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The Yeast α2 and Mcm1 Proteins Interact through a Region Similar to a Motif Found in Homeodomain Proteins of Higher Eukaryotes

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Homeodomain proteins are transcriptional regulatory factors that, in general, bind DNA with relatively low sequence specificity and affinity. One mechanism homeodomain proteins use to increase their biological specificity is through interactions with other DNA-binding proteins. We have examined how the yeast (*Saccharomyces cerevisiae*) homeodomain protein α2 specifically interacts with Mcm1, a MADS box protein, to bind DNA specifically and repress transcription. A patch of predominantly hydrophobic residues within a region preceding the homeodomain of α2 has been identified that specifies direct interaction with Mcm1 in the absence of DNA. This hydrophobic patch is required for cooperative DNA binding with Mcm1 in vitro and for transcriptional repression in vivo. We have also found that a conserved motif, termed YPWM, frequently found in homeodomain proteins of insects and mammals, partially functions in place of the patch in α2 to interact with Mcm1. These findings suggest that homeodomain proteins from diverse organisms may use analogous interaction motifs to associate with other proteins to achieve high levels of DNA binding affinity and specificity.

Homeodomain proteins comprise a large family of sequence-specific DNA-binding transcription factors that regulate key developmental and cellular processes in organisms ranging from *Saccharomyces cerevisiae* to humans (14, 32). In vitro studies indicate that some homeodomain proteins recognize families of target sequences, suggesting that these proteins bind DNA with relatively low sequence specificity (2, 9, 13, 20, 37). This apparent lack of target specificity of homeodomain proteins has been difficult to reconcile with their proposed roles as regulators of development, which requires precise temporal and spatial gene expression. One explanation for this discrepancy is that in vivo homeodomain proteins achieve their DNA binding specificity, at least in part, through associations with other transcription factors (5, 11, 15, 26, 48). Consequently, protein-protein interactions serve important roles in directing homeodomain proteins to their proper gene targets.

A model for understanding how protein-protein interactions affect the target specificity of a homeodomain protein is provided by the transcriptional regulators that determine the mating type in the yeast *Saccharomyces cerevisiae*. In this system, α2, a homeodomain protein, functions as a repressor to turn off transcription of two distinct sets of cell-type-specific genes (19, 22). In haploid α cells, α2 combines with Mcm1, a MADS box protein, to bind to one set of target sites, repressing transcription of **a**-specific genes (*asg*) (27). In diploid **a**/α cells, α2 associates with **a**1, another homeodomain protein, to bind to a different set of sites, repressing transcription of haploid-specific genes (*hsg*) (11, 15). Although α2 binds to its target site on its own in vitro, it must associate with **a**1 or Mcm1 to repress transcription in vivo.

The α2 and Mcm1 proteins bind as a heterotetramer to a partially symmetric 31-bp site located in the promoters of **a**-specific genes (23, 27, 43). Mcm1 binds as a dimer in the center of the site to the sequence $CC(A/T)_6GG$ (33). The α2 protein binds cooperatively as a dimer to sequences flanking the Mcm1 site. Although the homeodomain of each α2 monomer binds specifically to symmetric TGTA sequences in each half site, it cannot discriminate among operator sites with altered spacing between the two half sites (46). α2 binds to these altered sites with wild-type affinity in vitro; however, these sites do not function as repression sites in vivo. It has therefore been suggested that one role of Mcm1 is to increase the sequence specificity of α2 by requiring specific spacing and orientation of the α2 and Mcm1 recognition sites. A second role for Mcm1 is to increase the binding affinity of α2. Although both α2 and Mcm1 can bind to the operator site on their own, together they interact with an apparent 500-fold increase in DNA binding affinity, indicating that there are strong cooperative interactions between the proteins (27).

The α2 protein contains two stable domains: an amino-terminal dimerization domain that interacts with Tup1 and is required for repression and a carboxy-terminal homeodomain that binds DNA (18, 28, 43). Deletion experiments demonstrate that a short, 20-amino-acid linker region connecting these domains is required for interaction of α2 with Mcm1 in vitro (51). This region does not appear to be involved in DNA binding or interaction with **a**1, since its removal does not affect the ability of the protein to bind to DNA on its own or to bind cooperatively with **a**1 in vitro (16, 43). The linker region is sufficient for interaction with Mcm1, since it can be grafted onto the amino terminus of the *Drosophila* engrailed homeodomain and the resulting fusion protein binds cooperatively with Mcm1 (51). These experiments therefore define a small, modular region in α2 that is required and sufficient for cooperative DNA binding with Mcm1.

In this paper, we demonstrate that α2 and Mcm1 proteins interact directly, in the absence of DNA. This interaction is dependent on a hydrophobic patch in the α2 linker region that is also required for cooperative DNA binding and transcriptional repression with Mcm1. Finally, we demonstrate that the association of α2 with Mcm1 requires a specific amino acid sequence in the linker region and that this protein-protein

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interaction motif may be similar to interaction motifs found in homeodomain proteins of higher eukaryotes.

MATERIALS AND METHODS

Yeast strains, media, and β-galactosidase assays. The methods used for measuring β-galactosidase activity were described previously (26). The *CYC1-lacZ* reporter assays of the altered *asg* operator sites were performed with the yeast *MAT*α strain AJ82 (α *trp1 leu2 ura3 his4*). The α2 mutants were assayed for α2-Mcm1 repression in the *mat*Δ strain, which contains an integrated copy of a *CYC1-lacZ* fusion with an α2-Mcm1 site (30). Repression with **a**1 was assayed with a *MAT***a** strain which contains an integrated copy of a *CYC1-lacZ* fusion gene with an **a**1-α2 site. For each mutation, the β-galactosidase values of three independent transformants were measured, and the values varied by less than 10%.

Plasmids. pJM130 contains the engineered α2 gene with the following silent and unique restriction sites starting at the beginning of the gene: *Pst*I (nucleotide 330), *Bcl*I (nucleotide 370), *Msc*I (nucleotide 400), *Nru*I (nucleotide 445), *Stu*I (nucleotide 480), *Bam*HI (nucleotide 520), and *Nhe*I (nucleotide 590). Specific silent codon changes were made to maintain approximately the same level of codon usage, and sites were chosen to provide unique restriction sites every 30 to 70 bp. A complete sequence of all changes that were made is available upon request. Unique restriction sites were introduced into the linker region, homeodomain, and carboxy-terminal tail of the α2 gene by recursive PCR (39). pJM130 is a derivative of pAV115 (30), a *CEN LEU2* yeast plasmid with a 4.3-kb *Hin*dIII fragment that contains the entire *MAT*α locus.

pTBA23 (2μm *URA3* Amp^r) contains a *CYC1-lacZ* fusion vector and was constructed by insertion of a *Bgl*II linker into the unique *Xho*I site of pAV73 (49). The promoter reporter plasmids containing the different α2-Mcm1 DNA-binding sites were constructed by inserting double-stranded oligonucleotides into the *Xho*I site between the TATA box and the UAS site of pTBA23. pHZ13 contains the *STE6* operator (5′-TCGACATGTAATACTAATAGGGAAATT TACACG-3′), and pJM120 contains a consensus symmetric α2-Mcm1 DNA-binding site (5′-TCGACATGTAATTACCTAATTAGGTAATACATG-3′). Reporter plasmids pJM122 and pHZ3 contain the consensus symmetric operator with 1-bp (CS + 1) and 2-bp (CS + 2) insertions between the α2 half sites and the Mcm1 site, respectively (5′-TCGACATGTAATTAACCTAATTAGGTTA ATTACATG-3′ and 5′-TCGACATGTAATTATACCTAATTAGGTATAATT ACATG-3′ [inserted bases are underlined]). pHZ69 contains the operator with a 1-bp deletion, 3,3 (CS − 1), between the α2 half sites and the Mcm1 site (TCGACATGTAATTCCTAATTAGGAATTACATG).

The α2 expression vector, pJM163, has a T7 promoter driving expression of the engineered full-length α2 gene with six His residues fused in frame at the N terminus. pJM163 was constructed by PCR amplification of pJM130 (engineered α2 vector) with a 5′ primer containing an *Nde*I site and six His residues and a 3′ primer containing a *Xho*I site. The amplified fragment was cloned into the pET21a vector (Novagen).

The Mcm1 expression vector, pTBA25, has a P_{tac} promoter driving the expression of the fusion protein MBP (maltose binding protein)-$Mcm1_{1–96}$. This vector was constructed by insertion of a PCR-generated fragment encoding the sequence of Mcm1 residues 1 to 96 into a derivative of pMAL-c2 (New England Biolabs). The region between MBP and Mcm1 contains thrombin and factor X cleavage sites. To generate the expression vector (pJM206) for the hemagglutinin (HA)-tagged Mcm1 protein, three copies of the HA epitope were introduced into the *Bam*HI site of pTBA25. All constructs were verified by restriction digestion and complete DNA sequencing of the genes to ensure that no secondary mutations were introduced during the cloning steps.

α2 mutants. To make the alanine point mutations in α2, complementary oligonucleotides with either *Pst*I-*Bcl*I or *Bcl*I-*Msc*I compatible ends containing the alanine codon GCA in place of residues 112 to 131 in the linker region of α2 were annealed, phosphorylated, and cloned into the appropriate sites of pJM130. For the randomization of the residues within the hydrophobic patch region, an oligonucleotide extending from *Pst*I-*Msc*I sites containing either NN(G,C) codons for completely randomized residues or NT(G,C) for randomized hydrophobic codons at target positions was annealed with a short compatible primer that hybridizes to one end of the randomized oligonucleotide. The complementary strand was synthesized with Klenow polymerase, and the double-stranded oligonucleotides containing the random substitutions were digested with *Pst*I and *Bcl*I and cloned into pJM130.

Protein purification and DNA binding assays. Full-length wild-type and mutant α2 proteins containing the six-His fusion at the N terminus were expressed in bacteria and purified by Ni^{2+} affinity chromatography (Novagen). Briefly, pJM163 was expressed in BL21(DE3)/pLysS cells, induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at an optical density of 0.7 at 600 nm, and harvested 3 h later. Cells were sonicated in α2 lysis buffer (100 mM Tris [pH 8.0], 0.1 mM EDTA, 1 mM 2-mercaptoethanol, 500 mM NaCl, 2 M urea, 0.1% [vol/vol] Nonidet P-40). The cleared lysate was added to the Ni^{2+} column in binding buffer containing 5 mM imidazole, washed with 60 mM imidazole, and eluted in 1 M imidazole. Purified wild-type and mutant α2 proteins were dialyzed in S + 500 buffer (500 mM NaCl, 50 mM Tris [pH 7.4], 10 mM 2-mercaptoethanol, 1 mM EDTA) with two buffer changes, and the protein concentrations were determined as described previously (50). Each preparation was >90% homogeneous as assayed for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

$Mcm1_{1–96}$ and HA-Mcm1 proteins were purified from bacteria that were transformed with pTBA25 or pJM206, grown in ampicillin-supplemented Luria broth, and induced with 0.5 mM IPTG at an optical density of ~0.7 at 600 nm. Three hours after induction, cells were harvested and sonicated in column buffer (20 mM Tris [pH 7.5], 200 mM NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol). Amylose resin (New England Biolabs) affinity chromatography was used to isolate ~90% pure fusion protein. The Mcm1 proteins were separated from the MBP by cleavage with thrombin protease (Sigma) for 48 h at 4°C with addition of $CaCl_2$ to a final concentration of 2 mM. The reaction mixture was then loaded onto a heparin-Sepharose (Pharmacia) column, and Mcm1 protein was eluted with a gradient from 0.2 to 1.0 M NaCl. The proteins isolated consisted of two nonnative amino acid residues at the N terminus, Gly-1–Ser-2, followed by Mcm1 residues 1 to 96, and were >95% homogeneous as assayed by SDS-PAGE.

DNA binding assays were performed as described previously (50). The labeled wild-type *STE6* operator fragment used as a probe in the mobility shift experiments was generated by isolating the 86-bp fragment from an *Eco*RI and *Hin*dIII restriction digest of pCK1 (26) and filling in the 5′ overhangs with ^{32}P by using Klenow polymerase. Mobility shift gels were exposed to a phosphor screen and scanned and quantitated on a model 425E Molecular Dynamics PhosphorImager. The binding affinity for each α2 mutant was determined by measuring the percentage of the total probe that was bound in the α2-Mcm1 complex. The percentage bound was determined for several different concentrations of each protein, and these values were compared with the percentage bound by the wild-type protein at the same concentration.

Coimmunoprecipitation assays. For coimmunoprecipitation assays, wild-type and mutant α2 proteins (900 ng) were incubated with HA-Mcm1 (350 ng) in 100 μl of gel shift assay buffer (20 mM Tris [pH 7.5], 0.1 mM EDTA, 5 mM $MgCl_2$, 10 mg of bovine serum albumin [BSA; fraction V] per ml, 5% glycerol, 0.1% Nonidet P-40, 10 μg of sheared salmon sperm DNA per ml) with 1 μg of anti-HA antibody (12CA5; Boehringer Mannheim Corporation) and 1 μg of DNA (annealed oligonucleotides containing the α2-Mcm1 consensus symmetric DNA-binding site) for 3 h. Fifty microliters of a 20% protein A-Sepharose suspension (Sigma) was added, and the reaction mixtures were rocked overnight at 4°C. Precipitated complexes were washed seven times with assay buffer without BSA, electrophoresed on an SDS–15% polyacrylamide gel, and transferred (30 V overnight) to nitrocellulose. The blot was blocked with 10% powdered milk in 1× TBS (0.2 M Tris [pH 7.5], 2.5 M NaCl), washed in 1× TBS, and hybridized with an anti-rabbit polyclonal α2 antibody and goat anti-rabbit immunoglobulin G horseradish peroxidase-conjugated secondary antibody (Bio-Rad). Coimmunoprecipitation experiments were also performed in the absence of salmon sperm DNA with similar results.

RESULTS

Alanine scanning reveals a hydrophobic patch within the linker of α2 that is required for interaction with Mcm1. To analyze the protein-protein interactions between α2 and Mcm1, we made substitutions in the α2 linker region and tested their effects on α2-Mcm1 repression. We constructed a wild-type α2 gene that contains unique restriction sites for introduction of site-specific and randomized mutations. These α2 mutant genes were transformed into yeast cells for analysis of transcriptional repression in vivo (Fig. 1). α2 mutants were expressed in a *mat*Δ strain from the native α2 promoter on the low-copy CEN plasmid, pJM130 (Fig. 1A). This strain contains an integrated *CYC1-lacZ* reporter gene that is under the control of an α2-Mcm1 (*asg*) binding site. Under nonrepressing conditions in the absence of α2, we measured approximately 150 U of β-galactosidase activity. Full repression of the reporter gene by wild-type α2 results in approximately 6 U of β-galactosidase activity, indicating that α2 represses the promoter 22-fold. To ensure that mutations in the linker region do not affect α2 expression, stability, DNA binding, or the ability to interact with **a**1, the α2 mutants were also assayed for repression with the **a**1 protein. For this assay, **a**1 is supplied from a chromosomal copy of *MAT***a** and the integrated reporter contains an **a**1-α2 (*hsg*) site (Fig. 1B). α2 expressed from the engineered gene represses transcription from the reporter promoter at the same level as wild-type α2, indicating that the codon changes we made in the gene do not affect the level of expression (data not shown).

To identify specific residues within the linker region of α2

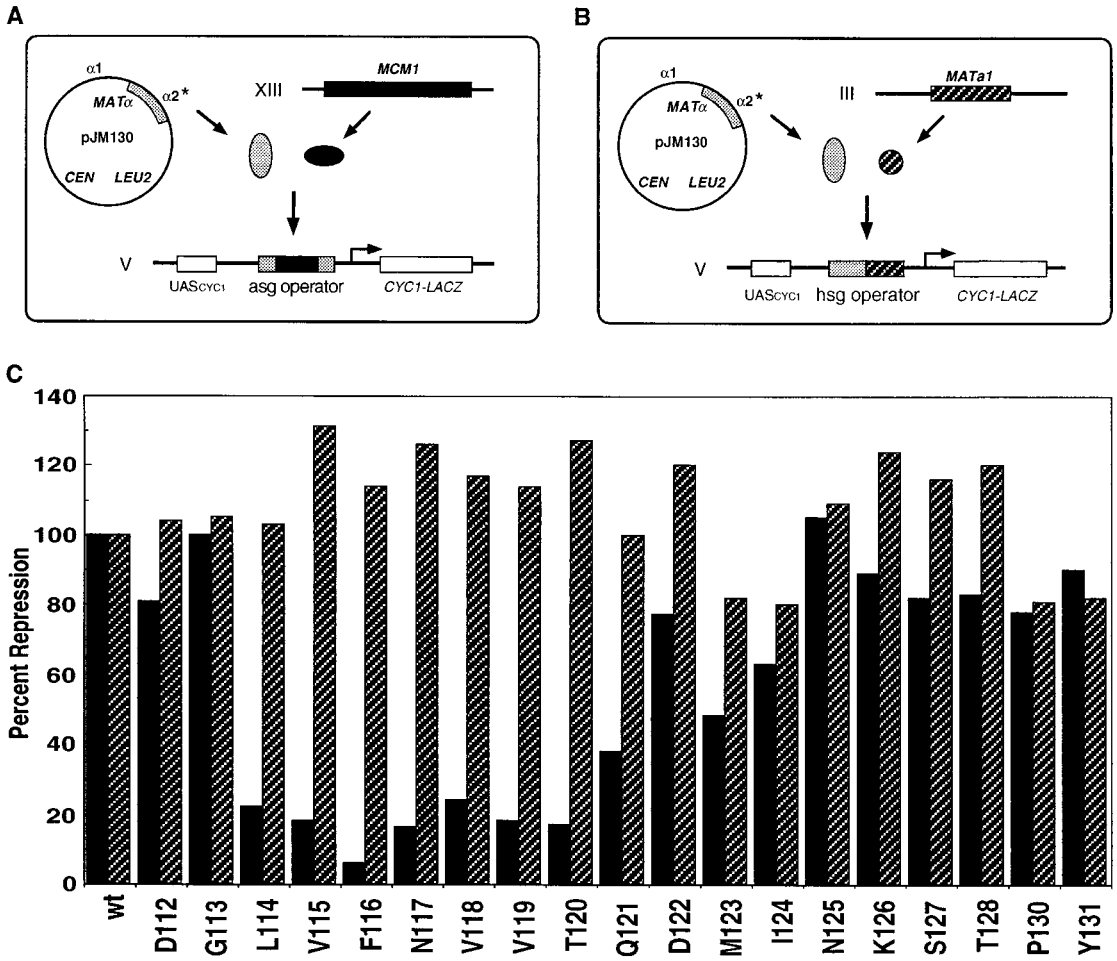

FIG. 1. Assays for transcriptional repression and alanine scanning of the α2 linker region. (A) Assay for **a**-specific gene (*asg*) repression by α2 mutants. pJM130, a *CEN LEU2* plasmid, contains the engineered wild-type (wt) α2 gene inserted into the *MAT*α fragment. Oligonucleotides with site-specific or randomized mutations were cloned into pJM130 (designated here as α2*) and transformed into a *mat*Δ strain that carries an integrated copy of the *CYC1-lacZ* gene under control of the α2-Mcm1 DNA-binding site (*asg*) as described previously (50). The Mcm1 protein is expressed from its endogenous gene on chromosome XIII. Nonrepressed conditions (tester strain transformed with pAV114, a blank control vector) produced ≈150 ± 10 U of β-galactosidase activity. Full repression of the reporter promoter (tester strain transformed with pJM130) gave an average of 6 ± 1 U of β-galactosidase activity or ≈22-fold repression of the reporter promoter. (B) Control assay for haploid-specific gene repression of α2 mutants. **a**1-α2-mediated repression by α2 mutants was measured by a similar strategy with a *MAT***a** tester strain that contains an integrated copy of the *CYC1-lacZ* promoter containing a haploid-specific (*hsg*) site. (C) Alanine scan of α2 linker region. Mutants with alanine point mutations (residues 112 to 131) were assayed for repression with Mcm1 (solid bars) and **a**1 (hatched bars). Each alanine substitution was cloned into pJM130 and transformed into either a *mat*Δ strain for Mcm1-mediated repression or a *MAT***a** strain for **a**1-mediated repression. The percentage of repression was calculated by comparison of β-galactosidase values of each of the mutants with those obtained with wild-type α2. Each bar represents the average of three transformants. For each mutation, the β-galactosidase values of the three transformants varied by less than 10%. The figure shows alanine substitutions of all positions within the linker region of α2 except for residue K-129.

that mediate cooperative interaction with Mcm1, we used a strategy called alanine-scanning mutagenesis (8). For this analysis, each amino acid of the α2 linker (residues 112 to 131) was systematically replaced by an alanine residue so that the contribution of each side chain could be determined independently (Fig. 1C). The results from these experiments reveal that alanine substitutions of amino acids 114 to 120 have the greatest effects on repression with Mcm1. The amino acids within this region are predominantly hydrophobic (LVFN VVT) and are clustered together in a patch. In contrast, alanine substitutions of the residues outside of this region have little or no effect on Mcm1-mediated repression. All mutants, including those that show weak repression with Mcm1, repress to wild-type or near-wild-type levels with **a**1. In addition, immunoblots show that these proteins are present at wild-type levels (data not shown). These results indicate that none of these substitutions dramatically affects expression or stability of the protein. These results identify a patch of hydrophobic residues within the linker region of α2 (residues 114 to 120) that is required for transcriptional repression with Mcm1.

Interaction between α2 and Mcm1 requires a specific sequence that is functionally similar to a *Drosophila* homeodomain interaction motif. Given that α2 and Mcm1 proteins interact through a hydrophobic patch, we wished to determine whether this interaction requires a specific amino acid sequence or whether random combinations of hydrophobic residues would also maintain the interaction. To test this, we made two groups of mutants. In one group (Fig. 2A, ran-11 to -14), residues of the patch were randomized for all amino acids. These mutants were compared with a second group of mutants (Fig. 2A, hran-1 to -5) in which the same residues were randomized exclusively for hydrophobic residues. Both

	α2 Mutant	Sequence	% Repression
	WT	G L V F N V V T Q	100
A	ran-11	G **R R E V S T T** Q	5
	ran-12	G **T L R E R R T** Q	6
	ran-13	G **I P K Q S W T** Q	3
	ran-14	G **Y N P R T R T** Q	3
	hran-1	G **L F L F L V M** Q	5
	hran-2	G **V L L I L L V** Q	5
	hran-3	G **L V V M I V M** Q	7
	hran-4	G **V L M M L L V** Q	5
	hran-5	G **L V V I L L V** Q	7
B	YPWM	G **Y P W M** V V T Q	25
	AAAA	G **A A A A** V V T Q	5
	ran4-1	G **F M V F** V V T Q	3
	ran4-2	G **F L V L** V V T Q	3
	ran4-3	G **L L I L** V V T Q	3
	ran4-4	G **L M M L** V V T Q	3
	ran4-5	G **I V L M** V V T Q	2
	ran4-6	G **V I V I** V V T Q	3

FIG. 2. Transcriptional repression by random patch mutants. α2 mutants were assayed for Mcm1-mediated repression as described in the legend to Fig. 1. (A) In mutants ran-11 to -14, residues 114 to 120 of the hydrophobic patch of α2 were randomized for all amino acids. In mutants hran-1 to -5, residues 114 to 120 of α2 were randomized exclusively for the hydrophobic residues F, L, I, M, and V. (B) The YPWM and AAAA mutants contain these residues in place of residues 114 to 117 (LVFN) in α2. In the ran4-1 to -6 mutants, residues 114 to 117 in α2 had random substitutions of the hydrophobic residues F, L, I, M, and V. Residues that were randomly substituted are represented by boldface letters.

groups of mutants were assayed for transcriptional repression with Mcm1 in vivo. The results from these experiments indicate that all of the random patch mutants fail to repress with Mcm1, indicating that the interaction requires a specific sequence beyond a random combination of hydrophobic residues.

It has been noted that within the amino-terminal extensions of several *Drosophila* and mammalian homeodomain proteins is a highly conserved sequence (25, 31, 52) that mediates interactions with other transcription factors (6, 24, 38). The amino acid sequence within this motif, Tyr-Pro-Trp-Met (YPWM), is also hydrophobic, and we wondered if it may function similarly to the hydrophobic patch in α2. We therefore substituted YPWM residues for residues 114 to 117 (LVFN) of the hydrophobic patch and assayed this mutant for transcriptional repression with Mcm1 (Fig. 2B). Our results indicate that the YPWM mutant has partial function, repressing transcription to 25% of wild-type levels, while a mutant containing alanine residues (AAAA) in the same positions fails to repress with Mcm1. We then compared repression of the YPWM mutant with that of mutants for which the corresponding four residues (LVFN) of α2 were randomly substituted with hydrophobic residues. We found that these random mutants (Fig. 2B), like the random patch mutants (Fig. 2A), do not repress with Mcm1. In comparison, all of the mutants repress to wild-type levels with **a**1 and are detected in Western blots (immunoblots) at the same levels as wild-type α2, indicating that these mutants are expressed at appropriate levels, bind DNA, and can function as repressors (data not shown). These results demonstrate that the interaction of α2 with Mcm1 requires specific sequence information within the patch region beyond that provided by a random combination of hydrophobic residues. The fact that we observed partial repression with a conserved hydrophobic protein-protein interaction motif (YPWM) that is located in an analogous region of *Drosophila* and mammalian homeodomain proteins suggests that, although there are likely differences in their specific interactions, the hydrophobic patch of α2 may function similarly to a protein-protein interaction motif of higher eukaryotes.

α2 linker mutants bind DNA but show decreased cooperative interactions with Mcm1. The experiments described above indicate that the hydrophobic patch in the linker region of α2 is required for repression with Mcm1. To determine whether mutations within the linker region affect DNA binding or cooperative interaction with Mcm1, full-length α2 mutants were expressed in *Escherichia coli*, purified to >90% homogeneity, and assayed for binding to a *STE6* (*asg*) site by electrophoretic mobility shift assays. Figure 3A shows DNA binding of wild-type α2 protein, two randomized patch mutants (ran-11 and ran-12), and the YPWM and F116A (change of F-116 to A) mutants to the *STE6* (*asg*) site. The DNA binding affinity of each of these α2 proteins and of all other mutants that we have tested is similar to that of wild-type α2. These results indicate that mutations within the linker region do not affect the affinity of α2 to bind DNA on its own.

Wild-type and mutant α2 proteins were also tested for cooperative binding with Mcm1 to the *STE6* site (Fig. 3B). Our results indicate that cooperative binding of the ran-11 and ran-12 mutants with Mcm1 is reduced at least 25-fold compared with that of wild-type α2. This decrease in binding is comparable to the 20-fold decrease in repression that we ob-

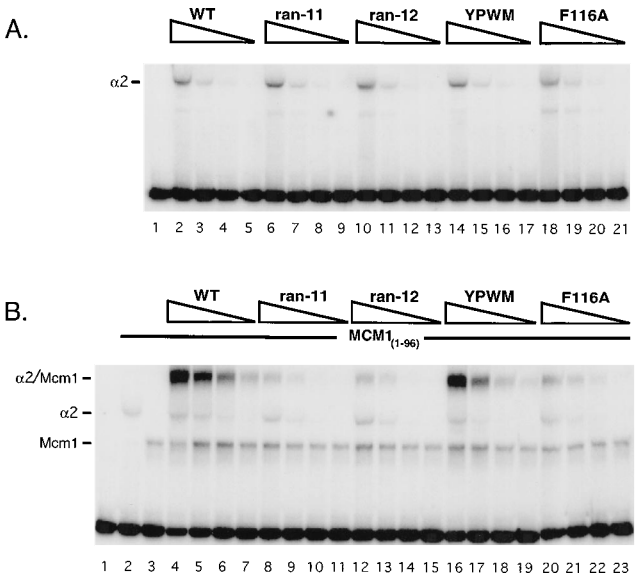

FIG. 3. DNA binding of α2 linker mutants with or without Mcm1. Shown is a PhosphorImage of gel mobility shift assays of wild-type (WT) α2, two randomized patch mutants (ran-11 and ran-12), the YPWM motif mutant (YPWM), and the phenylalanine 116-to-alanine substitution mutant (F116A) in the absence (A) or presence (B) of Mcm1 protein. (A) The concentrations of α2 proteins are varied by fivefold dilutions from 4×10^{-7} M (lanes 2, 6, 10, 14, and 18) to 3.2×10^{-9} M (lanes 5, 9, 13, 17, and 21). (B) The concentrations of α2 proteins vary by fivefold from 8×10^{-8} M (lanes 4, 8, 12, 16, and 20) to 6.4×10^{-10} M (lanes 7, 11, 15, 19, and 23). Note that lane 3 in panel A has the same concentration of α2 as lane 2 in panel B. Lanes 4 to 23 contain 2×10^{-10} M Mcm1 protein.

served in the in vivo transcription assay (Fig. 2). The F116A mutant, which exhibits a 20-fold decrease in repression with Mcm1 in vivo, demonstrates an approximate 13-fold decrease in cooperative binding with Mcm1. The YPWM mutant displays only a twofold decrease in cooperative binding with Mcm1, which is consistent with the fourfold reduction obtained in our repression assay (Fig. 2). We also assayed many of the mutants containing alanine point mutations for DNA binding with Mcm1 and found that DNA binding by these mutants, like that by the mutants shown in Fig. 3, correlates with in vivo repression values (data not shown). These results therefore confirm that the hydrophobic patch within the linker region of α2 is required for cooperative DNA binding with Mcm1 protein.

Although substitutions in the patch significantly reduce cooperative binding by α2 and Mcm1, the intensity of the α2-Mcm1 shift in the randomized mutants is higher than expected. If there are absolutely no cooperative interactions between α2 and Mcm1, then α2 should bind with equal affinity to free probe and to probe that is bound by Mcm1. If this is true, then the ratio of α2-Mcm1 binding to binding by Mcm1 alone should be the same as the ratio of α2 binding to free probe. We found that the ratio of the binding shift produced by α2-Mcm1 to that produced by Mcm1 is significantly larger than the ratio of the α2 shift to that of the free probe, suggesting that there is some cooperativity even when the patch is completely randomized. We observed the same amount of residual cooperativity with a fragment of α2 that contains only the homeodomain and carboxy-terminal tail (data not shown). These results suggest that a small amount (approximately 20%) of the cooperativity between α2 and Mcm1 is independent of the linker region but that the hydrophobic patch is the major determinant for the cooperative interactions between α2 and Mcm1.

α2 and Mcm1 interact in the absence of DNA, and this interaction is mediated by the hydrophobic patch of α2. The mobility shift assay (results shown in Fig. 3B) demonstrates that α2 and Mcm1 bind DNA in a highly cooperative manner and that substitutions in the hydrophobic patch of α2 destroy most of this cooperative interaction. We therefore wished to determine whether α2 and Mcm1 interact directly, in the absence of DNA, and whether mutations in the patch of α2 would disrupt this interaction. To test this, we assayed whether purified α2 would coimmunoprecipitate with an HA-tagged Mcm1 protein in the presence and absence of DNA. Results from this experiment indicate that wild-type α2 is specifically immunoprecipitated with Mcm1 at approximately the same level in the presence and in the absence of a DNA fragment containing an α2-Mcm1 DNA-binding site (Fig. 4, lanes 1 and 2). The immunoprecipitation of α2 is specific, since it is not detected in reactions in the absence of HA-Mcm1 or HA antibody (lanes 7 and 8). This result demonstrates that α2 and Mcm1 interact directly and that this interaction does not require DNA.

This assay was used to determine the effect of mutations in the linker region on this protein-protein interaction. We observed that the randomized patch mutant ran-11 immunoprecipitates with Mcm1 in the presence of DNA (lane 3). This result was expected, since the mobility shift assay indicates that randomized patch mutants bind DNA with wild-type affinity. This mutant is immunoprecipitated in the presence of an α2-Mcm1 *asg* site, because it is tethered to Mcm1 through the DNA. In the absence of DNA, however, the ran-11 mutant fails to interact with Mcm1, suggesting that the hydrophobic patch of α2 mediates the direct interaction with Mcm1 (lane 4). The YPWM mutant, like wild-type α2, immunoprecipitates with Mcm1 in the presence and absence of DNA (lanes 5 and 6).

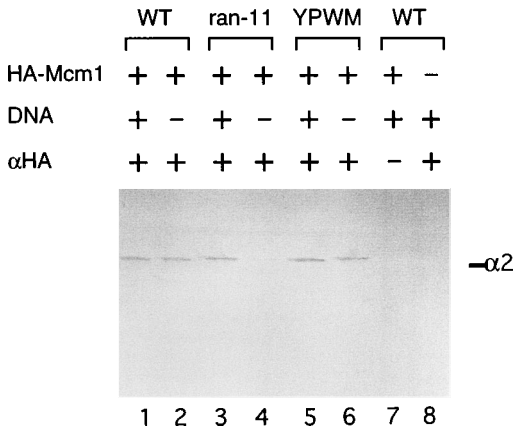

FIG. 4. Coimmunoprecipitation of wild-type (WT) and mutant α2 proteins with HA-Mcm1 in the presence or absence of DNA. The same concentrations of wild-type α2 (lanes 1 and 2), a randomized patch mutant (lanes 3 and 4), and the YPWM motif mutant (lanes 5 and 6) were coimmunoprecipitated with HA-tagged Mcm1 protein in the presence or absence of an oligonucleotide containing the consensus symmetric α2-Mcm1 DNA-binding site. Complexes were precipitated with 1 μg of anti-HA mouse monoclonal antibody (αHA) and protein A-Sepharose. Precipitated complexes were washed, electrophoresed by SDS-PAGE, transferred to nitrocellulose, and probed with an α2 antibody. Control reaction mixtures (lanes 7 and 8) contained wild-type α2 in the absence of αHA or HA-Mcm1, respectively.

Effect of altering the spacing between α2 and Mcm1 sites on transcriptional repression. Protease sensitivity and operator spacing experiments suggest that the linker region of α2 is somewhat flexible and unstructured (43). To investigate this apparent flexibility and to assess its role in the interaction of α2 with Mcm1, we tested the transcriptional repression of mutant operators that have altered spacing between the Mcm1 and α2 half sites. The wild-type *STE6* site (Fig. 5), as well as most other *asg* sites, is asymmetric, with a 4-bp spacing between the conserved α2 homeodomain TGTA binding sequence and the Mcm1 CC(A/T_6)GG sequence on one half site and a 5-bp

FIG. 5. Effect of varying the spacing between α2 and Mcm1 DNA-binding sites. Operators with altered spacing between the α2 and Mcm1 half sites are shown. The fold repression was calculated by comparing the β-galactosidase activities (average of three transformants) of a *CYC1-lacZ* promoter containing an α2-Mcm1 (α2/Mcm1) site with those of the parent vector, which does not contain a site. pTBA23 expressed 250 U of β-galactosidase activity, while a construct containing the *STE6* site produced 2 U. STE6, natural α2-Mcm1 site of the *STE6* gene; CS, consensus symmetric sequence for *asg* genes; CS + 1 bp, construct with an A inserted between the left α2 half and Mcm1 sites and a T inserted at the symmetric position of the right half site; CS + 2 bp, construct with AT and TA base pairs inserted at symmetric positions in the left and right half sites, respectively; CS − 1 bp, construct missing 1 bp at symmetric positions between the α2 half site and Mcm1 sites.

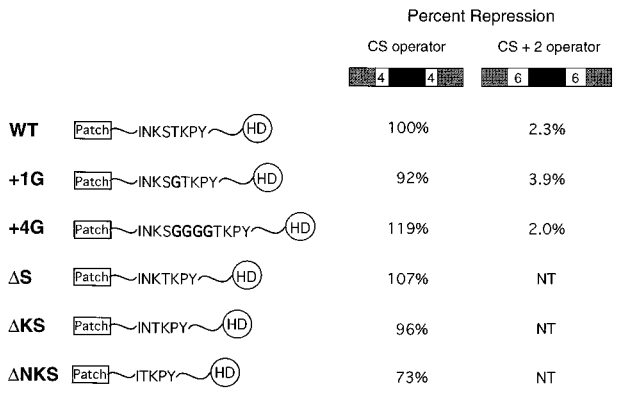

FIG. 6. Repression by insertion and deletion α2 linker mutants on wild-type (WT) and mutant α2-Mcm1 sites. Wild-type and mutant α2 proteins were assayed for repression with a reporter gene construct that contains either a consensus symmetric α2-Mcm1 site (CS) or a mutant site with 2 bp inserted between the α2 and Mcm1 DNA-binding sites (CS + 2). The following α2 mutants were tested for repression at these sites: mutants with 1 (+1G) or 4 (+4G) glycine residues introduced between residues S-127 and T-128 and mutants with deletion of S-127 (ΔS); K-126 and S-127 (ΔKS); and N-125, K-126, and S-127 (ΔNKS). Values are the average results of three transformants. The relative positions of the hydrophobic patch and homeodomain of α2 are indicated by Patch and HD, respectively. NT indicates mutants were not tested.

spacing on the other half site. In order to determine if there was a preference for the spacing between the half sites, we tested two consensus symmetric operators with either 4,4 (CS) or 5,5 (CS + 1) base pair spacing between the α2 and Mcm1 sites. The *STE6* site represses transcription 122-fold when cloned into a *CYC1-lacZ* reporter promoter (Fig. 5). Our results indicate that the 4,4 (CS) and 5,5 (CS + 1) spacing sites repress transcription as well as the wild-type *STE6* site. However, repression is destroyed by more than 5 bp (CS + 2) or less than 4 bp (CS − 1) between the α2 and Mcm1 half sites. These results suggest that there is a modest amount of flexibility between the spacing of α2 and Mcm1 proteins bound to DNA.

Insertions and deletions in the α2 linker have slight effects on repression with a wild-type α2-Mcm1 site but are unable to rescue an altered-spacing operator mutant. The altered-spacing operator experiment described above suggests that there is a small amount of flexibility in the spacing requirements of α2 and Mcm1 proteins. Another way to address this issue is to determine the effect of altering the spacing between the hydrophobic patch and the homeodomain of α2. We therefore inserted and deleted residues within the linker region, between the hydrophobic patch and homeodomain, and tested the ability of these mutants to repress transcription with Mcm1 on a symmetric α2-Mcm1 site (Fig. 6). Our results indicate that as many as four glycine residues can be introduced between residues S-127 and T-128 of the linker region with no deleterious effects on repression. We also tested +2 and +3 glycine and +1 and +4 alanine mutants and found that these mutants repress transcription with Mcm1 as well as with wild-type α2 (data not shown). In addition, we found that we could delete up to two residues, S-127 and K-126 (ΔKS), without affecting *asg* repression. In fact, we did not see a significant effect on repression levels until three residues, N-125, K-126, and S-127 (ΔNKS), had been deleted. These results are consistent with the idea that the region between the hydrophobic patch and the homeodomain is unstructured and flexible, since it can accommodate relatively large changes in length without affecting repression with Mcm1.

We next examined whether α2 insertion mutants could rescue the mutant CS + 2 operator with 6 bp of spacing between the α2 and Mcm1 sites. Wild-type α2 cannot repress transcription with Mcm1 on the mutant CS + 2 site (Fig. 5). We therefore assayed repression of the +1 and +4 glycine linker insertion mutants with this mutant operator site. Our results indicate that the +1 and +4 glycine mutants are unable to suppress the effects of the CS + 2 mutant operator (Fig. 6). To rule out the possibility that either protein (α2 or Mcm1) is unable to recognize or bind the mutant operator, we used the mobility shift assay to test the ability of the +4 glycine α2 mutant and Mcm1 proteins to bind alone and together to this mutant operator in vitro. Our results indicate that both proteins bind to the CS + 2 site with wild-type affinity on their own but are unable to bind cooperatively (data not shown). On the basis of these results, we conclude that although there is some flexibility within the spacing of α2 and Mcm1 proteins on the DNA and with respect to the length of the α2 linker, the α2 homeodomain must be in close proximity to Mcm1 to achieve cooperative DNA binding and transcriptional repression.

DISCUSSION

The α2 repressor requires additional proteins, **a**1 or Mcm1, to bind to its proper target sites and to repress transcription in vivo. In order to gain an understanding of how homeodomain proteins combine with other classes of DNA-binding proteins to increase their DNA binding affinity and specificity, we have mapped the region of α2 that mediates cooperative interaction with the MADS box protein, Mcm1. Using alanine-scanning mutagenesis, we have identified a small patch of predominantly hydrophobic residues (LVFNVVT) adjacent to the homeodomain of α2 that mediates direct interaction with Mcm1. This interaction requires a specific sequence of hydrophobic residues as determined in vitro with mobility shift and immunoprecipitation assays and in vivo with a transcription repression assay.

There are several significant parallels between our findings with the α2-Mcm1 interaction and those described for the *Drosophila* HOM proteins and their mammalian Hox counterparts. The HOM and Hox family of homeodomain proteins specify segmental identity in the developing embryo by regulating downstream target genes. Like many homeodomain proteins, HOM and Hox family members have relatively low levels of DNA binding specificity in vitro (1, 7, 10, 12, 37), suggesting that these transcriptional regulators require cofactors to achieve their functional specificity in vivo. Recently, several groups have identified two proteins, *Drosophila* exd and its mammalian homolog Pbx, which interact with specific HOM and Hox proteins (5, 35, 41, 42). These HOM and Hox proteins combine with their partners (exd and Pbx) to bind DNA cooperatively and regulate transcription of appropriate target genes in much the same way that α2 and Mcm1 combine to repress transcription of **a**-specific genes in yeast cells. Interaction among the *Drosophila* and mammalian regulatory proteins appears to be dependent on a highly conserved hydrophobic peptide sequence, YPWM, that is located in amino-terminal extensions of the homeodomains of many HOM and Hox proteins (25, 31, 52). Substitutions within this motif destroy the ability of these cofactors to bind DNA cooperatively (6, 24, 38). The YPWM protein-protein interaction motif appears to be similar in many ways to the hydrophobic patch that we identified in the linker region of α2. In fact, we found that this HOM and Hox motif can partially function in place of residues in the hydrophobic patch to interact with Mcm1 both in vitro and in vivo. Moreover, we have shown that random combinations of

hydrophobic residues in the linker region are not sufficient for cooperative interactions between α2 and Mcm1.

Although the YPWM element functions in α2, there are some significant differences between the HOM and Hox cofactor interactions and the interactions of α2 and Mcm1. The most obvious difference is that the α2-Mcm1 interaction involves a homeodomain and MADS box protein rather than two homeodomain proteins, as is the case for the HOM-exd and Hox-Pbx protein complexes. Another difference is that in vitro, the HOM and Hox proteins appear to require DNA for interaction with exd and Pbx (6, 48). In contrast, our results show that α2 and Mcm1 interact in the absence of DNA and that this interaction is dependent on the hydrophobic patch in α2. These results indicate that the hydrophobic patch is involved in protein interactions between α2 and Mcm1 and that this interaction may be stronger than the interactions between the HOM and Hox proteins and their cofactors.

In many of the HOM and Hox proteins, the YPWM motif is found in a region adjacent to the amino-terminal arm of the homeodomain. The distance between this interaction motif and the homeodomain varies among these proteins, and it has been suggested that these differences may contribute to the affinity and/or specificity of the interaction between the proteins (3, 24). For example, there are several isoforms of *Drosophila* Ubx with different spacings between the YPWM element and homeodomain, and these isoforms appear to have differential affinity for exd as shown by the two-hybrid assay (24). It is not clear, however, whether it is the specific sequence in this region or the spacing between the YPWM motif and homeodomain that is important for the interaction of exd with Ubx. Our results indicate that the spacing between the hydrophobic patch and homeodomain of α2 is not critical for cooperative interaction and repression with Mcm1. We found that insertion of up to four glycine or alanine residues or deletion of up to three residues in this region had little or no effect on transcriptional repression or cooperative DNA binding with Mcm1. This observation, together with the fact that single alanine substitutions of residues outside of the patch region had very little effect on Mcm1-mediated repression, suggests that the spacing and specific sequence between the patch and homeodomain of α2 are not important for the interaction with Mcm1. In addition, the ability of this region to accommodate large changes in length suggests that it is flexible and that the hydrophobic patch may function independently from the α2 homeodomain. This model is supported by the finding that the hydrophobic patch in α2 can be grafted onto another homeodomain and can confer cooperative interaction with Mcm1 (51). Our results are also in agreement with those of other studies, which have found that regions upstream of other homeodomain proteins appear to be unstructured (40).

Since the α2 linker region is flexible, we might expect that insertions between the patch and homeodomain would suppress the effects of a mutant *asg* site with increased spacing between the α2 and Mcm1 half sites. In fact, however, we found that mutants with insertions in the linker, like wild-type α2, fail to repress with *asg* sites that have more than 5 bp between the α2 and Mcm1 half sites. This result is in contrast to what is observed for the interaction of α2 with **a**1. The **a**1 and α2 homeodomain proteins, which bind cooperatively as a heterodimer to *hsg* sites, interact via a carboxy-terminal tail in α2 that tethers the **a**1 and α2 homeodomains together (11, 29, 30). Amino acid insertions in the extended region of the α2 tail suppress defective **a**1-α2 sites with increased spacing between the **a**1 and α2 half sites (21). The spatial constraints between α2 and Mcm1 are also in contrast with the distance requirements of the human ETS domain proteins, Elk-1 and SAP-1, which bind cooperatively with the MADS box protein, serum response factor (SRF) (47). In these complexes, changes in the spacing and orientation of the binding sites for the ETS proteins and SRF have little or no effect on the ability of these proteins to bind DNA cooperatively in vitro. One explanation for the strict spacing constraints for the α2-Mcm1 complex may be that the α2 and Mcm1 proteins are linked together by the hydrophobic patch of α2. It is possible that this region adopts a folded structure that contacts residues in both the MADS domain of Mcm1 and the homeodomain of α2. According to this model, alterations in the spacing between the DNA-binding sites would destroy the contacts made by the hydrophobic patch with either domain. If this model is correct, one would predict that mutations in some of the solvent-exposed residues of the α2 homeodomain reduce the cooperative interactions between α2 and Mcm1.

The fact that we observed a small amount of cooperativity between α2 random patch mutants and Mcm1 in our mobility shift assays (Fig. 3B) suggests that there may be a second component to the α2-Mcm1 interaction in addition to the contribution provided by the hydrophobic patch. We envision two possibilities for these findings. The first is that although the hydrophobic patch provides most of the energy requirements for the interaction of α2 with Mcm1, there may be additional protein-protein contacts between the α2 homeodomain and the MADS domain of Mcm1 that stabilize this interaction. An argument against this possibility is suggested by the recent crystal structure of the SRF MADS domain bound to DNA (36). Mcm1 is 72% identical to SRF within the MADS domain (34), and these related proteins can recognize the same DNA sequence (17, 33). It is therefore likely that the two proteins have similar structures and bind DNA in a similar manner. On the basis of the separate crystal structures of SRF (36) and the α2 homeodomain binding to DNA (53), Pellegrini et al. have proposed a model of the α2-Mcm1-DNA complex. According to this model, which has the α2 and SRF proteins positioned on their respective DNA-binding sites, the α2 homeodomain and the SRF MADS domain do not appear to be close enough to directly contact each other.

A second possible source for the residual cooperativity between α2 and Mcm1 proteins involves DNA bending. Circular permutation assays have shown that binding by Mcm1 induces a 120° bend in the *STE6* operator and that in combination, α2 and Mcm1 bend the DNA 145° (45). The crystal structure of the SRF-DNA complex also shows significant bending of the DNA that is localized in the region adjacent to the conserved CArG box (36). If Mcm1 bends the DNA in the same relative position as SRF, the bend would be positioned between the Mcm1 and α2 DNA-binding sites, which is the region in the minor groove that is contacted by the amino-terminal arm of α2. The effect of this bend is to narrow the major groove and widen the minor groove. The bend produced by Mcm1 may facilitate binding of α2 to its flanking half sites by permitting additional or more favorable protein-DNA contacts in this region. These contacts may account for the residual cooperativity we observed in the absence of a functional patch. The existence of bend-dependent DNA contacts could also explain the rigid spacing constraints of the α2-Mcm1 DNA-binding sites. Separation of the α2 and Mcm1 sites would change the position of the bend relative to the α2 sites and could therefore interfere with protein-DNA contacts that are dependent on the bend.

The identification of a specific sequence in α2 that interacts with Mcm1 and the determination of the SRF structure allow us to speculate about how α2 and Mcm1 proteins interact. The crystal structure of SRF provides a good model for the α2-

Mcm1 interaction, since α2 can bind cooperatively with SRF to an *asg* site in vitro, indicating that the residues involved in the interaction with α2 are conserved between Mcm1 and SRF (50). Two mutations in Mcm1 that destroy interaction with α2 (S73R and V81F) (4) correspond to residues in SRF (A-198 and M-205) located in the second strand of the β-sheet and the adjacent coil (36). Within this region of SRF, there are a number of hydrophobic residues that correspond to the residues F-77, I-80, V-81, and T-82 in Mcm1. One possible model for the interaction of α2 and Mcm1 is that residues in the hydrophobic patch of α2 contact a hydrophobic pocket in Mcm1 that is located between the β-sheet and the adjacent coil. Although all seven residues within the hydrophobic patch of α2 (LVFNVVT) are required for repression with Mcm1, it is unlikely that they all directly contact Mcm1. Some of these residues may be required for the patch to adopt a properly folded structure that contacts Mcm1.

In addition to interacting with α2, Mcm1 also binds DNA cooperatively with the Ste12 and Matα1 proteins to regulate different sets of genes (19). Mutations in Mcm1 that affect cooperative interactions with α1 lie in the same region as mutations that destroy interactions with α2 (4). However, substitutions at many of the positions in this region have different effects on the interactions with α1 and α2, suggesting these proteins may use slightly different mechanisms to interact with Mcm1. We are unable to find any significant regions of sequence similarities between the α2 hydrophobic patch and either α1 or Ste12, suggesting either that these proteins interact with Mcm1 in a different manner or that the protein interaction motif is degenerate. It has been observed, however, that the α2 hydrophobic patch has some sequence similarity to the B-box region of the human ETS proteins Elk-1 and SAP-1 (44). Point mutations and deletions in this region destroy the ability of Elk-1 to bind with SRF, showing that this region also has a function similar to that of the α2 hydrophobic patch. The hydrophobic patch within the linker of α2 may therefore represent a generalizable motif that specifies interaction of transcriptional regulatory proteins with different partners to achieve high levels of DNA binding affinity and specificity.

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