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MCM1 Point Mutants Deficient in Expression of α-Specific Genes: Residues Important for Interaction with α1

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Complexes formed between MCM1 and several coregulatory proteins—α1, α2, and STE12—serve to govern transcription of the a- and α-specific gene sets in the yeast *Saccharomyces cerevisiae*. The N-terminal third of MCM1, MCM1(1–98), which includes a segment homologous to mammalian serum response factor, is capable of performing all of the functions necessary for cell-type-specific gene regulation, including DNA binding and interaction with coregulatory proteins. To explore the mechanisms by which MCM1(1–98) functions, we isolated point mutants that are specifically deficient in α-specific gene expression in vivo, anticipating that many of the mutants would be impaired for interaction with α1. Indeed, in vitro DNA binding assays revealed that a substantial number of the mutants were specifically defective in the ability to bind cooperatively with α1. Two other mutant classes were also found. One class, exemplified most clearly by substitutions at residues 22 and 27, exhibited a general defect in DNA binding. The second class, exemplified by substitutions at residues 33 and 41, was proficient at DNA binding and interaction with α1 in vitro, suggesting that these mutants may be defective in achieving an α1-mediated conformational change required for transcription activation in vivo. Most of the mutants defective for interaction with α1 had substitutions within residues 69 to 81, which correspond to a region of serum response factor important for interaction with its coregulatory proteins. A subset of the mutants with changes in this region were also defective in the ability to bind with STE12 to DNA from an a-specific gene, suggesting that a common region of MCM1(1–98) mediates interaction with both α1 and STE12. This region of MCM1 does not seem to constitute an independent domain of the protein, however, because some substitutions within this region affected DNA binding. Only two of the MCM1(1–98) point mutants showed significant defects in the ability to form complexes with α2, suggesting that the mechanism by which MCM1 interacts with α2 is distinct from that by which it interacts with α1 and STE12.

The MCM1 protein has the ability to interact with several coregulatory proteins, a feature that is crucial for its central role in regulating transcription of cell-type-specific genes in the yeast *Saccharomyces cerevisiae*. The two haploid cell types of *S. cerevisiae*, **a** and α, each transcribe a specific set of genes whose products enable them to communicate and mate with cells of the other type. This differential pattern of transcription is the result of combinatorial interactions between MCM1 and the coregulators α1, α2, and STE12. Distinct regulatory complexes involving these proteins form on upstream control regions of **a**- and α-specific genes and mediate activation or repression of the two gene sets (for review, see references 8, 19, and 49).

Transcription activation of **a**-specific genes in **a** cells is achieved primarily by the action of MCM1 binding to nearly palindromic versions of the MCM1 binding site, the P box (Fig. 1) (2, 11, 20, 23, 24, 29, 38). However, maximal transcription activation also requires the protein STE12, which makes a modest contribution to the basal level of transcription and also mediates induced transcription in response to pheromone (7, 13, 14, 20, 48). These STE12 activities are conferred by DNA elements, termed pheromone response elements (PREs), located within the upstream control regions of **a**-specific genes (9, 12, 29, 54). STE12 does not bind well to isolated PRE sequences but binds cooperatively with MCM1 at composite control elements containing a P box and a nearby PRE (Fig. 1) (12). Protein-protein interactions between MCM1 and the C-terminal domain of STE12 contribute to the cooperative formation of STE12-MCM1 complexes at P-box/PRE sequences (12, 27).

While the cooperative binding of MCM1 and STE12 results in optimal transcription of **a**-specific genes in **a** cells, complexes formed between MCM1 and α2 mediate repression of **a**-specific genes in α cells (Fig. 1). The P boxes of **a**-specific genes are flanked by α2 binding sites. MCM1 and α2 bind cooperatively to the combined α2/P-box/α2 sites, and the ternary complex mediates repression (18, 20, 24–26, 41). Recent studies show that a small region of α2 adjacent to its DNA binding domain mediates the protein-protein interactions with MCM1 required for cooperative binding (55).

Complexes formed between MCM1 and α1 serve to activate transcription of α-specific genes in α cells (Fig. 1) (1, 15, 21–23, 43). The control regions of α-specific genes contain versions of the P box in which one half of the palindrome is degenerate. Adjacent to the degenerate side of the P element is a 10-bp sequence, the Q box, which is thought to be the binding site for α1 (2, 17). MCM1 and α1 bind cooperatively to QP elements, forming complexes containing a dimer of MCM1 and a single molecule of α1 (17, 38). Alone, MCM1 binds weakly to the QP element and α1 does not bind at all (1, 2, 16, 17, 52). Thus, in addition to interactions with the P and Q sequences, there are presumably protein-protein interactions between MCM1 and α1 that are critical for formation of these complexes. Moreover, α1 is thought to induce a conformational change in MCM1, thereby converting it to a more proficient transcription activator (53).

STE12 contributes to the basal and pheromone-induced expression of α-specific genes, but this contribution probably

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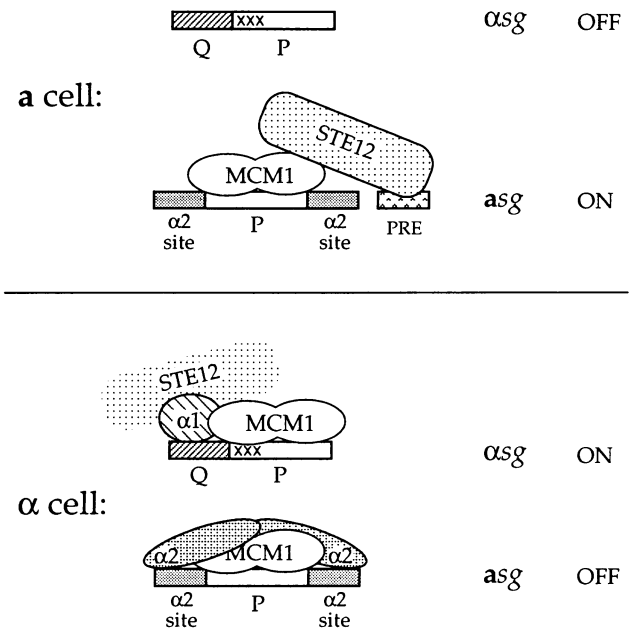

FIG. 1. Models for regulatory complexes formed between MCM1 and the coregulators α1, α2, and STE12. MCM1 binds as a dimer to the nearly palindromic P-box sequences in the control regions of **a**-specific genes and mediates transcription activation in **a** cells. In addition to the α2 sites flanking the P boxes at **a**-specific genes, binding sites for STE12, called PREs, are found nearby. STE12 does not bind well to isolated PRE sequences but binds cooperatively with MCM1 to the combined PRE/P-box sites and increases the levels of transcription of **a**-specific genes in **a** cells. In α cells, dimers of MCM1 and α2 bind to the composite α2/P-box/α2 sites and mediate transcription repression. The control regions of α-specific genes contain degenerate versions of the P box and an adjacent 10-bp sequence, the Q box. MCM1 binds weakly to the degenerate P sequences within QP elements, and, consequently, α-specific genes are not transcribed in **a** cells. In α cells, MCM1 and α1 together bind cooperatively to QP sequences, leading to transcription activation. STE12, which can increase the levels of transcription from α-specific genes, has been shown to contact α1 in solution; however, its presence in α1-MCM1-QP complexes has not yet been demonstrated (see text).

does not involve the same type of interaction with MCM1 as occurs at **a**-specific genes (Fig. 1) (13, 14, 20). In particular, the upstream control regions of α-specific genes do not contain good matches to the PRE (20, 23), and STE12-α1-MCM1-DNA complexes have not been detected in vitro. Recent studies show that the STE12 and α1 proteins can interact in solution (56), suggesting that in vivo STE12 may join α1-MCM1-DNA complexes via protein-protein interactions. In any event, it seems likely that STE12 and MCM1 interact differently at **a**-specific and α-specific control elements.

How does MCM1 carry out all of the activities summarized above? Although MCM1 is a relatively small protein, 286 amino acids in length, deletion analysis of MCM1 has shown that an even smaller version encompassing residues 1 to 98 is capable of high-affinity DNA binding, transcription activation, and interaction with coregulatory proteins, including α1, α2, and STE12 (5, 6, 38). The amino acid sequence of this region of MCM1 is very similar (68% identity) to a region of serum response factor (SRF), a mammalian protein that mediates the early transcriptional response to growth factors (35). Similar regions have also been found in other yeast (10, 36), human (37), frog (33), and plant (32, 42) proteins, and this region has been termed the MADS box domain. Like MCM1, these proteins seem to be versatile DNA binding proteins that regulate transcription through interactions with coregulatory proteins.

In order to begin to understand the mechanisms by which the N-terminal third of MCM1 interacts with coregulatory proteins, we screened randomly generated point mutants of MCM1(1–98) for ones that were deficient in activation of an α-specific reporter gene but were capable of activation from a palindromic P box in vivo, expecting that mutants with this phenotype may be deficient for interaction with α1. Indeed, in vitro DNA binding assays showed that many of the mutants were capable of high-affinity binding to palindromic P boxes but were deficient in their ability to form α1-MCM1-DNA complexes. Two other classes of mutants were also found. These did not seem to be impaired for the ability to interact with α1, but rather they appeared to have general defects in DNA binding or in achieving a conformation necessary for activation of α-specific genes. Many of the mutants that affected interaction with α1 at α-specific elements were also deficient in binding with STE12 at **a**-specific elements, suggesting that a common region of MCM1(1–98) mediates interaction with both α1 and STE12.

MATERIALS AND METHODS

Strains and media. The yeast strains used in this research were derived from YY1888 [*MAT*α *leu2-3,112 trp1-1 ura3 his3-11,15 ade2-1 can1-100 mcm1*::*LEU2*/pSL1483 (*MCM1* on a *CEN/ARS-TRP1* vector)] (5). To screen the pool of mutagenized *MCM1* plasmids, a two-reporter-gene strain, YY1901, was constructed. The P(PAL)-*lacZ* reporter gene construct carried on plasmid pSL1477 (5) was introduced into YY1888 by two-step gene replacement (39) at the *FUS1* locus to create YY1895. Next, the *STE3-HIS3-STE3* reporter construct was used to replace the *STE3* locus of YY1895 to create YY1897. The *STE3-HIS3* reporter, derived from plasmid pSL1501 (provided by B. Stevenson), has the coding region of *HIS3* embedded within *STE3* sequences such that *HIS3* expression is under control of the *STE3* promoter (17). Plasmid pSL1574, which contains *MCM1* on a *CEN/ARS-URA3* vector (5), was then introduced into YY1897, and plasmid pSL1483 (5) was lost by passage on nonselective medium to create YY1901.

Strains containing individual UAS-*lacZ* reporter gene constructs integrated at the *FUS1* locus, YY2049 [*MAT*α QP (*STE3*)-*lacZ*::*fus1*/pSL1574] and YY2052 [*MAT***a** P(PAL)-*lacZ*::*fus1*/pSL1574], were derived from YY1888 as described previously (5). Note that the P(PAL)-*lacZ* reporter construct was previously called P(PAL-16)-*lacZ* (5).

Plasmid pSL2190, expressing wild-type MCM1(1–98), was introduced into each of the reporter gene-containing strains, and pSL1574 was lost by using the plasmid shuffle technique (3) to create SY2656 [*MAT*α *STE3-HIS3* P(PAL)-*lacZ*], SY2654 [*MAT***a** P(PAL)-*lacZ*], and SY2531 [*MAT*α QP-(*STE3*)-*lacZ*]. *ste12*Δ::*URA3* versions of these strains were created by one-step gene replacement with plasmid pSL1311 (50). *mat*α*1*Δ versions of SY2656 and SY2531 were created by two-step gene replacement with pSL1711 (39).

The following *Escherichia coli* strains were used and have been described previously: SB115 (20), DH5F′ (Bethesda Research Laboratories), and CJ236 (30). Standard media and methods were used for propagating yeast and bacterial strains (40, 46). YEPD and SD media have been described elsewhere (23, 46). Plasmid-containing yeast strains were grown in synthetic selective media (e.g., SD-Trp).

Plasmids. The plasmids used in this research were constructed by standard methods (40). Plasmid pSL2190 contains *MCM1(1–98)* under control of the *MCM1* upstream promoter region in vector pRS314, a *CEN/ARS-TRP1* yeast shuttle vector (47). A blunt-ended fragment that extends from the *Sph*I site ~860 bp upstream of the *MCM1* ATG through the sequence coding for residues 1 to 98 and terminating with a stop codon and 13 nucleotides of polylinker sequences was introduced into the *Sma*I site of the polylinker in pRS314 such that transcription of *MCM1(1–98)* is directed in the opposite direction of the *bla* gene of the vector. This construct, pSL1940, expresses the MCM1(1–98) protein at levels similar to those of full-length MCM1 expressed from the normal chromosomal location (5). A *Bsp*EI site was introduced at nucleotide 288, relative to the ATG, by site-directed mutagenesis such that the sequence from codon 96 to the stop codon is GCT CCG GAC TAG. To facilitate PCR manipulations, a *Bgl*II site was introduced at nucleotide −15 by site-directed mutagenesis with oligomer D2071 (see below) to produce pSL2190. The UAS-containing fragments used in the bandshift assays were obtained from plasmids pSL1874 [P(PAL)], pSL1121 [QP(*STE3*)], pSL1702 [QP(PAL)], pSL2377 (*STE2*), and pAJ21 (*STE6*). pSL1874, pSL1121 (5), and pSL1702 (17) have been described previously. pSL2377 contains a 97-bp *Ava*II (Klenow fill-in)-*Hin*dIII fragment, containing *STE2* upstream promoter sequences from position −268 to position −177 (relative to the ATG), ligated into *Sma*I-*Hin*dIII-cut vector Bluescript KS+ (Stratagene). pAJ21 was provided by the A. Johnson laboratory (University of California, San Francisco) and contains a synthetic *STE6* operator fragment containing α2/P-box/α2 sequences in pUC18 (25). The sequences of QP(*STE3*), P(PAL), and QP(PAL) are as follows:

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QP(STE3), CTGTCATTGTGACACTAATTAGGAAA
  P(PAL),           TTTCCTAATTAGGAAA
 QP(PAL), CTGTCATTGTTTTCCTAATTAGGAAA
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PCR-mediated mutagenesis of *MCM1(1–98)*. The sequences of the oligomers used for the PCR were 5′-CAATATTATAG ATCTCCCAGCAAAAATG-3′ (D2071) and 5′-GCGGAAT TCAGCCCGGCCGCTCTAGAACTA-3′ (D2153). For PCR-mediated mutagenesis, the coding sequence of *MCM1(1–98)* in pSL2190 was amplified in a modified PCR buffer containing $MnCl_2$, which reduced the fidelity of *Taq* polymerase. The PCR primers used were D2071, which contains a *Bgl*II site, and D2153, which contains an *Eco*RI site. PCRs were performed essentially as described previously (31), and the reaction mixtures contained 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris (pH 8.8), 6.7 mM EDTA, 170 μg of bovine serum albumin per ml, 6.1 mM $MgCl_2$, 100 mM β-mercaptoethanol, 10% dimethyl sulfoxide, 1 mM each deoxynucleoside triphosphate, 0.5 mM $MnCl_2$, 6 μg (each) of D2071 and D2153 per ml, and ~100 ng of *Pvu*II-cut pSL2190 per ml. Reactions were performed in a 100-μl volume with 5 U of *Taq* polymerase (added after heating to 94°C) with 15 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 4 min. PCR-generated fragments were purified by agarose gel electrophoresis, cut with *Bgl*II and *Eco*RI, repurified by agarose gel electrophoresis, ligated into purified *Bgl*II- and *Eco*RI-cut vector pSL2190, and transformed into *E. coli*. Four separate PCRs were used to generate independent pools of mutagenized *MCM1(1–98)*-containing plasmids, each prepared from ~10^6 *E. coli* transformants.

Site-directed mutagenesis of codon 70. A library of mutated plasmids encoding MCM1(1–98) with all possible amino acids at residue 70 was created by site-directed mutagenesis of pSL2190 with an oligonucleotide degenerate at the three positions of codon 70. A novel restriction site was engineered into the oligonucleotide so that mutated products could be identified.

β-Galactosidase and ATZ assays. Plasmid-bearing yeast strains were grown at 30°C overnight to late log phase in synthetic selective medium. The cells were diluted to a density of ~10^7 cells per ml in fresh selective medium and grown for two doublings (~4 h). The cells were prepared and assayed, and β-galactosidase activity was calculated as previously described (23).

A semiquantitative assessment of the abilities of the MCM1(1–98) point mutants to promote expression of the *STE3-HIS3* reporter was made by testing strains for resistance to 3-amino-1,2,4-triazole (ATZ), which is a competitive inhibitor of the *HIS3* gene product (28, 51). Strains grown to saturation in SD-Trp medium were diluted in SD medium, and droplets, each containing ~1,000 cells, were spotted on a series of SD-Trp-His plates containing 0, 1, 3, 5, 8, 10, 15, 20, and 40 mM ATZ. The test spots were incubated at 30°C for 48 h and scored qualitatively for growth on the basis of the highest concentration of ATZ on which significant growth was observed.

Bandshift assays. Radioactively labelled UAS-containing DNA fragments for bandshift assays were prepared as follows. *Hin*dIII-*Sst*I fragments from pSL1874, pSL1121, pSL1702, and pSL2377 and a *Hin*dIII-*Bam*HI fragment from pAJ21 were purified by agarose gel electrophoresis and electroelution and subsequently were quantitated spectrophotometrically (40). The sizes of the fragments are as follows: P(PAL), 148 bp; QP(*STE3*) and QP(PAL), 158 bp; *STE2*, 140 bp; *STE6*, 68 bp. Fragments were labelled by filling in the 5′-overhanging ends with the Klenow fragment of DNA polymerase and [α-^{32}P]dATP (6,000 Ci/mmol; New England Nuclear).

Crude protein preparations were made from yeast strains (SY2654 background) bearing wild-type and mutant versions of MCM1(1–98) as described previously (5). Protein concentrations were determined with a Bio-Rad protein assay kit. Recombinant maltose binding protein-α1 fusion protein (MBPα1) was expressed and purified from *E. coli* as described previously (17). Recombinant α2 protein, expressed and purified from *E. coli*, was generously donated by the A. Johnson laboratory. For bandshift experiments involving MBPα1 and α2, a protein concentration was used such that a linear relationship existed between the amount of protein added and the amount of ternary complex formed with wild-type MCM1(1–98).

Bandshift reaction mixtures were incubated for 25 min at room temperature in a final volume of 15 to 20 μl containing 20 mM $NaPO_4$ (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 120 mM NaCl, 5 μg (or amounts indicated in figure legends) of crude yeast extract, 15 μg of double-stranded poly(dI-dC) (Pharmacia) per ml, and 100 pM labelled DNA fragment. α1 or α2 protein (amounts indicated in the figure legends) was mixed with the yeast extract on ice before addition of DNA.

Gels for resolving protein-DNA complexes were 4% (or 6% for experiments with the *STE6* fragment) polyacrylamide–bisacrylamide (38:1.25) containing 10% glycerol in 1× TGE (5). Gels were prerun for 30 min at 150 V, 15 to 20 μl of each binding reaction mixture was loaded as the gel was running, and electrophoresis was continued for 2 h. For quantitation, regions of the gels containing free DNA and protein-DNA complexes were excised from the gel, and the radioactivity present at each region was measured by scintillation counting. The ability of each of the MCM1(1–98) mutants to form protein-DNA complexes on the various DNA fragments was

quantitated relative to that of wild-type MCM1(1–98) as shown in Tables 2 and 3. Independent quantitative bandshift experiments were performed at least twice with each DNA fragment for all of the MCM1(1–98) mutants, and the results of these experiments generally differed by less than 10%. The values presented in Tables 2 and 3 are from a series of experiments in which extracts for each mutant were made and quantitated in parallel.

RESULTS

Isolation of point mutants of MCM1(1–98) specifically deficient for activation of α-specific genes. The N-terminal 98 residues of MCM1 are capable of carrying out all of the functions necessary for proper regulation of **a**- and α-specific genes, including DNA binding, transcription activation, and interaction with α1, α2, and STE12 proteins (5, 6, 38). To begin to investigate how this small domain interacts with different coregulator proteins, we sought to isolate point mutants of MCM1(1–98) that are specifically deficient for activation of α-specific genes. It is conceivable that the consequences of such alterations could be somewhat different in the context of the full-length protein. For example, a polyacidic stretch of amino acids from residue 99 to 117 is thought to contribute modestly to α-specific gene expression (5, 6). Nonetheless, MCM1(1–98) can carry out all known MCM1 functions in vivo and is therefore a suitable subject for mutational analysis. A library of random point mutants was screened for ones that showed decreased activation of an α-specific reporter gene, *STE3-HIS3*, but were capable of activation of a non-cell-type-specific reporter gene controlled by the perfectly palindromic P-box sequence, P(PAL)-*lacZ*. Expression of P(PAL)-controlled genes requires the DNA binding and transcription activation abilities of MCM1, while expression of the *STE3-HIS3* reporter requires that MCM1 also interact with α1.

To create a library of point mutants, the coding sequence of MCM1(1–98) was amplified by PCR under low-fidelity conditions, ligated into a *CEN/ARS-TRP1* vector containing the *MCM1* promoter, and introduced into *E. coli*. Four independent pools of mutagenized plasmid DNA representing separate PCRs were purified from ~10^6 *E. coli* transformants each. Because MCM1 is an essential gene, the pool of mutagenized plasmids was introduced into the yeast strain containing integrated *STE3-HIS3* and P(PAL)-*lacZ* reporter constructs, YY1901 (see Materials and Methods), by the plasmid shuffle technique (3). Transformants were replica plated to 5-fluoroorotic acid (5-FOA)-containing media twice in succession, ensuring that only colonies containing versions of MCM1(1–98) that supplied the essential functions of MCM1 were alive. Of the original ~10^4 transformants, 5,500 survived and were replica plated to SD-His-Trp media containing 0 or 10 mM ATZ (see Materials and Methods) to assess expression of the *STE3-HIS3* reporter. Three hundred thirty colonies were chosen that showed decreased ability to express *STE3-HIS3* as judged by their growth on these plates. β-Galactosidase assays of these isolates were then performed to quantitate expression of the P(PAL)-*lacZ* reporter. One hundred thirty mutants that expressed P(PAL)-*lacZ* to at least 50% of the level expressed by wild-type MCM1(1–98) were chosen for sequencing.

Sequencing of plasmid DNA isolated from these strains revealed that all had mutations in the coding sequence of MCM1(1–98). Counting only nonsilent changes, 50% of the mutants had a single mutation, 40% had two mutations, and 10% had three to five mutations. Among the complete collection of mutations sequenced, there were approximately equal numbers of transition and transversion mutations, as expected for the PCR conditions used (31), and all possible types of nucleotide changes were represented. Many of the specific amino acid substitutions were isolated multiple times either as single changes or with other changes. For example, 12 isolates of Y70H (mutant with change of Y-70 to H) were sequenced—5 had a single mutation and 7 had multiple changes—all of which had the same phenotype. Given that ~10^9 molecules of DNA were used to amplify the coding sequence of MCM1(1–98) in the PCRs and that ~10^4 yeast transformants were screened, it seems unlikely that the multiple isolates of specific nucleotide changes were the result of amplification of mutations occurring early in the PCR. Furthermore, in all cases in which multiple mutants with specific amino acid substitutions were isolated, at least some of them were demonstrably independent, because they had different nucleotide changes and/or were isolated from more than one of the four independent mutagenized pools of plasmid DNA.

TABLE 1. MCM1(1–98) point mutants and in vivo phenotypes

MCM1(1–98) strain (no. of isolates with each amino acid change)[a]	Highest concn (mM) of ATZ at which growth of *STE3-HIS3* was observed[b]	β-Galactosidase activity (%)[c] of:	
		QP(*STE3*)-*lacZ*	P(PAL)-*lacZ*
Wild type	20	100	100
matα1Δ	0	0	ND[e]
ste12Δ	3	81	ND
E22K (5)	1	9	79
E22V (2)	1	9	110
E27G (3)	5	48	96
E27K (2)	3	22	80
E27V (5)	5	10	85
H33D (1)	0[d]	12	135
H33L (6)	5	31	109
H33Q (5)	5	119	112
H41L (6)	1	8	78
H41Q (3)	1	14	107
H41R (9)	1	7	65
H41Y (1)	3	14	78
V52A (3)	3	25	75
V69A (1)	5	31	72
V69I (8)	1	18	81
Y70H (12)	0[d]	4	74
T71A (1)	0[f]	18	53
T71S (2)	5	32	83
S73C (3)	3	32	78
S73R (3)	0[d]	1	56
E78K (6)	0[f]	6	96
E78V (15)	3	18	94
V81F (1)	0[f]	5	48

[a] Numbers in parentheses include both isolates with single changes and those with additional changes that did not appear to affect the phenotype.
[b] Constructs expressing wild-type and mutant versions of MCM1(1–98) were transformed into YY1901 by the plasmid shuffle technique (3). Expression levels of the *STE3-HIS3* construct are reported as the highest concentration of ATZ on which significant growth was observed, as described in Materials and Methods.
[c] Constructs expressing wild-type and mutant versions of MCM1(1–98) were transformed into YY2049 [QP(*STE3*)-*lacZ*] and YY2052 [P(PAL)-*lacZ*] by the plasmid shuffle technique, and β-galactosidase activities were determined (see Materials and Methods). β-Galactosidase activity is presented as a percentage of the activity measured for strains expressing wild-type MCM1(1–98). Differences between independent transformants were less than 10%.
[d] No growth on media lacking histidine was observed.
[e] ND, not determined.
[f] Some growth on media lacking both histidine and ATZ was observed.

Quantitative analysis of reporter gene expression. The collection of MCM1(1–98) mutants chosen for further analysis is listed in Table 1. To quantitate the ability of the mutants to express α-specific and P(PAL)-controlled reporter genes, plasmids containing single point mutations were introduced into

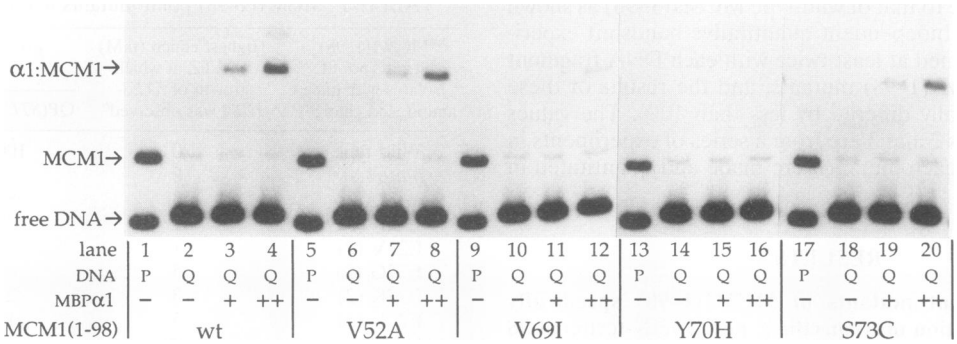

FIG. 2. Binding of MCM1(1–98) mutants to P(PAL) and QP(*STE3*). Bandshift reactions were performed with the DNA fragments indicated in each lane of the figure as described in Materials and Methods. P represents P(PAL), and Q represents the QP(*STE3*) fragment. Five micrograms of extract containing the wild-type (wt) or mutant versions of MCM1(1–98) was used in each reaction mixture as indicated. Buffer (−) or 50 (+) or 100 (++) ng of partially purified MBPα1 protein was mixed with the extracts prior to addition of DNA.

yeast strains YY1901 (*MAT*α *STE3-HIS3*), YY2049 [*MAT*α QP(*STE3*)-*lacZ*], and YY2052 [*MAT***a** P(PAL)-*lacZ*]. Expression of the QP(*STE3*)-*lacZ* reporter is controlled by a minimal 26-bp QP sequence from *STE3*, whereas the *STE3-HIS3* reporter in YY1901 includes the entire *STE3* upstream sequence. Expression of the QP(*STE3*)-*lacZ* and P(PAL)-*lacZ* reporters was determined by quantitative β-galactosidase assays (Table 1). As expected, all of the MCM1(1–98) point mutants showed a greater deficiency in activation of the α-specific reporters than in activation of the non-cell-type-specific reporter, P(PAL)-*lacZ*. The mutant phenotypes ranged from a 2-fold reduction in expression of the α-specific reporter to a 50-fold reduction.

The phenotypes of these mutants imply that they are defective for α1-dependent transcription. However, because STE12 is required for maximal expression of α-specific reporters but not for expression of P(PAL)-controlled reporters, the mutants could be defective for STE12-dependent transcription instead. As shown in Table 1, a *ste12*Δ strain bearing the *STE3-HIS3* reporter was resistant to 3 mM ATZ, while an isogenic wild-type strain was resistant to 20 mM ATZ (see Materials and Methods). Northern (RNA) blot analysis showed that the amount of *STE3-HIS3* transcript expressed in the *ste12*Δ strain was approximately fourfold lower than that produced by the wild-type strain (data not shown), an effect consistent with that previously observed for expression of the native *STE3* transcript in a *ste12*Δ strain (13). The level of expression of the QP(*STE3*)-*lacZ* reporter, however, was relatively unaffected in a *ste12*Δ strain (Table 1). Presumably, differences in the sequence context of the QP control elements are responsible for the subtly different effects of STE12 on the two reporters. In any event, with only one exception (H33Q), the MCM1 point mutants were significantly more deficient for expression of the QP(*STE3*)-*lacZ* reporter than was the *ste12*Δ mutant. Therefore, it seems unlikely that the deficiencies of the point mutants in expression of α-specific genes can be explained entirely by a decreased ability to carry out STE12-dependent transcription.

In vitro DNA binding assays. The defect in expression of α-specific reporters exhibited by the mutants listed in Table 1 could be due to a defect in interaction with α1. That is, these MCM1 mutants may be impaired for the ability to bind cooperatively with α1 to α-specific promoters such as QP(*STE3*). However, subtle defects in the ability of MCM1 to bind to DNA or to carry out its transcription activation function could in principle lead to the same phenotype. To distinguish between these possibilities, we used quantitative bandshift analysis to determine the DNA binding properties of the mutants. For each mutant, we compared its ability to bind cooperatively with α1 to QP(*STE3*) DNA with its ability to bind alone to P(PAL) DNA. As a second assessment of the ability of the point mutants to interact with α1, we performed bandshifts with a synthetic element, QP(PAL), which contains the Q box from *STE3* adjacent to a perfectly palindromic P box (described in reference 17). MCM1 binds this fragment with high affinity, as for P(PAL), and the addition of the Q box to P(PAL) allows formation of α1-MCM1-QP(PAL) complexes (17).

Figure 2, lanes 1 to 4, shows a series of bandshift experiments in which wild-type MCM1(1–98), prepared as a crude extract from yeast **a** cells, was incubated with P(PAL) and with QP(*STE3*) either alone or with purified MBPα1. Previous work established that MBPα1 behaves like native α1 in bandshift assays (17). As expected, MCM1-P(PAL) complexes formed readily but very little MCM1-QP(*STE3*) complex was detected. When increasing amounts of MBPα1 were added, increasing amounts of α1-MCM1-QP(*STE3*) complexes were observed. A similar set of bandshift experiments are shown for representative point mutants in Fig. 2. Quantitation of bandshift experiments for each point mutant with P(PAL), QP(*STE3*), and QP(PAL) is presented in Table 2.

The amount of MCM1-DNA complex formed reflects both the binding affinity of MCM1 for DNA and the concentration of MCM1 in the extract. Thus, an amino acid substitution that reduces the amount of complex formed could do so by reducing the affinity of MCM1 for DNA or by reducing the stability of the protein, resulting in a lower concentration of MCM1 in the extracts used for bandshift analysis. Scatchard-type analysis of bandshift experiments with P(PAL), as described in reference 5, was done for representative point mutants to distinguish between these possibilities. For all mutants tested (E27V, H33L, H41R, H41Q, V52A, V69I, Y70H, T71A, S73C, E78K, and E78V), the primary effect of the amino acid substitution was to reduce binding affinity rather than to reduce protein concentration (data not shown). Inspection of the in vivo and in vitro properties of the mutants summarized in Tables 1 and 2 suggests that it is useful to divide the mutant phenotypes into three broad categories. Below, we use this framework to describe the characteristics of the mutants in detail. We note that not all mutants fall neatly into a single category and that different substitutions at the same

TABLE 2. Quantitation of in vitro binding to P(PAL) and with α1 to QP(*STE3*) and QP(PAL)[a]

MCM1(1–98) strain	% of complex formed by mutant			
	QP(*STE3*) (α1-MCM1)	P(PAL) (MCM1)	QP(PAL)	
			α1-MCM1	MCM1
Wild type	100	100	100	100
E22K	16	21	19	13
E22V	19	44	27	43
E27G	38	48	34	51
E27K	6	16	11	10
E27V	41	44	30	42
H33D	92	175	142	139
H33L	37	108	18	120
H33Q	140	102	25	123
H41L	79	71	155	69
H41R	39	41	5	35
H41Q	70	105	50	111
H41Y	98	65	221	67
V52A	72	95	70	92
V69A	73	71	71	78
V69I	23	79	12	86
Y70H	2	77	6	83
T71A	26	26	69	17
T71S	39	93	8	99
S73C	69	109	27	107
S73R	15	110	8	107
E78K	37	72	14	74
E78V	42	51	42	47
V81F	17	91	4	93

[a] The amounts of DNA bound in MCM1-DNA and α1-MCM1-DNA complexes from bandshift experiments like those shown in Fig. 2 were quantified as described in Materials and Methods. The amount of complex formed by each mutant was converted to a percentage relative to the amount of complex formed by wild-type MCM1(1–98), designated as 100%.

residue sometimes yield mutants that fall into different categories.

In vitro DNA binding activities: mutants defective for DNA binding. A subset of the mutants showed a significant decrease (more than twofold) in binding to P(PAL) and a similar decrease in ability to form α1-MCM1-QP(*STE3*) and α1-MCM1-QP(PAL) complexes, indicating that they may have general defects in DNA binding. All mutants with changes at E-22 and E-27 (E22K, E22V, E27V, E27G, E27K) showed this pattern, suggesting that E-22 and E-27 may be important for DNA binding. The mutants H41R, T71A, and E78V also showed defects in DNA binding ability, although other alleles at these residues, H41Q, T71S, and E78K, showed greater defects in binding with α1 than when binding alone to P(PAL) (described below).

In vitro DNA binding activities: mutants not defective for binding to QP(*STE3*). The second group of mutants formed α1-MCM1-QP(*STE3*) complexes as well as (or nearly so) wild-type MCM1(1–98) (>80% of wild-type binding). Mutants at residues H-33 and H-41 are particularly noteworthy because four different substitutions had this phenotype—H33D, H33Q, H41L, and H41Y (Table 2). Moreover, some substitutions at these residues exhibited unusual DNA binding properties. For example, H33Q formed α1-MCM1-QP(*STE3*) complexes more readily than wild-type MCM1(1–98) but showed a marked decrease in ability to form α1-MCM1 complexes on QP(PAL). Likewise, H41R, which showed a general reduction in binding ability (described above), showed an eightfold decrease in ability to form α1-MCM1-QP(PAL) complexes compared with MCM1-QP(PAL) or α1-MCM1-QP(*STE3*) complexes. H41L and H41Y, on the other hand, formed α1-MCM1-QP(PAL) complexes more efficiently than MCM1-QP(PAL) complexes. These results imply that the DNA sequence at which the H-33 and H-41 mutants are bound affects their ability to interact with α1, suggesting that the conformation of DNA-bound MCM1 is affected by substitutions at H-33 and H-41. The other two mutants at these positions, H33L and H41Q, showed binding activities similar to those shown by the third group of mutants, discussed below.

In vitro DNA binding activities: mutants defective for interaction with α1. Many of the mutants showed a significant decrease in ability to form α1-MCM1-QP(*STE3*) complexes compared with MCM1-P(PAL) complexes, suggesting that they may have defects in interacting with α1 (Table 2). Y70H showed the greatest difference, binding ~35-fold less well with α1 to QP(*STE3*) than autonomously to P(PAL). In addition, V69I, S73R, and V81F showed four- to sevenfold reductions, and H33L, H41Q, V52A, T71S, S73C, and E78K (and perhaps E22V) showed modest but reproducible reductions (50 to 70%) in ability to form α1-MCM1-QP(*STE3*) complexes compared with MCM1-P(PAL) complexes.

The QP(PAL) fragment was used to test further the ability of these mutants to interact with α1 in vitro. The use of this fragment allows the relative ability of the mutants to form α1-MCM1-DNA and MCM1-DNA complexes to be assessed in a context in which MCM1 can interact with the same DNA sequence. The mutants that showed defects in binding with α1 to QP(*STE3*)—H33L, H41Q, V52A, V69I, Y70H, T71S, S73C, S73R, E78K, and V81F—were also defective in the ability to recruit α1 to QP(PAL) (Table 2). In general, the magnitudes of the defects in binding with α1 to QP(PAL) were greater than those observed for the formation of α1-MCM1-QP(*STE3*) complexes. T71S was especially deficient (more than fourfold) at forming α1-MCM1-QP(PAL) complexes compared with α1-MCM1-QP(*STE3*) complexes, and in this property, T71S resembles the H33Q and H41R substitutions discussed above.

Isolation and analysis of other mutants at Tyr-70. As discussed above, the mutant Y70H was isolated repeatedly from the screening of random point mutants to the exclusion of other changes at this residue. The finding that Y70H had one of the strongest α-specific phenotypes in vivo and strongly affected interaction with α1 in vitro prompted analysis of the effect of other amino acid substitutions at this position. Site-directed mutagenesis with an oligonucleotide degenerate at the three positions of codon 70 was used to generate a pool of mutagenized MCM1(1–98) DNA with all possible amino acid changes at Y-70. This pool was transformed into YY1901 by the plasmid shuffle technique, and the mutant versions of MCM1(1–98) were analyzed for their ability to maintain viability and promote expression of the reporter genes as described above. Interestingly, ~45% of the transformants were inviable upon loss of the wild-type MCM1-containing plasmid. Of the remaining (viable) colonies, only 2% showed decreased expression of the *STE3-HIS3* reporter, while the remaining colonies showed no detectable phenotype.

Sequencing of plasmid DNA from these strains showed that all mutants with α-specific phenotypes had histidine codons at position 70. Plasmid DNAs from seven viable isolates that contained the restriction site incorporated by the oligonucleotide used in the mutagenesis (see Materials and Methods) but showed no detectable phenotype were also sequenced. Four of these had the wild-type tyrosine codon at position 70, two had phenylalanine codons, and one had a tryptophan codon at residue 70. Further analysis of the ability of the Y70F and Y70W mutants to express the *STE3-HIS3*, QP(*STE3*)-*lacZ*,

TABLE 3. Phenotypes of additional substitutions at Tyr-70

MCM1(1–98) strain	Viability[a]	Reporter gene expression[b]		
		STE3-HIS3 (mM)	QP(*STE3*)-*lacZ* (%)	P(PAL)-*lacZ* (%)
Wild type	+	20	100	100
Y70H	+	0[c]	4	74
Y70F	+	20	130	100
Y70W	+	8	40	72
Y70G	−	ND[d]	ND	ND
Y70C	−	ND	ND	ND
Y70D	−	ND	ND	ND
Y70S	−	ND	ND	ND

[a] Constructs expressing wild-type and mutant versions of MCM1(1–98) were transformed into YY1901 by the plasmid shuffle technique (see Materials and Methods). A + indicates that the transformants were capable of growing on 5-FOA-containing media; a − indicates that the transformants were unable to grow on 5-FOA-containing media, implying that the MCM(1–98) mutant plasmid was unable to support life.

[b] Reporter gene assays were performed as described in Table 1, footnote *c*. Values for Y70H are repeated here from Table 1.

[c] No growth on media lacking histidine was observed.

[d] ND, not determined.

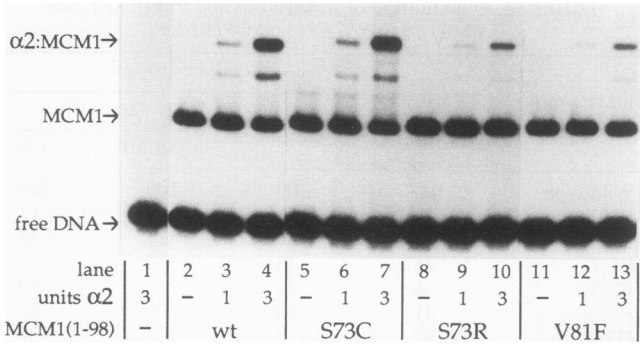

FIG. 3. Binding of MCM1(1–98) mutants and α2 to *STE6* DNA. Bandshift reactions were performed with a *STE6* fragment that contains α2 binding sites flanking the P box as described in Materials and Methods. Five micrograms of extract containing the wild-type (wt) and mutant versions of MCM1(1–98) was used in each reaction mixture as indicated. Buffer (−) or 1 or 3 nM (final concentration) α2 protein was mixed with the extracts prior to addition of DNA.

and P(PAL)-*lacZ* reporter genes (Table 3) revealed that Y70W was somewhat defective for expression of all three reporter genes. However, the defect of Y70W was not particularly α specific, and it would not have been chosen for further study from the original screening. Of 12 inviable MCM1(1–98) mutants that were sequenced, three Y70S substitutions, two Y70D substitutions, two Y70C substitutions, and one Y70G substitution were found. Three of the remaining four inviable mutants had stop codons at position 70, and one had a 2-bp deletion.

These results demonstrate that, at least for residue 70, bias towards particular nucleotide changes in the PCR-mediated mutagenesis did not limit the spectrum of amino acid substitutions identified. Instead, Y70H seems to be the only substitution at position 70 that results in an α-specific defect in gene expression. More-conservative changes (Y70F and Y70W) produce little or no defect in gene expression. Less-conservative changes generate versions of MCM1(1–98) that are incapable of carrying out the essential functions of MCM1, either because the mutant proteins are not stable or because they are deficient in a particular MCM1 activity.

To explore the basis of the lethal phenotype, bandshift experiments were performed with extracts expressing both full-length MCM1 and the inviable versions of MCM1(1–98). Y70S and Y70C showed severely reduced (<5%) ability to bind P(PAL) as homodimers, whereas Y70G and Y70D showed no detectable binding to P(PAL) as homodimers (data not shown). Interestingly, all four inviable mutants showed some ability to bind P(PAL) as heterodimers with full-length MCM1. Y70S showed levels of heterodimer formation equivalent to that of wild-type MCM1(1–98). Thus, for Y70S, the inviable phenotype is not due to a lack of the mutant protein in the cells, e.g., caused by proteolysis of improperly folded mutant MCM1, but is more likely due to reduced DNA binding affinity. The Y70G, Y70D, and Y70C mutants showed reduced heterodimer formation, compared with that of the wild type, indicating that the inviable phenotype of Y70G, Y70D, and Y70C could be due to decreased binding or dimerization ability and/or decreased protein concentration.

In vitro DNA binding with α2 and STE12. The results of the bandshift studies presented above suggest that many of the point mutants in our collection affect the ability of MCM1(1–98) to interact with α1. These substitutions may therefore define a region or surface of MCM1 that makes direct contact with one of its coregulators. To investigate whether the same region is involved in interaction with other coregulators, we asked whether these substitutions also affected interaction with α2 and STE12, which bind cooperatively with MCM1 at **a**-specific gene control elements.

We examined the relative ability of the MCM1(1–98) point mutants to interact with α2 protein by using an *STE6* DNA fragment that contains an α2/P-box/α2 sequence. As expected, wild-type MCM1(1–98) bound well to the P box in the *STE6* fragment (Fig. 3, lane 2), and α2-MCM1-DNA complexes formed in the presence of added α2 protein (Fig. 3, lanes 3 and 4). Compared with their ability to bind to the *STE6* fragment autonomously, only two of the mutants, S73R and V81F, were impaired in the ability to bind in conjunction with α2 (Table 4). Most of the mutants defective for interaction with α1 were relatively unaffected in the ability to bind in conjunction with α2.

We used a DNA fragment containing the P-box and PRE sequences from the *STE2* gene to assess the ability of the MCM1(1–98) mutants to form complexes with STE12. Two complexes formed on this fragment: one containing MCM1 and one containing both MCM1 and STE12 (Fig. 4). The latter complex was absent when extract from a *ste12*Δ strain was used (Fig. 4, compare lanes 2 and 3 and 4 and 5) and had altered mobility when extract from a strain expressing full-length MCM1 was used (data not shown), confirming that the complex contained both proteins.

S73R and E78K showed marked decreases (10- and 40-fold, respectively) in the ability to bind the *STE2* fragment in conjunction with STE12 compared with their ability to bind alone, suggesting that they may have a defect in the ability to interact with STE12 (Fig. 4 and Table 4). Other mutants—H41R, H41Y, V52A, V69I, E78V, and V81F—exhibited approximately two- to threefold reductions in the ability to bind with STE12. The collection of mutants defective for interaction with STE12 is a subset of the mutants that are defective for interaction with α1. Two mutants that had a marked decrease in the ability to interact with α1, Y70H and T71S, were not impaired in the ability to interact with STE12. It should be emphasized that a defect in MCM1-STE12 interaction detected on *STE2* DNA need not indicate that STE12 function is

TABLE 4. Quantitation of in vitro binding with STE12 and α2[a]

MCM1(1–98) strain	% of complex formed by mutant			
	STE2		*STE6*	
	STE12-MCM1	MCM1	α2-MCM1	MCM1
Wild type	100	100	100	100
E22K	14	26	5	8
E22V	72	55	45	50
E27G	50	54	25	32
E27K	6	17	1	5
E27V	44	53	31	36
H33D	356	150	136	91
H33L	191	119	158	167
H33Q	140	101	106	136
H41L	49	84	79	53
H41R	12	43	35	46
H41Q	116	112	77	83
H41Y	35	74	68	53
V52A	32	106	53	67
V69A	50	85	75	63
V69I	38	92	72	84
Y70H	104	75	62	43
T71A	45	26	13	14
T71S	130	89	72	85
S73C	161	101	107	107
S73R	12	115	43	105
E78K	2	84	38	62
E78V	33	61	48	43
V81F	34	93	45	81

[a] The amounts of DNA bound in MCM1-*STE2*, STE12-MCM1-*STE2*, MCM1-*STE6*, and α2-MCM1-*STE6* complexes from bandshift experiments like those shown in Fig. 3 and 4 were quantified as described in Materials and Methods. The amount of complex formed by each mutant was converted to a percentage relative to the amount of complex formed by wild-type MCM1(1–98), designated as 100%.

impaired at α-specific genes because, as discussed in the Introduction, MCM1 and STE12 probably interact differently at **a**-specific and α-specific control elements.

To determine whether the observed defects of these MCM1 mutants in binding cooperatively with STE12 had consequences in vivo, we measured expression of an integrated *STE2-lacZ* reporter containing the same *STE2* fragment used in the bandshift experiments. β-Galactosidase activity was two- to threefold lower in *ste12*Δ cells than in wild-type cells (data not shown), an effect comparable to the reduction in transcription of the chromosomal *STE2* locus seen in *ste12*Δ mutants (13). However, none of the MCM1 mutants was more deficient at expression of *STE2-lacZ* than at expression of P(PAL)-*lacZ* (data not shown). Thus, the in vitro bandshift assay appears to be more sensitive than the in vivo reporter gene assay for detecting defects in MCM1-STE12 interaction.

DISCUSSION

The N-terminal 98 residues of MCM1 can carry out a remarkable array of biochemical functions, including site-specific DNA binding, transcription activation, and interaction with coregulatory proteins. Interactions with three coregulators in particular—α1, α2, and STE12—modulate MCM1's activity at cell-type-specific genes (see Introduction). To begin to understand the means by which MCM1 interacts with coregulators, point mutants of MCM1(1–98) that are specifically deficient for activation of α-specific genes were isolated from a library of random mutants.

The in vivo phenotypes of >90% of the mutants could be attributed to single changes at residues E-22, E-27, H-33, H-41, V-52, V-69, Y-70, T-71, S-73, E-78, and V-81 (Table 1). On the basis of quantitative bandshift analysis of their relative ability to bind autonomously to P(PAL) and to bind in conjunction with α1 to QP(*STE3*) and QP(PAL), most of the mutants could be divided into three broad classes: (i) those that had general deficiencies in DNA binding, (ii) those that may affect the conformation of DNA-bound MCM1, and (iii) those that were specifically deficient in ability to bind in conjunction with α1. A subset of the positions important for interaction with α1 at α-specific control elements were also important for interaction with STE12 at **a**-specific control elements, indicating that the regions of MCM1 that contact α1 and STE12 may overlap. Interestingly, particular substitutions within this region also affect the ability of MCM1(1–98) to bind DNA. The implications of these findings are discussed below.

Mutants defective in DNA binding. All mutants with changes at E-22 and E-27 were impaired (2- to 10-fold reductions) in the ability to bind DNA at high-affinity sites, such as P(PAL). Particular changes at other residues—H41R, T71A, and E78V—also showed general defects in DNA binding (described below). These mutants were capable of forming

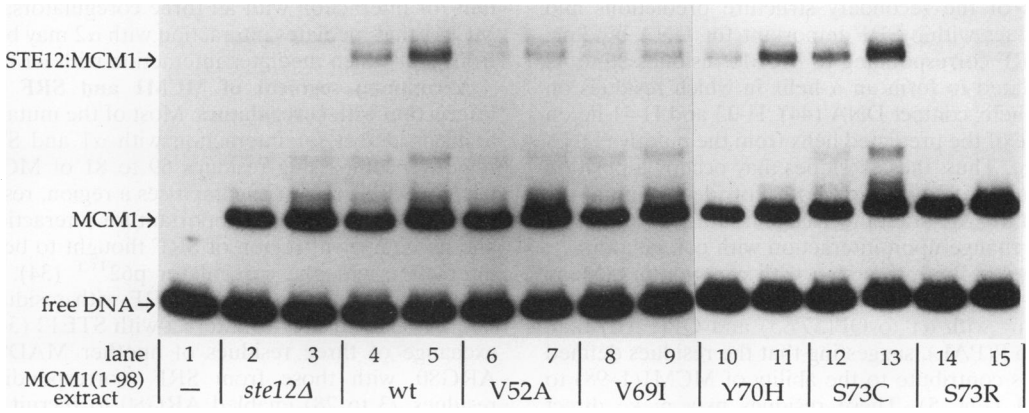

FIG. 4. Binding of MCM1(1–98) mutants and STE12 to *STE2* DNA. Bandshift reactions were performed with a *STE2* fragment that contains P-box and PRE sequences as described in Materials and Methods. Lanes: 1, no extract; 2 to 15, 5 (even-numbered lanes) and 10 (odd-numbered lanes) μg of extract from strains carrying wild-type (wt) and mutant versions of MCM1(1–98) as indicated. Extract used in lanes 2 and 3, *ste12*Δ, was from a strain carrying wild-type MCM1(1–98) and a *ste12*Δ::*URA3* disruption (see Materials and Methods).

α1-MCM1-QP(*STE3*) complexes at levels equivalent to their ability to form MCM1-P(PAL) complexes, suggesting that defects in DNA binding rather than defects in interaction with α1 caused their deficiencies in activating α-specific genes. Why might a general defect in DNA binding affect expression of QP(*STE3*)-driven reporters more than P(PAL)-driven reporters? MCM1-P(PAL) and α1-MCM1-QP(*STE3*) complexes form in vitro at very different rates (halftimes of <2 min versus 14 min [4]). Perhaps this difference leads to substantial differences in the efficiency of formation of transcription complexes at P(PAL) and QP(*STE3*) in vivo if the binding affinity of MCM1 is also reduced. Alternatively, it is conceivable that defects other than the DNA binding deficiencies of these mutants are responsible for their α-specific phenotypes. For example, perhaps some of the residues targeted by these substitutions have a direct role in activating transcription of α-specific genes but have only a minor role in activating transcription of P(PAL)-controlled genes. Either of these explanations may also apply to mcm1-1, the defining MCM1 mutant (6, 36), which is deficient for expression of α-specific genes but appears to have as its primary biochemical defect a reduced ability to bind DNA rather than to interact with α1 (4, 26, 38).

Mutants with an altered conformation. Mutants at positions H-33 and H-41 had unusual and intriguing phenotypes. Many of these mutants bound autonomously to P(PAL) and with α1 to QP(*STE3*) as well as or better than wild-type MCM1(1–98), yet they were deficient for expression of the QP(*STE3*) reporter. Moreover, some of the mutants showed a marked difference in their ability to bind with α1 to QP(PAL) compared with their ability to bind to QP(*STE3*). One possible explanation for the reduced ability of these mutants to express α-specific genes is that they are bound to the DNA in an altered conformation that prevents transcription activation of α-specific genes. Indeed, it has been proposed that MCM1 has a different conformation when bound alone at degenerate P boxes than when bound to palindromic P boxes (53). Thus, in addition to recruiting MCM1 to bind DNA, α1 may induce a conformational change in MCM1 that converts it to a form that is more competent to activate transcription from degenerate P boxes. We suggest that particular substitutions at H-33 and H-41 alter the conformation of MCM1 and make it unable to undergo the α1-induced conformational change. In some cases, the altered conformation of the H-33 and H-41 mutants even diminishes their ability to interact with α1 when bound to palindromic P boxes.

On the basis of the secondary structure predictions and analysis of residues within SRF important for DNA binding, the region of SRF corresponding to MCM1 residues 28 to 42 has been postulated to form an α-helix in which residues on one face of the helix contact DNA (44). H-33 and H-41 lie on the opposite side of the predicted helix from the putative DNA binding interface. Thus, these residues may occupy a position where they could be important both for determining the conformation of MCM1 bound to DNA and for transmitting a conformational change upon interaction with coregulators.

Mutants defective for interaction with coregulators. Many of the MCM1(1–98) point mutants showed significantly greater defects in binding with α1 to QP(*STE3*) and QP(PAL) than autonomously to P(PAL), suggesting that the residues defined by these mutants contribute to the ability of MCM1(1–98) to interact with α1 (Fig. 5). These residues may make direct contacts with α1, or they may play a structural role in maintaining the integrity of the surface of MCM1(1–98) that contacts α1. The defects of the V69I, Y70H, S73R, and V81F mutants at interaction with α1 were particularly strong (more than fourfold) and are likely to account for their deficiency at expression of α-specific genes in vivo. On the other hand, the H33L, H41Q, V52A, T71S, S73C, and E78K mutants had relatively weak α1 interaction defects, and these defects may not account fully for their in vivo phenotypes.

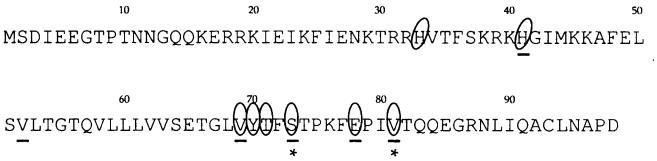

FIG. 5. Summary of residues in MCM1(1–98) important for interaction with coregulators. The locations of residues of MCM1 at which substitutions lead to a decrease in the ability to form protein-DNA complexes in vitro with α1 (circled), STE12 (underlined), and α2 (*) are shown on the primary amino acid sequence of MCM1(1–98). H-33 and H-41 are indicated (slanted circles) because the effect of most of the substitutions at these residues on interaction with α1 in vitro is dependent upon the sequence at which MCM1 is bound (see text).

Bandshift analysis of the ability of the mutants to form complexes in conjunction with STE12 at an **a**-specific control element revealed that many of the MCM1 residues important for interaction with α1 are also important for interaction with STE12 (Fig. 5). The H41Y, V69I, and V81F mutants showed modest defects (~2-fold) in interaction with STE12, whereas the H41R, V52A, S73R, and E78K mutants showed strong defects (3- to 10-fold). The finding that many of the same positions in MCM1 are important for interaction with both α1 and STE12 suggests that the mechanisms by which MCM1(1–98) interacts with the two proteins are similar. However, two mutants, Y70H and T71S, were strongly defective for interaction with α1 but were proficient at formation of STE12-MCM1-DNA complexes. Conversely, other mutants, for example, E78K, were substantially more deficient for interaction with STE12 than for interaction with α1. Thus, although α1 and STE12 probably interact with a common surface of MCM1, there may be residues that provide specificity for each interaction.

Only two mutants with reduced ability to interact with α1 and/or STE12 were also impaired in the ability to bind in conjunction with α2 in vitro. These two mutants, S73R and V81F, showed approximately twofold decreases in the ability to bind with α2. Thus, although some residues may be important for interaction with all three coregulators, the surface of MCM1 that mediates interaction with α2 may be quite distinct from that which mediates interaction with α1 and STE12.

A common segment of MCM1 and SRF is involved in interaction with coregulators. Most of the mutants that exhibit a strong defect for interaction with α1 and STE12 fall in a region encompassing residues 69 to 81 of MCM1(1–98). Intriguingly, this region encompasses a region, residues 73 to 78, of MCM1 thought to be important for interaction with STE12 and an equivalent region of SRF thought to be important for interaction with the coregulator $p62^{TCF}$ (34). Replacing the corresponding amino acids of SRF with residues 73 to 78 of MCM1 enabled SRF to interact with STE12 (34). Conversely, exchange of three residues of another MADS box protein, ARG80, with those from SRF (corresponding to MCM1 residues 73 to 78) enabled ARG80 to recruit $p62^{TCF}$ into a ternary complex (34). Finally, many changes within residues 177 to 205 of SRF, which correspond to MCM1 residues 52 to 80, were required to eliminate interaction of SRF with $p62^{TCF}$ (45). Thus, our findings provide further evidence that the same

segments of MCM1 and SRF are important for interaction with their respective coregulators.

Previous studies of SRF and MCM1 have led to the conclusion that the N-terminal half of the MADS box domains within these proteins (up to approximately residue 42 in MCM1) mediates DNA binding, while the C-terminal half mediates dimerization and interaction with coregulators (35, 38, 44). Our findings are consistent with this view but suggest refinements to the model as well. Substitutions at positions E-22 and E-27 of MCM1 affected DNA binding, and most of the substitutions that affected interaction with coregulators lay in the region from residue 69 to residue 81. However, some substitutions in this latter region, notably T71A and E78V, also caused a modest two- to fourfold reduction in DNA binding. It is conceivable that the binding defects of these mutants are due to defects in dimerization rather than defects in recognition of DNA. In fact, substitutions at hydrophobic residues of SRF corresponding to positions 59, 61, and 72 of MCM1 strongly affected dimerization of SRF, suggesting that dimerization of MADS box proteins is mediated by a hydrophobic interface made up of residues in this region (44). Thus, residues that mediate dimerization seem to be interspersed with residues important for coregulator interactions. However, analysis of other MCM1(1–98) mutants with stronger phenotypes indicates that this region may affect direct interaction with DNA rather than, or in addition to, dimerization. The finding that inviable Y-70 mutants (e.g., Y70S and Y70D) exhibit a severe defect in the ability to bind DNA as a homodimers but are capable of binding as heterodimers with full-length MCM1 (see Results) suggests that some substitutions in this region affect DNA binding affinity rather than dimerization. Interestingly, the SRF Y195D substitution, which is analagous to MCM1 Y70D, had no effect on the ability of SRF to bind DNA (44). Thus, although the means by which SRF and MCM1 bind DNA and interact with coregulators may be similar, there are probably distinct differences in the manner in which different regions of the folded proteins relate to one another.

In summary, the point mutants analyzed in this study define a stretch of ~13 amino acids (residues 69 to 81) that seems to be important for interaction with α1 and STE12. A homologous region of another MADS box protein, SRF, is important for mediating interaction with coregulatory proteins like $p62^{TCF}$. This region of MCM1 does not seem to constitute an independent domain of the protein, however, because mutations within this region can also affect DNA binding and/or dimerization. Thus, although some functions of MCM1 appear to be grossly organized in a fashion concordant with the primary sequence—DNA binding in the N-terminal half of residues 1 to 98, dimerization, and interaction with coregulators in the C-terminal half—MCM1(1–98) appears to fold into a compact structure in which regions important for DNA binding, dimerization, transcription activation, and interaction with coregulators are in close proximity to one another.

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