et al. 1981). Therefore, CYC7-H2 expression in strains with different STE12 alleles was quantified on the basis of its gene product, iso-2-cytochrome c. The STE12-M and STE12-transformed strains produced amounts of iso-2-cytochrome c that were greater than the basal amount produced in the ste12 deletion strain (Table 1). The results demonstrate that the STE12-668M product is fully functional with respect to its ability to promote Ty1-regulated transcription. However, a quantitative difference was found be-
Figure 3. Nucleotide sequence of the STE12 locus. The nucleotide sequence of the coding strand is shown. The amino acid sequence for the open reading frame corresponding to the predicted STE12 protein is shown below the nucleotide sequence (nucleotide positions 1127-3190). The standard single-letter amino acid code is used. The end of another open reading frame upstream of the STE12 gene at position 567 is indicated (orf stop). The function of the protein encoded by the open reading frame is not known. The 5' map positions for the STE12 mRNA are indicated by asterisks (*| above the nucleotide sequence at positions 831, 832, 839, and 840. Five short sequences homologous to the PRE (underlined or overlined) are found in the presumed promoter region. Restriction sites are indicated for those enzymes referred to in this study: XbaI at positions 634 and 1766, NcoI at position 2529, BamHI at position 2539, and ScaI at positions 677 and 3129.
between the \textit{STE12}- and \textit{STE12}-469M-transformed strains.

The amount of iso-2-cytochrome c was 17-fold above the background when \textit{STE12} was expressed on a low-copy centromeric plasmid (pSC4). By comparison, the amount of iso-2-cytochrome c was 13-fold above background when \textit{STE12}-469M was expressed on the low-copy centromeric plasmid (pNC204) and only 8-fold above background when overexpressed on the high-copy plasmid (pGA1635).

The result with the \textit{STE12}-469M fusion product is somewhat unexpected in light of observations reported by Fields and Herskowitz [1987]. They found that a \textit{STE12} fragment with a breakpoint at the \textit{BamH}I site located within the coding region was unable to comple-

Table 1. The \textit{STE12-M} products are functional for mating and activation of gene expression in yeast

| Strain   | Plasmid | Relevant genotype | Matinga | Amount of cytochromeb |
|----------|---------|------------------|----------|-----------------------|
| E929-6C-6| none    | \textit{ste12}\Delta | −        | 1 [−]                 |
| E929-6C  | none    | \textit{STE12} + | +        | 20 [3]                |
| E929-6C-6| pSC4    | \textit{ste12}\Delta::\textit{LEU2} \textit{STE12} | +        | 17 [3]                |
| E929-6C-6| pNC228  | \textit{ste12}\Delta::\textit{LEU2} \textit{STE12}-668M | +        | 23 [3]                |
| E929-6C-6| pNC204  | \textit{ste12}\Delta::\textit{LEU2} \textit{STE12}-469M | +        | 13 [3]                |
| E929-6C-6| pGA1635 | \textit{ste12}\Delta::\textit{LEU2} \textit{STE12}-469M | +        | 8 [2]                 |

\textsuperscript{a}Mating competence was determined by the patch test [Sprague and Herskowitz 1981]. (+) Ability to mate and (−) inability to mate is indicated.

\textsuperscript{b}Low-temperature spectroscopic examination of intact cells was used to estimate the relative amount of iso-2-cytochrome c [Sherman and Slonimski 1964]. In the case of transformed strains, values are the average of determinations on at least 10 independent transformation isolates. Values given in parentheses are the standard deviations. One unit is the amount produced by standard \textit{CYC7 +} strains.
In vitro function of the tagged STE12 products

The ability of protein extract prepared from STE12-469M and STE12-668M strains to form specific complexes with the STE12-dependent regulatory elements was determined by a gel mobility shift binding assay. In one set of experiments, the CYC7-H2 Ty1 fragment A5, which was previously shown to form a specific STE12-dependent protein complex, was used as a probe for the binding assay (Company et al. 1988). No complex formed with the Ty1 probe when extract from a ste12Δ strain was used in the binding reaction. The STE12-dependent complex C1 formed with the Ty1 probe when extract from the STE12-, STE12-469M-, or STE12-668M-transformed strains was used in the binding reactions (Fig. 5, No Ab). It is evident that the complex formed with the STE12-469M extract migrated with a slightly greater mobility than did the complex with the STE12 extract. This observation is consistent with the hypothesis that the STE12-469M product is a component of the protein-DNA complex. The DNase I footprint [one strand] for the Ty1 DNA complex that forms with STE12-469M extract was compared with that for the STE12 extract [data not shown]. The protected sites observed with the two different extracts were essentially the same as those published previously (Company et al. 1988). This result indicates that truncation of the STE12 product does not alter protein contacts on the Ty1 regulatory element.

In a second set of experiments, the 97-bp STE2 UAS was used as a probe in binding assays with extracts from strains producing the normal or tagged STE12 proteins (Fig. 6, No Ab). The STE12-dependent complex C1, as well as complex C2, was observed when extracts prepared from the STE12 or STE12-668M strains were used in the binding reactions. Furthermore, the DNase I-protected sites observed for complexes C1 and C2 were identical in extracts prepared from STE12 or STE12-668M strains [Fig. 2B]. However, only the STE12-independent complex C2 was observed when extracts from the ste12Δ or STE12-469M strains were used in the binding reactions. Because the STE12-469M fusion complements the mating defect of a ste12 deletion, it is possible that the conditions of the in vitro assay are not sufficiently sensitive to detect interactions that may still occur in vivo. Nonetheless, the finding that the STE12-469M product does not support the in vitro formation of complex C1 with the STE2 UAS suggests that requirements for this STE12-dependent complex are different from those necessary for formation of the complex with the Ty1 probe.

The STE12 product is a component of DNA-protein complexes

The STE12 product is critical for formation of a DNA-protein complex with the STE12-dependent elements from Ty1 and STE2. The simplest interpretation of the above results is that the STE12 product is a component of the DNA-protein complex. If this hypothesis is correct, the antibody specific for the peptide tag could be used to determine whether the STE12-469M and STE12-668M products are a component of the DNA-protein complexes that form with the Ty1 and STE2 UAS fragments. If an antibody recognizes a DNA-binding protein, it is expected to cause an alteration in the gel mobility shift pattern. The antibody-protein interaction could interfere with complex formation and abolish the signal. Alternatively, the antibody-protein interaction may not interfere with complex formation but will lead to a further retardation of the complex during electrophoretic fractionation.

Results consistent with the latter possibility were obtained with the Ty1 probe [Fig. 5]. Preincubation of extract containing either STE12-469M or STE12-668M product with the Myc1-9E10 antibody specific for the
prior to protein-DNA-binding reactions (Anti-PRTF). Preincubation with 1.5 µl of anti-PRTF serum at a 1:25 dilution performed, the mixtures were incubated with 2 µg of mouse protein-DNA complexes were formed under one of the following genetic background, as indicated. Pro-

469M, STE12-668M or labeled 97-bp UAS. DNA-binding reactions were performed with extract prepared from yeast with stel2A, STE12, STE12-668M and STE2 Probe

Figure 6. STE12-M complex formation with the STE2 UAS. Probe DNA in each binding reaction was ~0.5 ng of the end-labeled 97-bp STE2 UAS. DNA-binding reactions were performed with extract prepared from yeast with ste12Δ, STE12, STE12-469M, or STE12-668M genetic background, as indicated. Protein–DNA complexes were formed under one of the following conditions: Binding reactions were performed in the absence of antibody (No Ab); after protein–DNA-binding reactions were performed, the mixtures were incubated with 2 µg of mouse monoclonal antibody Myc1-9E10 (Anti-myc); extracts were preincubated with 1.5 µl of anti-PRTF serum at a 1:25 dilution prior to protein–DNA-binding reactions (Anti-PRTF).

peptide tag (Anti-myc) decreased the mobility of the Ty1 probe to a greater extent than extract not incubated with antibody (Fig. 5, No Ab). Preincubation of extract containing the unmodified STE12 product with the antibody did not affect the mobility of the complex. In addition, preincubation of extracts with another monoclonal antibody (Myc1-3C7) that does not recognize the peptide tag did not affect probe or complex mobility (data not shown) [also see Fig. 5, anti-PRTF]. The same results were obtained if the protein–DNA complexes were allowed first to form and then were incubated with the antibody [data not shown]. From these results we conclude that the STE12-469M and the STE12-668M products are a component of the Ty1 protein complex.

The antibody specific for the peptide tag also was used to determine whether the STE12-668M product is a component of the STE12-dependent protein–DNA complex C1 that forms with the STE2 UAS. When the antibody specific for the peptide tag was preincubated with extract, formation of complex C1 was abolished with STE12-668M extract but not with STE12 extract [data not shown]. When the protein–DNA complexes first were allowed to form and then incubated with the anti-myc antibody, the complex C1 that formed with the STE12-668M extract was detectable but was present in lower amounts. This suggests that complex C1 still was disrupted significantly by the antibody interaction. The important observation is that the residual amount of complex C1 was retarded to a greater extent than when extract was not incubated with antibody [Fig. 6]. In contrast, the amount and mobility of complex C1 that formed with extract containing the normal STE12 product was not affected by incubation with the anti-myc antibody. Incubation of binding reactions with the anti-myc antibody did not affect the mobility of the STE12-independent complex C2 that formed with each of the extracts tested. We conclude that the STE12–668M product is a component of the complex C1 that forms with the STE2 UAS.

The DNase I footprint at the P box of the STE2 UAS suggests that PRTF is a component of both complexes C1 and C2. To test this, a rat polyclonal antibody produced against purified PRTF (anti-PRTF) was used in the gel shift assays. In these experiments, extracts from ste12Δ, STE12, and STE12-668M strains were incubated with or without anti-PRTF prior to the DNA-binding reactions. In the case of the STE2 DNA probe, preincubation of extracts with the anti-PRTF antibody severely retarded both complexes C1 and C2 relative to extracts without antibody [Fig. 6]. The results are also consistent with the possibility that complex C2 is retarded, whereas complex C1 is disrupted by interaction of PRTF with the antibody. According to either interpretation, these results demonstrate that PRTF is a component of both complexes C1 and C2 that form with the STE2 UAS. In the case of the Ty1 DNA probe, anti-PRTF antibody had no effect on complex mobility [Fig. 5]. We conclude that PRTF is not a component of the complex that forms with the Ty1 regulatory sequence.

Cross competition analysis

If the STE12 product binds directly to DNA, e.g., at the PRE-binding site common to the Ty1 and STE2 regulatory elements, the two fragments are expected to compete with each other for protein–DNA complex formation. However, we found no evidence for such competition [Fig. 7]. As expected, the signal corresponding to complex C1 using STE12 extract with the labeled Ty1 probe was inhibited progressively by addition of a 5- to 150-fold molar excess of the unlabeled Ty1 DNA fragment. The same results were obtained for complexes C1 and C2 when the STE2 probe was competed with a 5- to 150-fold molar excess of the unlabeled STE2 fragment. In contrast, the unlabeled STE2 fragment did not inhibit complex formation with the Ty1 probe even when present in amounts that completely abolished the signal when the Ty1 fragment was used as competitor. Similarly, no inhibition was observed when the Ty1 frag-
Figure 7. Competition analysis for Ty1 and STE2 factor binding. Probe DNA in each binding reaction was ~0.5 ng of end-labeled Ty1 fragment A5 (Ty1) or the 97-bp STE2 UAS (STE2), as indicated. The same Ty1 and STE2 fragments were added as competitors at the indicated (0–150) molar excess. Competitor DNAs were added to binding reactions prior to addition of extract from a STE12 yeast strain.

Discussion

The STE12 protein is a component of two distinct DNA–protein complexes

The aim of these experiments was to define the function of the protein encoded by the STE12 gene. For this purpose, we constructed two fusion genes, STE12-469M and STE12-668M, in which the STE12 product was tagged with an immunologically recognizable peptide. One concern was whether the STE12-M products could substitute for the normal STE12 product. Our results showed that both of these fusion genes carried on extrachromosomal plasmids were sufficient to restore the biologically relevant function of mating in a ste12 deletion strain. No difference between the normal and STE12-668M proteins was detected for their ability to promote expression of the Ty1 reporter gene, CYC7–H2. Only a quantitative difference (~25%) in CYC7–H2 expression was detected for the STE12-469M protein in which the carboxy-terminal 219 amino acids of the normal protein are deleted.

The results of gel shift assays in the presence of specific antibodies clearly show that the STE12-469M and STE12-668M products are components of specific protein–DNA complexes that form with Ty1 and STE2 transcriptional control elements. These experiments do not distinguish between the possibilities that the STE12 product binds on its own to DNA or that it requires cooperative interaction with other proteins to form the complex. It is not even clear that STE12 makes direct contact with DNA. Nonetheless, STE12-dependent complex formation in vitro correlates with gene expression in vivo, so we conclude that the STE12 protein constitutes an integral part of active transcription complexes on the Ty1 and STE2 regulatory elements [Figs. 1 and 6; Company et al. 1988]. However, our results indicate the STE12 protein complex that forms with Ty1 DNA is different from that which forms with STE2 DNA. First, the two regulatory elements do not compete with each other for specific DNA-binding proteins. Second, the complex with the STE2 UAS contains PRTF but that with Ty1 does not. Third, the truncated STE12-469M product supports complex formation in vitro with the Ty1 probe but not with the STE2 probe. Finally, the anti-myc antibodies appear to interfere with the complex that forms with the STE2 probe but not with the Ty1 probe.

Models for STE12 interaction in protein–DNA complexes

The failure to observe competition between the Ty1 and the STE2 UAS fragments for the formation of STE12-dependent protein–DNA complexes puts restrictions on any model to account for STE12 interaction with DNA. Considering this result, we find it unlikely that the STE12 protein binds independently to both the Ty1 and STE2 regulatory elements [Fig. 8A]. One would have to argue that the STE12 protein contains DNA-binding domains with different sequence specificity. This situation has a precedent in the case of the HAP1 protein [Pfeifer et al. 1987]. However, the absence of competition in our case imposes the restriction that such domain structures are locked into mutually exclusive and stable conformational states of the protein.

Another model is that the STE12 protein interacts cooperatively with different DNA-binding proteins in different sequence environments [Fig. 8B]. Here, we propose that STE12 interacts at the PRE sequence common
Figure 8. Models for STE12-dependent complex formation. (A) Direct DNA binding model. According to this model, STE12 (X) binds directly to the PRE sequences (hatched boxes) common to the STE2 and Ty1 elements. (B) Cooperative protein DNA binding model. According to this model, STE12 requires cooperative interaction with another DNA-binding protein for DNA complex formation. The cooperative binding protein is proposed to be PRTF (P), which binds at the P box (solid box) in the STE2 UAS. The cooperative binding protein for complex formation with the Ty1 regulatory element is an unknown protein [X]. (C) Non-DNA binding model. According to this model, STE12 does not contact DNA directly but stabilizes complexes that form with different DNA-binding proteins on the Ty1 (X and Y) and STE2 (P and Y) elements.

Does STE12 interact with PRTF?

If STE12 interacts with other DNA-binding proteins, PRTF is an obvious candidate for one such factor. It is the major factor responsible for DNA complex formation at the P-box element present in the promoter region of cell-type-specific genes. It has already been shown to bind cooperatively with two different proteins, the cell-type-specific regulators α1 and α2. In both cases, the factors interact directly with PRTF (Keleher et al. 1988; Tan et al. 1988). Although we have not proved the existence of direct interactions between the STE12 protein and PRTF, both are present in the complex C1 that forms with the STE2 UAS. A functional relationship between the two proteins is suggested by the finding that a double point mutation in the P box (Fig. 1) abolishes protein-binding activity of the 47-bp STE2 UAS and also destroys the in vivo activation functions of this highly STE12-dependent fragment (G. Ammerer, in prep.). In addition, the experiments with the truncated STE12-469M product suggest that the 219-amino-acid carboxy-terminal domain may be important for interactions between STE12 and PRTF at the STE2 UAS.

Is STE12 a distal element of the signal transduction pathway?

Genetic evidence established that the STE12 gene is necessary for physiological responses to pheromone and may specify, directly or indirectly, a component of the signal pathway that functions downstream of the α-subunit of G protein (Hartwell 1980; Nakayama et al. 1988). We propose that the STE12 protein is a nuclear target of the pheromone induction signal. Our data show clearly that the STE12 protein is related functionally to the transcriptional PRE. This sequence element is protected against DNase I digestion in the STE12-containing protein–DNA complex with the STE2 UAS. On the basis of DNase I protection and methylation interference analyses, the PRE sequence also is involved in complex formation with the Ty1 regulatory element (Fig. 5 in Company et al. 1988). As discussed above, either the STE12 protein binds to the PRE or it interacts with another factor that binds to this sequence. Because two other gene products (STE7 and STE11) that are required for response to pheromone show significant similarities to protein kinases (Teague et al. 1986; B. Errede, unpubl.), an obvious hypothesis is that the induction signal may affect STE12 function in transcription complexes by a
modification such as protein phosphorylation. The proposal rests partly on the observation that extracts from a ste7 or ste11 deletion strain show reduced binding activity with the Ty1 fragment [Company et al. 1988; C. Adler and B. Errede, unpubl.] Additionally, the STE7, STE11, and STE12 gene products appear to act at the same point in the pathway. This view is supported by the observation that a deficiency in any one of the three gene products causes reduced expression of target genes and that there is not a more severe reduction in target gene expression when all three gene products are deficient [Fields et al. 1988].

The finding that STE12 is part of a DNA-protein complex also has implications for other responses to pheromone such as G1 arrest. The STE12 protein plays a role in the pathway that leads to cell-cycle arrest induced by pheromone. This conclusion is based on the observation that loss of STE12 function relieves the growth defect caused by a mutation in the a-subunit of the G protein [Nakayama et al. 1988]. Therefore, one prediction from our findings is that at least one gene required for G1 arrest whose transcription is dependent on the STE12 product.

Materials and methods
Plasmids
Plasmid constructions to test STE12-dependent regulatory activity of STE2 UAS elements were made with the promoter-disabled, CYC1-lacZ vector pLGA178. pLGA178 was constructed from pLG669-Z [Guarente and Ptashne 1981] by deletion of the 433-bp XhoI fragment, which includes both UAS1 and UAS2 from the CYC1 promoter region [Guarente et al. 1984]. The 97-bp STE2 UAS fragment was isolated as an AvaI–HindIII fragment (nucleotides 266–362; Nakayama 1985). The fragment was end-repaired and blunt-end-ligated into the end-repaired XhoI site of pLGA178 to give pGA1680. Oligonucleotides corresponding to each strand of the 47-bp STE2 fragment (Fig. 1A) were synthesized with the addition of XhoI-compatible ends. The annealed oligonucleotides were inserted at the XhoI site of pLGΔ178 to give pGA1702.

The DNA sequence of the STE12 locus was determined from subclones of pLB2124 [Fig. 4A]. The plasmid pLB2124 was a gift from L. Bell and is a yeast genomic DNA clone that was able to complement a ste12 mutation. The 3.2- and 2.3-kb BamHI fragments from pLB2124 were subcloned into the BamHI site of pUC4 to give pLB204 and pLB205, respectively.

Two different STE12 fusion genes were constructed. For STE12–469M, the STE12 product was fused at amino acid 469 with an antigenic peptide tag using DNA fragments from pLB204 and pMCT61. The plasmid pLB204 carries a BamHI fragment encompassing the amino-terminal 472 amino acids of STE12 (Fig. 4A). The plasmid pMCT61, provided by H. Pelham, contains a fragment of the human c-myc gene that codes for a 10-amino-acid antigenic peptide. The c-myc sequence is flanked by a NcoI site on the 5’-end which provides an in-frame ATG codon, and a filled-in EcoRI site on the 3’-end, which adds one amino acid and provides an in-frame termination codon [Fig. 4B] [Munro and Pelham 1987]. In addition, pMCT61 contains ~700 bp of unspliced sequence from the herpes simplex thymidine kinase gene and SV40 DNA. The unspliced sequence is located downstream of the c-myc-coding segment, and its end point is defined by an Sp6I site. The 3.2-kb EcoRI–NcoI fragment that encodes 469 amino acids of STE12 from pLB204 and the 700-bp NcoI–Sp6I fragment from pMCT61 were cloned into SphI–EcoRI-cut pUC19 to give the plasmid pGA1630. This STE12–469M fusion was transferred as a BamHI–HindIII fragment into the vector pGA1618 to give the 2-μm plasmid pGA1635. The vector pGA1618 is identical to pSEY101, except that the polylinker region contains a HindIII site (Douglas et al. 1984). The STE12–469M fusion gene also was transferred as an EcoRI–Sp6I fragment from pGA1635 to the vector YCp50 to give the centromeric plasmid pNC204.

For STE12–668M, the STE12 product was fused at amino acid 668 with the antigenic peptide tag using DNA fragments from the plasmids pSC4 and pNC212. The plasmid pSC4 contains the STE12 gene cloned at the BamHI site of YCp50 and was provided by S. Fields [Fields and Herskowitz 1987]. A 4.1-kb Sp6I–ScaI fragment from pSC4 encompassing the amino-terminal 668 amino acids of the STE12 gene was cloned into the SphI–ScaI site of pNC161 to give the plasmid pNC205 [Fig. 4A]. The plasmid pNC161 is a centromeric yeast–Escherichia coli shuttle vector. It is the same as the previously published plasmid pNC98, except that the polylinker from M13mp19 replaces the CYC7 sequences [Company and Errede 1987]. Site-directed mutagenesis was used to insert an oligonucleotide encoding the c-myc epitope between the last codon and the stop codon of STE11 to give the plasmid pNC212 [C.N. Rhodes II and B. Errede, unpubl.]. A 1.1-kb PstI fragment from pNC212 was isolated. This fragment includes codons for 9 amino acids from STE11, the 10-amino-acid peptide tag, and 1 carboxy-terminal amino acid. The coding segment is followed by a TGA stop codon and 3’-noncoding sequences from STE11. After appropriate end repair, the 1.1-kb fragment was inserted at the SacI site at codon 668 of STE12 to give the STE12–668M fusion in the plasmid pNC228.

The ste12Δ::LEU2 plasmid pSUL16 was provided by S. Fields and has been described previously [Fields and Herskowitz 1987]. The XbaI–PstI fragment from the plasmid pNC188 was the source of the TY1 DNA [fragment A5] used as a probe for DNA-binding reactions [Company et al. 1988]. The plasmid pGA1682 carries the AvaI–HindIII fragment from the 5’-noncoding region of STE2 [nucleotides 266–362] cloned into the Smal site of pUC13. An XbaI–EcoRI fragment from pGA1682 was the source of the STE2 UAS, which was used as a probe for DNA-binding reactions.

Standard recombinant DNA procedures were used for all plasmid constructions and preparations [Maniatis et al. 1982].

Sequencing
The STE12 nucleotide sequence was determined on both strands by the dideoxy sequencing method [Sanger et al. 1977]. A directed deletion strategy was employed with derivatives of pLB204 and pLB205 [Henikoff 1984].

Yeast strains and genetic procedures
Yeast strains used in these studies were E929-6C [MATa STE12+ cycl1 CYC7–H2 can1 leu2-3,112 trpl-M ura3-52], E929-6C-6, G2 [MATa leu2 trpl ura3], G2A12, and KZ8-1D [MATa cycl1 CYC7 his4 ura1]. E929-6C-6 and G2A12 are isogenic ste12Δ::LEU2 derivatives of E929-6C and G2, respectively. Each was constructed using the 6-kb SacI–Sp6I fragment from pSUL16 for gene replacement. E929-6C and E929-6C-6 carry the STE12-dependent reporter gene CYC7–H2 at its chromosomal locus. Media and genetic procedures were as described in Sherman et al. (1979).
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Antibodies

Mouse monoclonal antibody Myc1-9E10 was raised against a synthetic peptide comprising residues 409–439 of human c-myc, and mouse monoclonal antibody Myc1-3C7 was raised against a synthetic peptide comprising residues 171–188 of human c-myc [Evan et al. 1985]. Acetate fluid for each monoclonal antibody was a gift from G. Evan. Saturated [NH₄]₂SO₄ (100%) in buffer [20 mM HEFES (pH 8.0), 5 mM EDTA] was added to the crude acetate fluid to give a final [NH₄]₂SO₄ concentration of 40%. This mixture was incubated at 4°C with gentle agitation for 30 min. The protein precipitate was pelleted by centrifugation at 4°C for 10 min at 13,000g. Each protein pellet was suspended in 20 mM HEPES (pH 8.0), 5 mM EDTA, to a final protein concentration of 10 μg/μl. Protein concentrations were determined using the Bio-Rad Laboratories (Richmond, California) protein assay kit. The rat polyclonal antibody anti-PRTF was raised against the DNA affinity-purified PRTF protein (G. Ammerer, in prep.). The anti-PRTF serum was used without [NH₄]₂SO₄ fractionation at a 1 : 25 dilution.

Preparation of yeast cell extracts

Cultures of E929-6C-6, PGA1635, or pNC228 were grown in appropriate synthetic omission medium to maintain selection for the plasmids encoding the STE12–469M or STE12–668M products. Cultures of E929-6C or E929-6C-6 without plasmid were grown in complete synthetic medium. Extracts were prepared according to procedures described previously [Company et al. 1988]. The protein precipitate from the 40% ammonium sulfate fraction was used for both DNA-binding reactions and immunoblot analysis.

Gel electrophoresis, DNA-binding assays, and DNase I protection analysis

Protein–DNA-binding reactions were carried out in a 20-μl volume containing ~0.5 ng of probe DNA and 10–30 μg of protein extract in binding buffer [20 mM Tris (pH 8), 40 mM NaCl, 4 mM MgCl₂, 1 mM dithiothreitol, 5% glycerol, 2 μg of salmon sperm DNA]. Binding reactions were incubated for 7–10 min at room temperature. Conditions for competition assays were exactly the same as those used for binding assays, except that competitor DNAs were added to the reaction mix before addition of the protein extract. For experiments in which antibodies were used, either of two protocols was employed. In one, antibody was preincubated with cell extract for 7 min at room temperature. DNA-binding buffer and probe DNA were added to initiate the DNA–protein-binding reaction, and the mixture was incubated for an additional 8 min. In the other protocol, the standard DNA–protein-binding reactions were prepared and incubated for 8 min at room temperature. Then the antibodies were added, and the mixtures were incubated for an additional 7 min at room temperature. Gels for resolving protein–DNA complexes were 5% 19:1 polyacrylamide : bis-acrylamide gel in 0.5 × TBE. (1 × TBE is 89 mM Tris, 89 mM Borate, and 2.4 mM EDTA). Gels were run in 0.5 × TBE at 150 V for 4–4.5 hr without pre-electrophoresis. After electrophoresis, the gels were dried and autoradiographed with an intensifying screen at ~70°C. Procedures for DNase I protection assays were as described previously [Company et al. 1988], except that the experiments in this study used 3’-end-labeled DNA fragments and standard DNA-binding reactions were scaled up 10-fold.

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Note

Sequence data described in this paper have been submitted to the EMBL/GenBank Data Libraries.

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