Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 5703–5707, August 1989
Biochemistry

The yeast STE12 protein binds to the DNA sequence mediating pheromone induction

(transcription/yeast mating type/hormone induction/cell-type determination/*Saccharomyces cerevisiae*)

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Communicated by Ira Herskowitz, April 20, 1989 (received for review February 7, 1989)

ABSTRACT **The *STE12* gene product of *Saccharomyces cerevisiae* is required for the transcription of two sets of cell-type-specific genes: the a-specific genes (active only in a cells) and the α-specific genes (active only in α cells). We show that radiolabeled STE12 protein, prepared by *in vitro* transcription and translation, is capable of forming complexes with unlabeled DNA fragments from two a-specific genes. Wild-type yeast, but not a *ste12* mutant, produce a factor that forms complexes with labeled DNA from these same two genes. We use assays with yeast extracts to localize the binding site for the STE12-dependent activity. This site corresponds to the sequence identified as the pheromone induction element, which is responsible for increased transcription of genes when cells are exposed to α-factor or a-factor. Thus the STE12 protein may be an ultimate effector in the signal transduction pathway triggered by pheromone.**

Cell type in the yeast *Saccharomyces cerevisiae* is determined by the transcriptional regulation of different sets of genes (reviewed in refs. 1 and 2). The two haploid cell types, **a** and α, transcribe a-specific and α-specific gene sets, respectively. Products of these genes include the mating factors and receptors to these factors. These products allow **a** cells to mate with α cells to form the third cell type, the **a**/α diploid. The diploid represses transcription of a- and α-specific genes as well as that of haploid-specific genes, which are transcribed both in **a** and α cells. The mating type locus (*MAT*) is the primary determinant of cell type: **a** cells carry the *MAT***a** allele and α cells carry the *MAT*α allele. *MAT*α encodes two proteins: α1, which activates α-specific transcription, and α2, which represses a-specific transcription. The α1 protein binds with a protein designated pheromone/receptor transcription factor (PRTF) to sequences in the regulatory regions of α-specific genes (3). The α2 protein binds cooperatively with protein GRM (general regulator of mating type, which is the same as PRTF) to sequences in a-specific genes (4). *MAT***a** encodes the **a**1 protein, which in conjunction with α2 in the **a**/α cell represses haploid-specific transcription.

In addition to the *MAT*-encoded proteins and PRTF/GRM, the products of several *STE* genes are necessary for cell-type-specific transcription. Mutations in *STE7*, *STE11*, and *STE12* were obtained by selecting for **a** cells resistant to α-factor (5). These mutations were subsequently shown to decrease, although not abolish, transcription from both **a**- and α-specific genes, as well as from certain haploid-specific genes such as those due to Ty1 insertion (6, 7). A Ty1 regulatory sequence responsive to *STE7* and *STE12* has been identified, and this sequence binds a factor that is present in wild-type cells but that is reduced to low levels or absent in *ste7* and *ste12* mutants (8). Sequence analysis of the *STE7* and *STE11* genes indicates that they are likely to encode protein kinases (ref. 9; L. Connell and B. Errede, personal communication).

Transcription of cell-type-specific genes is also regulated by the pheromones **a**-factor and α-factor. The sequence TGAAACA or ATGAAACA has been implicated as an α-factor inducible element; multiple copies of this element are often found within the upstream region of α-factor inducible genes (10, 11). Studies with the haploid-specific gene *FUS1* indicate that this sequence may mediate induction by **a**-factor as well (12, 13). Such a common pheromone induction element is consistent with a common intracellular signal elicited by α-factor and **a**-factor (14).

We provide evidence here that the STE12 protein may play an important role in the response of cells to pheromone. We present the sequence of the *STE12* gene* and show that the encoded protein, when synthesized *in vitro*, is able to bind in a sequence-specific manner to sites present in two **a**-specific genes. Using extracts from yeast cells that overproduce the STE12 protein, we identify the STE12-dependent binding site as the pheromone induction element.

MATERIALS AND METHODS

DNA Sequence Analysis. DNA sequencing was performed by the dideoxy chain-termination method (15) using the deletion subcloning procedure of Dale *et al.* (16). A total of 44 M13 phages containing deletions of the *STE12* gene provided all of the sequence information of Fig. 1 for both strands except for 200 bases on one strand. This last region was sequenced by using a specific 16-mer oligonucleotide primer.

Strains and Plasmids. The **a** STE$^+$ yeast strain was EG123 (*MAT***a** *trp1 leu2 ura3 his4*) (17), and the **a** *ste12* strain was the isogenic derivative SF167-5a (7). The *MFA1* gene was contained on plasmid pSM18, which is pUC18 containing the 1.6-kilobase *Eco*RI/*Xba* I fragment (18). The *MFA2* gene was initially contained on plasmid pSM29 (18). The 209-base-pair (bp) region (see Fig. 2, fragment D) between the 5′ *Eco*RI site and an *Rsa* I site was subcloned into pUC18 to yield pRR6. Fragment E derives from an M13 clone containing base pairs 172–263 of the *MFA2* gene. pJD12 was constructed by ligating the *STE12* gene from the *Hin*cII site (nucleotide 257) to an *Eco*RI site in vector sequences downstream of the termination codon to the T7 φ10 promoter (19). pSY1 was generated by ligating the 5.5-kilobase *Cla* I fragment (using adjacent *Hin*dIII sites) containing the *STE12* gene (20) into YEp13 (21) cut with *Hin*dIII. pYF1 is essentially pSC4 (20) with the *Escherichia coli lacZ* gene inserted at the *Sac* I site near the end of the *STE12* coding sequence. pIR1 is pUC18 containing two copies of the pheromone induction element:

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Abbreviations: PRTF, pheromone/receptor transcription factor; GRM, general regulator of mating type.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M24502).

5′ GTCGACCATTTGAAACAAATGTCGACCATT-TGAAACAAATGTCGAC 3′.

DNA-Binding Assays. STE12-specific mRNA was prepared by *in vitro* transcription (22) of pJD12 DNA that had been linearized by restriction digestion. The RNA was purified by extraction with phenol and with chloroform followed by precipitation with ethanol. RNA (1.5–3.0 μg) was translated in a rabbit reticulocyte lysate (BRL) with Tran^{35}S-label (ICN) in 30-μl reaction volumes. Several parallel reaction mixtures were incubated at 30°C for 60 min, pooled, and then fractionated by adding saturated $(NH_4)_2SO_4$ to 40% saturation, which quantitatively precipitated the STE12 protein. The precipitated protein was then dissolved in 5 μl (per translation) of protein storage buffer [20 mM Hepes (pH 8.0), 5 mM EDTA, 7 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 20% (vol/vol) glycerol]. The ability of this protein to bind to DNA fragments was determined by a gel mobility shift assay (23). Each reaction contained 3 μl of $(NH_4)_2SO_4$-fractionated translation lysate, 1 μg of poly(dI-dC)·poly(dI-dC) (Pharmacia), various DNA fragments as indicated in Fig. 3, and binding buffer (24) in a total volume of 20 μl. The reaction mixtures were incubated at 30°C for 10 min and then fractionated by electrophoresis through a 4.5% nondenaturing polyacrylamide gel in 89 mM Tris/89 mM borate/2.5 mM EDTA at 150 V for 2.5 hr. The gels were fixed in 7.5% acetic acid, soaked in 1 M sodium salicylate (pH 7.0), dried, and fluorographed. Yeast extracts were prepared according to the method of Pfeifer *et al.* (24). DNA-binding reaction mixtures contained binding buffer, 2 μg of yeast extract, 1 μg of poly(dI-dC)·poly(dI-dC), and 0.1 ng of labeled gel-purified DNA fragment in a total volume of 20 μl. The reactions were incubated and subjected to electrophoresis as above, and the gels were dried and autoradiographed. For immunoprecipitation, extracts were prepared from *ste12* cells expressing the *STE12/lacZ* fusion. Binding reaction mixtures (50 μl) contained binding buffer, 5 μg of extract, 3 μg of poly(dI-dC)·poly(dI-dC), and 0.2 ng of labeled *Eco*RI/*Hin*fI fragments of pRR6. Reaction mixtures were incubated (10 min, 30°C), serum was added (10 min, 30°C), and IgSorb (The Enzyme Center, Malden, MA) was added (15 min, room temperature). Reaction mixtures were centrifuged, washed twice with 200 μl of 25 mM Tris Cl (pH 8), 150 mM NaCl, and 0.1% Nonidet P-40, and resuspended in 50 μl of 10 mM Tris Cl (pH 8), 25 mM NaCl, and 0.1% SDS. The samples were extracted with phenol, ethanol-precipitated, and separated on a 6% polyacrylamide gel.

RESULTS

Sequence of the *STE12* Gene. Complementation analysis of *ste12* mutants with cloned DNA fragments had indicated that a 2.5-kilobase *Sac* I fragment of yeast DNA could restore mating ability, albeit at reduced levels (20). This fragment was sequenced on both strands and translated in all possible reading frames. A single large open reading frame was present, with the first methionine 451 nucleotides downstream of one *Sac* I site and no termination codon before the distal *Sac* I site. Additional sequence was determined beyond this site, which predicted 19 more codons in this open reading frame before reaching a termination codon (Fig. 1). In the region upstream of the initiator ATG, no exact match is found to the sequence TATAAA, although numerous A+T-rich sequences are present, which may function as the TATA element. Two copies of the pheromone induction sequence, ATGAAACA and GTGAAACA, are found between nucleotides 16 and 64, with other related sequences also present within the upstream region, suggesting that transcription of the *STE12* gene may be induced by pheromone. Downstream of the termination codon, the sequence TAG . . . TATGT . . . TTT, implicated in transcription termination (25), is found between nucleotides 2770 and 2806.

The open reading frame predicts a hydrophilic protein of 688 amino acids and molecular mass of 77,800 Da (Fig. 1). The protein contains 12% acidic and 12% basic residues and

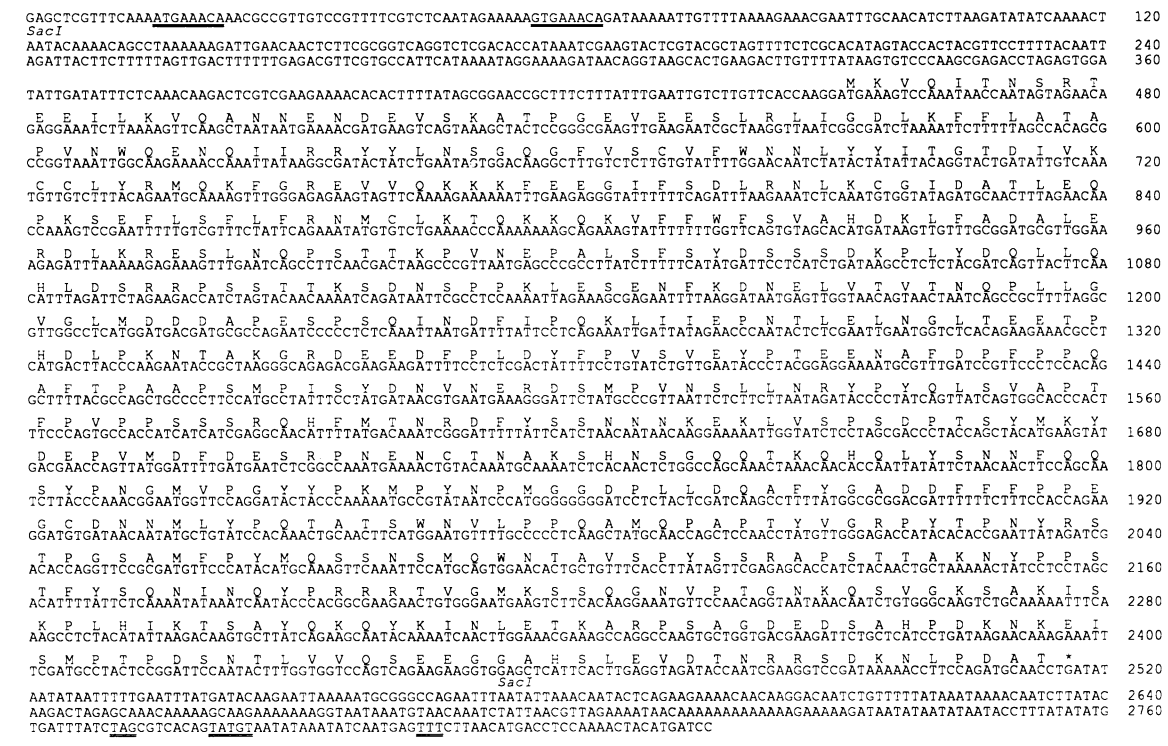

FIG. 1. Sequence of the *STE12* gene. The DNA sequence of the *STE12* gene and the deduced amino acid sequence are shown beginning with a *Sac* I site and continuing beyond the end of the open reading frame. The positions of the *Sac* I sites are shown. Underlined nucleotides in the 5′ upstream region represent matches to the pheromone induction consensus sequence, and underlined nucleotides in the 3′ downstream region represent matches to sequences implicated in transcription termination. *, termination codon. Amino acids are identified by the single-letter code.

has a high proline content (65 residues), but otherwise has no unusual features. Homology search using the FASTP program of Lipman and Pearson (26) did not detect any significant homology to proteins in the Protein Identification Resource (Natl. Biomed. Res. Found., Release 19.0). The predicted STE12 protein does not have an obvious homeodomain or "zinc finger" domain.

STE12 Protein Synthesized *in Vitro* Binds to DNA. We synthesized labeled STE12 protein by *in vitro* transcription of the *STE12* gene followed by translation of the mRNA in a rabbit reticulocyte lysate with ^{35}S-labeled amino acids. We then attempted to determine whether this protein is capable of sequence-specific DNA binding, by using as target substrates the two **a**-factor genes, *MFA1* and *MFA2*. Both of these genes are transcribed only in **a** cells, and their transcription shows a strong requirement for the *STE12* gene product (27). These genes have been isolated (Brake *et al.*, pp. 103–108 in ref. 28; ref. 18) and their sequences have been determined (A. Brake, personal communication). The *MFA1* and *MFA2* upstream regions and fragments used in DNA-binding assays are diagrammed in Fig. 2.

In Fig. 3, we provide evidence that STE12 protein synthesized *in vitro* binds specifically to fragments of the *MFA1* and *MFA2* genes. Unlabeled DNA fragments and ^{35}S-labeled protein were incubated and then fractionated by nondenaturing polyacrylamide gel electrophoresis (23). In this assay, the labeled protein remains at the origin of the gel unless it is bound to DNA, in which case it migrates much more rapidly. As expected, the labeled STE12 protein alone stays at the origin (Fig. 3, lane a) and does not change migration when incubated with *Hin*fI fragments of vector pUC18 (Fig 3, lane b). Upon incubation of the labeled STE12 protein with *Hin*fI-digested pSM18, which is pUC18 carrying the *MFA1* gene, a new band is apparent (Fig. 3, lane c). Work with yeast extracts (below) suggests that this complex contains fragment A (see Fig. 2) from the upstream region of the *MFA1* gene. Incubation of STE12 protein with a gel-purified fragment from the upstream region of the *MFA2* gene (Fig. 2, fragment C) results in the appearance of a different faster migrating

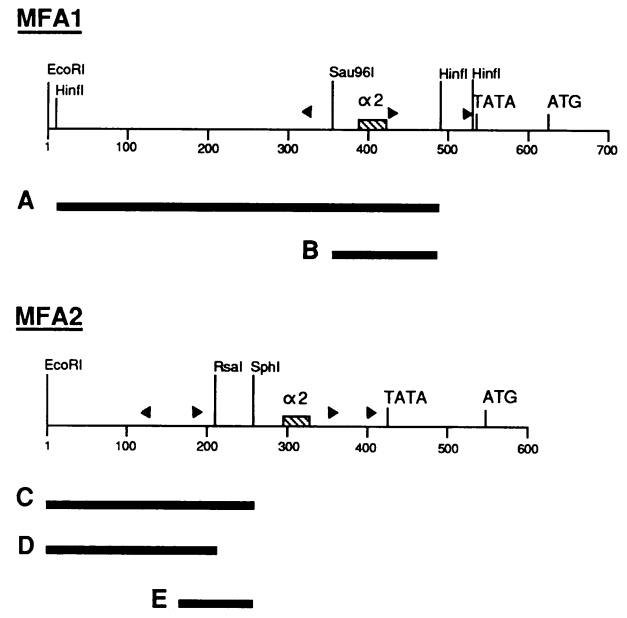

FIG. 2. Maps of DNA fragments used in gel mobility shift assays. The *MFA1* and *MFA2* restriction maps are from DNA sequences provided by A. Brake (personal communication), with base 1 in each case corresponding to an upstream *Eco*RI site. TATA, TATAAA sequence; ATG, translation start site; striped box (α2), binding site for α2 and PRTF/GRM; solid bars, DNA fragments used in gel shift assays; arrowhead, pheromone induction element.

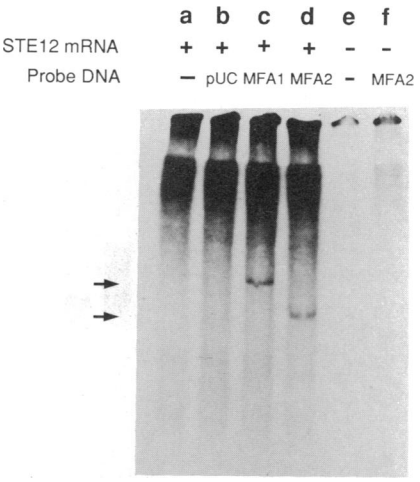

FIG. 3. *In vitro*-synthesized STE12 protein binds DNA in a sequence-specific manner. Labeled STE12 protein (+) or translation products from a lysate that had no added mRNA (−) were incubated with various DNA fragments and then fractionated by nondenaturing polyacrylamide gel electrophoresis. Probe DNA: −, no DNA fragments; pUC, addition of 1 μg of *Hin*fI-digested pUC18; MFA1, addition of 1 μg of *Hin*fI-digested pSM18; MFA2, addition of 50 ng of gel-purified fragment C from the *MFA2* gene. The arrows indicate the positions of the STE12 protein–DNA complexes.

species (Fig. 3, lane d). Translations that do not contain *STE12* templates show no fast-migrating species, either in the absence (Fig. 3, lane e) or presence (Fig. 3, lane f) of specific DNA fragments. Although this experiment demonstrates that the STE12 protein is capable of binding to specific sequences within the two **a**-factor genes, the binding reactions with *in vitro*-synthesized STE12 protein showed considerable variation with respect to the amount of binding activity. Under no conditions did more than a small percentage of the labeled protein form a detectable complex with DNA, suggesting that the STE12 protein may be modified in yeast to a form that binds more actively or that it may bind cooperatively with another yeast protein.

The STE12-Dependent Binding Site Corresponds to the Pheromone Induction Element. To determine whether the **a**-factor gene sequences could form a STE12-dependent complex with yeast proteins, we used gel mobility shift assays with labeled DNA and unlabeled proteins (29, 30). We prepared extracts from the following strains: **a** STE$^+$, **a** *ste12*, and **a** STE$^+$ transformed with pSY1, a high copy plasmid carrying the *STE12* gene, and we tested fragments from the *MFA1* and *MFA2* upstream regions. For the *MFA1* region, the most prominent STE12-dependent complex formed with the *Hin*fI/*Sau*96I fragment B, a subfragment of the *Hin*fI fragment A. Comparison of the complexes for this fragment (Fig. 4*A*) shows a slowly migrating species that is readily detectable in the extract from the pSY1 transformant, not present in the extract from the *ste12* strain, and detectable at a low level in the extract from the wild-type strain. A STE12-independent complex is also apparent, which migrates faster than the STE12-dependent complex and is present in all extracts.

For the *MFA2* gene, we assayed subfragments of fragment C, which was shown to form a complex with the *in vitro* synthesized STE12 protein. Both fragment C (data not shown) and fragment D (Fig. 4*B*) produce two complexes with the extract from the pSY1 transformant that are not present with the *ste12* extract. To further delineate the binding site, we assayed fragment E, which overlaps the 3′ 38 bp of fragment D. This fragment shows a single STE12-dependent complex (Fig. 4*C*). The presence of two STE12-dependent complexes with the larger fragment D suggests

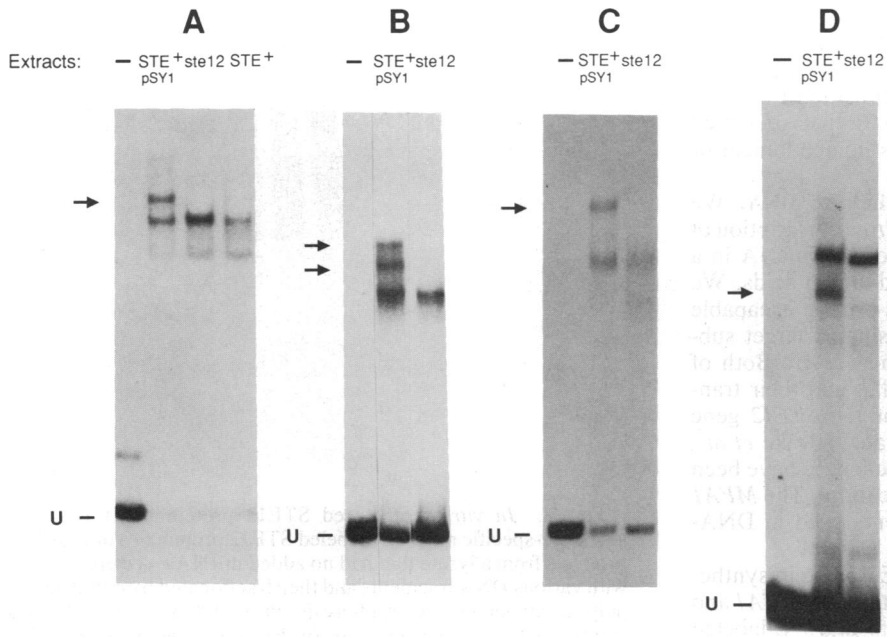

FIG. 4. STE12-dependent complexes formed with yeast extracts. Labeled DNA fragments were incubated with yeast extracts and fractionated by nondenaturing polyacrylamide gel electrophoresis. (*A*) The probe was fragment B (*MFA1* gene). (*B*) The probe was fragment D (*MFA2* gene). (*C*) The probe was fragment E (*MFA2* gene). (*D*) The probe was two copies of the induction element (from pIR1). Extracts: −, no extract; STE$^+$, pSY1, extract from a wild-type **a** strain carrying plasmid pSY1, which contains the *STE12* gene; ste12, extract from an **a** *ste12* mutant strain; STE$^+$, extract from a wild-type **a** strain. Arrows indicate the positions of STE12-dependent protein–DNA complexes; U, indicates the position of the unbound probe.

that there are likely to be two binding sites within this fragment. The slower migrating species would then represent occupancy of both sites, and the faster migrating species would represent occupancy of either site. Furthermore, one of these sites can be localized to the 38-bp overlap between fragments D and E. In addition to the STE12-dependent complexes, fragments D and E both produce a STE12-independent complex.

Examination of the sequences of fragments B, D, and E indicated that a 7 out of 8 base match to the pheromone induction element ATGAAACA is present twice within the D fragment (including once within the overlap with fragment E) and once within fragment B. We synthesized the 26-base oligonucleotide, 5′ GTCGACCATTTGAAACAAATGTCGAC 3′, and its complement. This oligonucleotide corresponds to 14 bases of fragment E, containing at their core the pheromone induction element (underlined), flanked by 6-nucleotide *Sal* I sites. The additional 3 bases of *MFA2* sequence on each side of the TTGAAACA element are not conserved among the *MFA1* and *MFA2* pheromone induction elements nor among other copies of this element within other genes (see ref. 10). We cloned into the pUC18 polylinker two copies of this oligonucleotide in a head-to-tail orientation, separated by the 6-nucleotide *Sal* I site. Digestion of this plasmid (pIR1) with *Eco*RI and *Hin*dIII yielded as probe the dimer sequence flanked by polylinker sequence. In a gel mobility shift assay, a single STE12-dependent complex is formed (Fig. 4*D*), suggesting that, in the absence of additional yeast sequences, two copies of the induction element can constitute a binding site.

To demonstrate that the STE12 protein is actually present in a STE12-dependent protein–DNA complex, we made use of a STE12/β-galactosidase fusion, which contains almost all of the STE12 protein sequence and which complements a *ste12* mutation (data not shown). An extract prepared from the **a** *ste12* strain carrying this fusion was incubated with a labeled digest of the plasmid carrying fragment D of the *MFA2* gene. Antibody to β-galactosidase was then used to immunoprecipitate the fusion, as well as any DNA fragments specifically bound to it (31). As shown in Fig. 5, the 230-bp *MFA2* fragment (containing fragment D plus polylinker sequence) is specifically precipitated, and this precipitation requires the presence of both the STE12/β-galactosidase fusion and antibody against β-galactosidase.

DISCUSSION

The transcriptional regulation that allows yeast **a**, α, and **a**/α cells to produce distinct sets of proteins is based on the combinatorial control of DNA-binding proteins encoded at the mating type locus and elsewhere in the genome. We sequenced the *STE12* gene, a component of this regulatory network, and demonstrated that the STE12 protein, when synthesized *in vitro*, is capable of binding specifically to sequences found in two STE12-responsive genes. In addition, extracts from wild-type yeast, but not a *ste12* mutant, are capable of forming complexes with DNA fragments containing these same sequences. The amount of these complexes is enhanced when extracts are prepared from a strain carrying the *STE12* gene on a high copy plasmid. We demonstrated directly that the STE12 protein from yeast can bind specifically to DNA by using a STE12/β-galactosidase fusion. This fusion selectively binds a fragment of the *MFA2* gene, which can be immunoprecipitated by antibody to β-galactosidase. The STE12 binding site corresponds to the pheromone induction element, (A)TGAAACA, identified by Kronstad *et al.* (10) and Van Arsdell and Thorner (11). We showed that a fragment containing essentially only two copies of this element is capable of forming a STE12-dependent complex, indicating that the element represents the minimal amount of sequence information necessary for such a complex.

Our identification of the STE12-binding site in two **a**-specific genes allows us to compare this site to sequences in STE12-dependent or pheromone-induced genes. Sites in the **a**-factor fragments that we have analyzed have a seven out of eight base match to the ATGAAACA consensus sequence (*MFA1*, ATGAAACC; *MFA2*, ATGAAACT and TTGAAACA). A Ty1 fragment of 40-bp, which confers dependence on *STE7* and *STE12* for transcription (8), contains the sequences CTGAAACG and CCAAAACA and has been shown to bind the STE12 protein (B. Errede and G. Ammerer, personal communication); however, it is not known whether either of these short sequences themselves are the binding site. For the *STE3* gene, a 43-bp fragment has been shown to be sufficient for α-specific transcription and **a**-factor induction (32). This fragment binds PRTF and α1 and contains two matches (six out of eight bases) to the ATGAAACA consensus sequence. However, STE12 binding to these sequences has not been demonstrated. This possible clustering of bind-

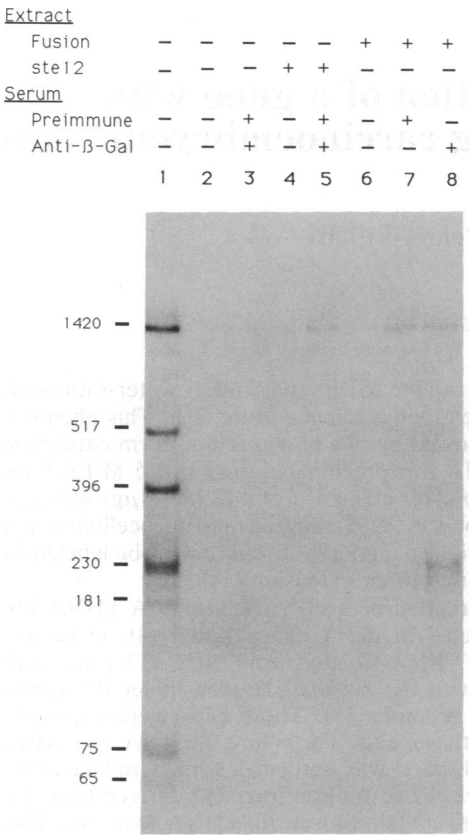

FIG. 5. Immunoprecipitation of DNA bound to the STE12/β-galactosidase fusion protein. Fusion, extract from *ste12* cells expressing STE12/β-galactosidase fusion protein; ste12, extract from *ste12* cells; preimmune, normal rabbit serum; anti-β-gal, anti-β-galactosidase antiserum. Lane 1 contains an aliquot of labeled DNA equal to 0.5% of the input DNA in each reaction. Lanes 2 and 3, no extract; lanes 4 and 5, *ste12* extract; lanes 6–8, fusion protein extract. Lanes 2, 4, and 6 received no serum; lanes 3 and 5 received both preimmune and anti-β-galactosidase antiserum; lane 7 received preimmune serum; and lane 8 received anti-β-galactosidase antiserum. Numbers on the left indicate size (in bp) of the input restriction fragments.

ing sites for different transcription factors suggests that STE12 protein may be binding cooperatively at these sequences with other proteins.

The *STE12* gene product is required for full constitutive transcription of the **a** and α-specific genes (7), suggesting that the STE12-responsive element has properties of a constitutive upstream activation site (UAS) and is not simply active in the presence of the appropriate pheromone. Analysis of the *BAR1* upstream region has shown that deletion of one pheromone induction element reduced constitutive expression to 37% of wild-type levels (10). This result indicates that the pheromone induction element can contribute to both constitutive and induced levels of transcription, with constitutive transcription possibly resulting from a pheromone-independent low level activity of the pheromone response pathway (see ref. 33). The requirement for the STE12 protein in constitutive transcription differs from gene to gene (27), as does the relative amount of transcriptional induction observed on exposure of cells to pheromone (see, for example, ref. 12). These differences may be due to such variables as the number, orientation, and location of STE12 binding sites. Clearly this binding site is not responsible for all UAS activity of STE12-responsive genes. For **a**- and α-specific genes, the total UAS activity may be due to a combination of different sequence elements and be dependent on other proteins such as PRTF/GRM and α1 (4, 10, 32). The mechanism by which the STE12 protein and MAT/PRTF (GRM) proteins act together and with the transcriptional machinery remains to be determined.

The effect of pheromone treatment on the STE12 protein is unknown. It is possible that binding of α-factor or **a**-factor to receptors on the yeast cell surface leads to phosphorylation of the STE12 protein, perhaps mediated by the *STE7* or *STE11* products. For the yeast GAL4 protein, increasing the overall negative charge of a domain required for transcriptional activation leads to increased levels of transcription (34). Thus the increased negative charge resulting from phosphorylation of the STE12 protein might lead to more efficient transcriptional activation.

Our demonstration that the *STE12* gene encodes a DNA-binding protein and is necessary for formation of a complex with the pheromone induction element suggests that the STE12 protein may be one of the targets in the cell's response to α-factor or **a**-factor. This finding may allow the further elucidation of the components of this pathway and the mechanism by which it functions.

We thank Susan Michaelis for the **a**-factor plasmids and Tony Brake for the **a**-factor sequences. We are grateful to Ira Herskowitz, Beth Grayhack, and Rolf Sternglanz for comments on the manuscript and to Anne Ostermeyer for technical assistance. This work was supported by Grant DCB 8601949 from the National Science Foundation and Public Health Service Grant 5 T32 CA09176 from the National Cancer Institute.

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