MOLECULAR AND CELLULAR BIOLOGY, Apr. 1997, p. 1881–1889 Vol. 17, No. 4
0270-7306/97/$04.00+0

DNA-Binding Specificity of Mcm1: Operator Mutations That Alter DNA-Bending and Transcriptional Activities by a MADS Box Protein

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Received 19 September 1996/Returned for modification 28 October 1996/Accepted 31 December 1996

The yeast Mcm1 protein is a member of the MADS box family of transcriptional regulatory factors, a class of DNA-binding proteins found in such diverse organisms as yeast, plants, flies, and humans. To explore the protein-DNA interactions of Mcm1 in vivo and in vitro, we have introduced an extensive series of base pair substitutions into an Mcm1 operator site and examined their effects on Mcm1-mediated transcriptional regulation and DNA-binding affinity. Our results show that Mcm1 uses a mechanism to contact the DNA that has some significant differences from the one used by the human serum response factor (SRF), a closely related MADS box protein in which the three-dimensional structure has been determined. One major difference is that 5-bromouracil-mediated photo-cross-linking experiments indicate that Mcm1 is in close proximity to functional groups in the major groove at the center of the recognition site whereas the SRF protein did not exhibit this characteristic. A more significant difference is that mutations at a position outside of the conserved $CC(A/T)_6GG$ site significantly reduce Mcm1-dependent DNA bending, while these substitutions have no effect on DNA bending by SRF. This result shows that the DNA bending by Mcm1 is sequence dependent and that the base-specific requirements for bending differ between Mcm1 and SRF. Interestingly, although these substitutions have a large effect on DNA bending and transcriptional activation by Mcm1, they have a relatively small effect on the DNA-binding affinity of the protein. This result suggests that the degree of DNA bending is important for transcriptional activation by Mcm1.

The MADS box family of DNA-binding proteins is a growing class of transcriptional regulatory proteins that are involved in the regulation of diverse cellular functions. Named for Mcm1, Arg80, Agamous, Deficiens, and serum response factor (SRF), MADS box proteins are grouped by their strong sequence similarity within a 90-residue DNA-binding and dimerization domain that is distinct from other known classes of DNA-binding proteins (3, 9, 24, 28, 40, 53). Currently, there are over 30 members of this class of proteins, which can be found in organisms ranging from the yeast *Saccharomyces cerevisiae* (Mcm1 and Arg80) to *Drosophila* (DRSF and MEF2) (1, 18), plants (Agamous, Deficiens, Globosa) (33, 40, 47, 53), and humans (SRF) (24, 45, 46). MADS box proteins are involved in a wide variety of functions ranging from transcriptional regulation of basic cellular processes, such as cell cycle control, to the regulation of developmental specific gene expression, such as floral development (1, 18–20, 22, 24, 40, 44, 53). MADS box proteins therefore contain a ubiquitous motif involved in many functions, much like the homeodomain and B-zip classes of transcriptional regulatory proteins (26). A thorough knowledge of the nature of the protein-DNA interactions exhibited by this class of transcription factors is therefore important for understanding the regulation of many cellular and developmental processes.

The yeast Mcm1 and human SRF proteins share almost 70% sequence identity in their MADS box regions and bind to similar DNA recognition sites (24, 31, 52). The recognition site of these proteins is frequently referred to as a "CArG" box, a 10-bp site with the general sequence characteristics of $CC(A/T)_6GG$. The high degree of similarity between the two proteins is further displayed by their ability to interact with each other's cofactors. For example, in humans, the SRF and $p62^{TCF}$ proteins form a ternary complex on the serum response element (SRE) (22, 35, 36), and Mcm1 can bind cooperatively with $p62^{TCF}$ to the SRE (22). Likewise, in yeast, the Mcm1 and α2 proteins bind cooperatively to sites upstream of **a**-specific genes (17, 29) and SRF can bind cooperatively with α2 to these sites (48).

The crystal structure of the SRF MADS domain in complex with DNA has recently been determined (30). The structure shows that three regions of the conserved DNA-binding and dimerization domain are involved in protein-DNA interactions. First, antiparallel coiled-coil α-helices align parallel to the DNA minor groove and make numerous phosphate as well as base-specific contacts in the major groove. Second, an N-terminal extension from the α-helices of each monomer provides contacts in the minor groove and makes several base-specific contacts at positions within the center of the recognition site. Finally a β-loop, protruding from the central dimerization region, provides several phosphate contacts outside of the CArG box within the major groove. In addition, the structural determination shows that the DNA-binding site is bent at an angle of 72°, with the majority of this perturbation occurring at the distal portion of the recognition site (30). The facts that Mcm1 and SRF have strong sequence similarity, bind similar DNA sites, and interact with each other's cofactors provide a strong rationale for using the SRF structure as a model for predicting how Mcm1 might recognize and bind DNA (30) (Fig. 1).

To more fully understand the nature of the protein-DNA interactions of Mcm1, we have used a mutational analysis of the Mcm1-binding site to determine the sequence-specific requirements for transcriptional regulation in vivo and DNA binding in vitro. Our results show that there are differences in the mechanism used by Mcm1 and SRF to contact the DNA. We also found that in addition to sequences within the CArG

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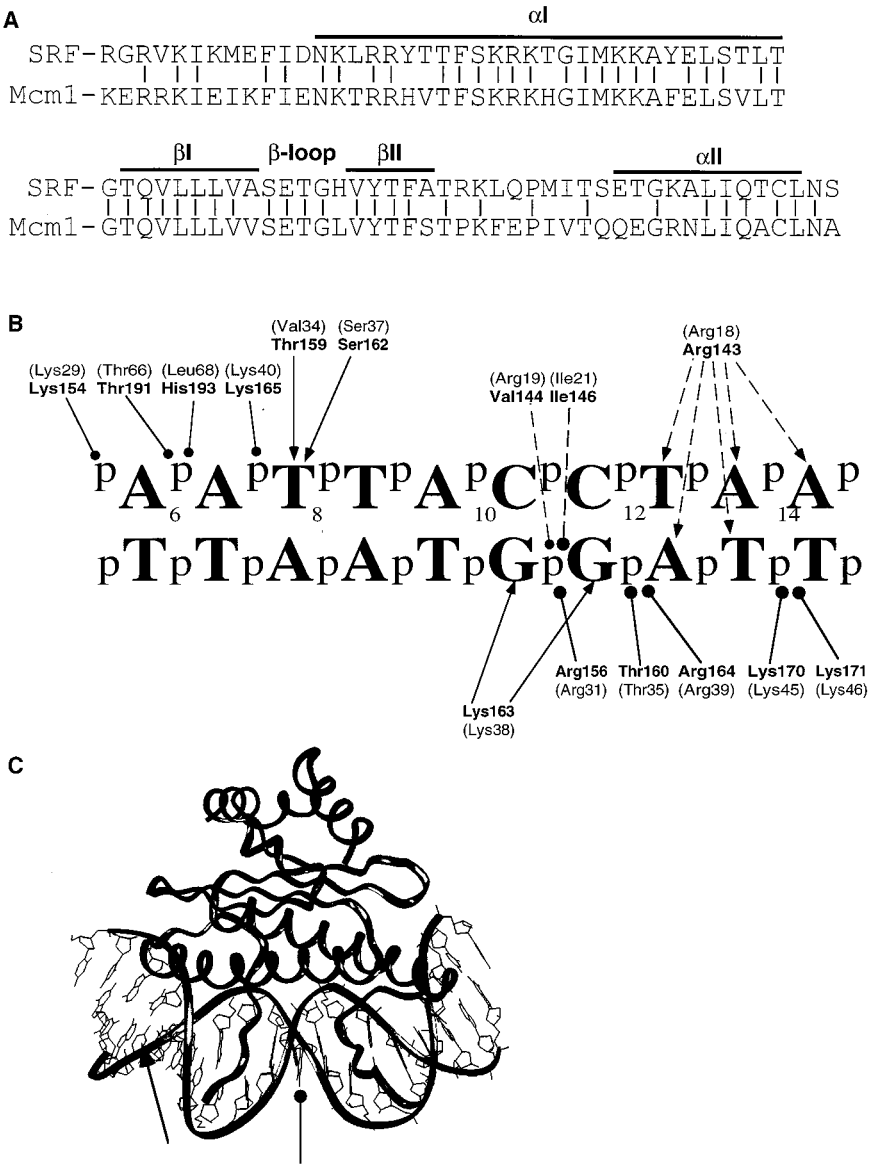


FIG. 1. Alignment of the amino acid sequences of SRF and Mcm1, schematic of predicted SRF-AMCS protein-DNA contacts, and representation of SRF-DNA cocrystal structure. (A) Amino acid alignment of the MADS box region of SRF (residues 141 to 221) and Mcm1 (residues 16 to 96). Above the SRF amino acid sequence are the secondary-structure characteristics found in the SRF cocrystal structure (29). (B) Schematic of the predicted protein-DNA contacts between SRF and one half-site of the AMSC based on the SRF cocrystal structure (29). The contacts in the major groove are delineated by solid lines, while minor-groove contacts are represented by broken lines. Residues in parentheses are the corresponding positions in Mcm1. The numbering of the base pairs corresponds to the position in the α2/Mcm1 symmetric consensus sequence. The arrows indicate base-specific contacts, and the circles indicate sugar-phosphate backbone contacts. (C) Structure of the MADS box domain of SRF in complex with DNA (29). The arrow indicates the thymine at position 9, which was shown to be important for Mcm1-dependent DNA bending. The ball and stick shows the location of BrU-mediated cross-linking at position 15.

box, sequences outside of this site play a critical role in transcriptional regulation by Mcm1. Although these substitutions have a large effect on DNA bending by Mcm1, they have a relatively small effect on the DNA-binding affinity of the protein, suggesting a link between DNA bending and transcriptional activation by Mcm1.

MATERIALS AND METHODS

Yeast, bacterial strains, and β-galactosidase assays. The yeast strain 246.1.1 (*MATα trp1 leu2 ura3 his4*) was used for measuring α2/Mcm1-mediated transcriptional repression (37). The yeast strains GY12 (*MAT***a** *his4-917 lys2-128 leu2 ura3-52 nrp2-1*) and the wild-type parent strain JF819 (*MAT***a** *his4-917 lys2-128 leu2 ura3-52*) were used in the measurement of Mcm1-mediated transcriptional activation (54). Constructs containing the different Mcm1-binding-site mutations were transformed into the appropriate yeast cell type and selected for growth in synthetic complete medium lacking uracil. Methods for measuring β-galactosidase activity were described previously (16). The strain of **a** cells used in the activation experiments (GY12) contains the *nrp2* mutation, which results in higher levels of activation by Mcm1 than a wild-type *NRP2* strain (54). To ensure that this mutant strain did not alter the DNA recognition characteristics of Mcm1, many of the operator mutants were assayed in the wild-type parent strain (JF819) and the mutant strain (GY12). Although the level of *lacZ* expression was increased in the mutant strain, the overall fold difference was nearly identical for all cases.

Cloning and protein expression were performed in *Escherichia coli* DH5α and BL21, respectively.

Oligonucleotides. The oligonucleotides used in the construction of the reporter plasmids and the 5-bromouracil (BrU)-mediated photo-cross-linking were synthesized on an ABI 392 oligonucleotide synthesizer. Oligonucleotides used for the construction of reporter plasmids were designed as a symmetric α2/

Mcm1-binding site based on a consensus of α2/Mcm1 sites found in the promoter regions of **a**-specific genes (55). The oligonucleotides were constructed such that symmetric mutations were introduced in both of the Mcm1 half-sites. Individual oligonucleotides were synthesized for each of the three possible substitutions at every position within the Mcm1-binding site. The α2/Mcm1 symmetric consensus site (AMSC) (5′-TCGACATGTAATTACCTAATTAGGTAATTACATG-3′) and mutant oligonucleotides were then self-annealed, leaving TCGA overhangs to allow cloning into the *Xho*I site of pTBA23. Oligonucleotides used in the cross-linking experiments were synthesized by using the photoreactive reagent, 5-bromo-2′-deoxyuridine-β-cyanoethylphosphoramidite (Glen Research, Inc.) in place of thymine phosphoramadite individually at thymine positions in the top DNA strand of MCMSCT (5′-GCCCTACCTAATTAGGTACCCG-3′) at base pairs corresponding to positions 8, 9, 10, 13, 14, and 15 of the AMSC site (55). The complementary bottom strand MCMCSB (5′-CGGGTACCTAATTAGGT AGGGC-3′), which did not contain BrU, annealed to the BrU-containing oligonucleotides to produce a double-stranded recognition site (MCMCS). All synthesis and further manipulations of the oligonucleotides were carried out in the dark.

Plasmid constructions. pTBA23 (2μm *URA3* Amp^r) is a derivative of pLGΔ312 (10), a *CYC1-lacZ* fusion described previously (21). Oligonucleotides containing the AMSC or symmetric mutants were kinase treated, self-annealed, and ligated into the *Xho*I site of pTBA23 to produce pTBA24 or its derivatives. Plasmids containing operator inserts were screened by restriction digestion and verified by sequencing. These constructs were used to measure Mcm1-dependent repression of the *CYC1* promoter. Plasmids used to measure Mcm1-dependent activation were constructed by digesting pTBA24 and its derivatives with *Bgl*II, followed by self-ligation to produce pTBA43 and the mutants. This step removes the *CYC1* upstream activation sequence (UAS) sites from the promoter region, leaving the α2/Mcm1 operator site and *CYC1* TATA box intact. Transformants were screened by restriction digestion and sequence analysis to ensure the *CYC1* upstream activation sequence sites fragments had been removed and the α2/Mcm1 operator site was correct.

Protein purification. The $Mcm1_{18–96}$ and $Mcm1_{1–96}$ proteins used in the cross-linking and gel shift assays were purified in the following manner. *E. coli* BL21 cells were transformed with either pTBA22 or pTBA25, protein expression vectors in which the sequences coding for Mcm1 residues 18 to 96 and 1 to 96, respectively, were fused in frame behind the maltose-binding protein. Luria-Bertani medium plus ampicillin (10 liters) was inoculated, and the solution was allowed to reach an optical density at 600 nm of ~0.7. Protein expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM. The cells were harvested by centrifugation and frozen at −70°C. The fusion protein was released from the cells by sonication in column buffer (CB) (20 mM Tris-HCl [pH 7.5], 200 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol, 25 mM sodium acetate [pH 7.0], 5% glycerol, 1.0 μM phenylmethylsulfonyl fluoride, 50 μM *N*-tolyl-L-phenylalanine chloromethyl ketone [TPCK]). The lysate was then centrifuged for 20 min at 10,000 × *g*, and the pellet was discarded. The supernatant was diluted fivefold and placed onto 100 ml of amylose resin (New England Biolabs). The fusion protein was eluted from the column with 5 mM maltose, and fractions containing the fusion protein were pooled. $CaCl_2$ was then added to the pool to a final concentration of 2.0 mM, and then 1,000 U of the thrombin protease (Sigma) was added. The protein pool was incubated on ice for 48 h or until the thrombin cleavage was complete. The cleavage reaction was loaded onto a heparin-Sepharose high-pressure liquid chromatography column (Pharmacia), which retained the Mcm1 protein and allowed the maltose-binding protein to flow through. The Mcm1 protein was eluted with a gradient of 0.2 to 1.5 M NaCl in CB. The proteins isolated by this procedure were >95% homogenous and consisted of two nonnative N-terminal amino acids, Gly_1-Ser_2, followed by the native Mcm1 residues 18 to 96 or 1 to 96.

EMSA. The DNA fragments used in the gel shift analysis were synthesized by PCR amplification of the operator sites of the transcriptional reporter plasmids. The oligonucleotides W-340 (5′-CACGCCTGGCGGATCTGC-3′), which was [γ-^{32}P]ATP end labeled and purified on a Sephadex G-25 spin column, and W341 (5′-GCCCACGCTAGGCAATC-3′) anneal on either side of the Mcm1 operator site in the *CYC1* promoter region and were used to amplify a 120-bp fragment containing the Mcm1 recognition site. The PCR-generated fragments were then purified by native polyacrylamide gel electrophoresis.

The circular permutation assays were performed with a derivative of pGD579, a pBR322-based construct which contains a tandem repeat of bp 1 to 375 separated by a 24-bp polylinker site (38). The oligonucleotides W1513 (5′-AAT TACCTAATTAGGTAATTAGCT-3′) and W1514 (5′-AATGACCTAATTAG GTCATTAGCT-3′), which are self-complementary and contain the wild-type and T_9G mutant Mcm1-binding sites, respectively, were cloned into the *Sac*I site of the pGD579 polylinker. Each construct was then cleaved with either *Bam*HI, *Eco*RI, *Nhe*I, or *Hin*dIII to produce circularly permuted 417-bp fragments. The fragments were labeled by incorporating [α-^{32}P]dATP or [α-^{32}P]dCTP with Klenow polymerase and gel purified. A schematic of the restriction fragments is found in Fig. 5. The apparent DNA-bending angles were calculated based on the Thompson and Landy relationship, $\mu_M/\mu_E = \cos \alpha/2$, where μ_M is the complex with the lowest mobility and μ_E is the complex with the highest mobility (43).

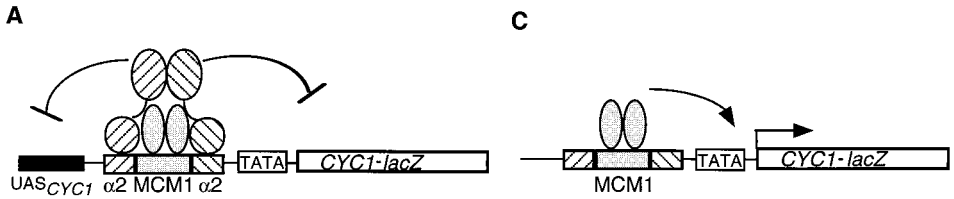
The purified $Mcm1_{1–96}$ proteins were incubated for 1 to 3 h at room temperature with the DNA fragment in 20 mM Tris (pH 7.6)–200 mM NaCl–5 mM $MgCl_2$–0.1 mM EDTA–0.1% Nonidet P-40, 10% glycerol, 10 μg of herring sperm DNA per ml. The circular-permutation electrophoretic mobility shift assay (EMSA) was performed with a 6% polyacrylamide gel (37.5:1) in 1× Tris-borate-EDTA (TBE) at 200 V, while all other EMSA were performed with a 6% polyacrylamide gel (37.5:1) in 0.5× TBE. EMSA gels were dried and visualized with a Molecular Dynamics 425E PhosphorImager. The binding affinity of Mcm1 for the various DNA sites was calculated by plotting the concentration of Mcm1 against the fraction of probe bound, and the equilibrium constant was determined by linear regression.

Cross-linking assays. The BrU-mediated protein-DNA cross-linking reactions were performed by incubating protein and DNA reaction mixtures for 1 h at 22°C in 20 mM Tris (pH 7.6)–300 mM NaCl–5 mM $MgCl_2$–0.1 mM EDTA–0.1% Nonidet P-40–10% glycerol–10 μg of herring sperm DNA per ml. The concentration of the $Mcm1_{18–96}$ protein in these reactions was 2 μM, while the ^{32}P-5′-end-labeled BrU-containing or non-BrU-containing recognition site DNA was at 1 μM in a total volume of 100 μl. Following incubation, the reaction mixtures were placed onto a siliconized glass plate and subjected to UV radiation for 90 s at 22°C in a Rayonet RPR100 photochemical reactor. The wavelength of irradiation was 254 nm (1280 ergs/mm^2/s). The irradiated samples were ethanol precipitated and then resuspended in 25 μl of 5 M urea–50 mM NaOH–0.5 mM EDTA–0.025% xylene cyanol–0.025% bromophenol blue. The samples were then electrophoresed on 10% polyacrylamide–8 M urea gels, the gels were dried, and the cross-linking efficiencies were calculated with a Molecular Dynamics 425E PhosphorImager.

RESULTS

Mutations of base pairs outside of the CArG box effect transcriptional regulation by Mcm1. One function of the Mcm1 protein is to regulate transcription of cell-type-specific genes in yeast (for reviews, see references 7, 13, 15, 29, and 41). In the **a** cell type, Mcm1 binds to P boxes, palindromic sites in the promoter regions of the **a**-specific genes (***a**sg*), to activate the transcription of these genes. The P-box sites are flanked on both sides by the recognition sequence of the yeast homeodomain protein α2, a repressor of transcription that is expressed only in the α and diploid cell types. In α and diploid cells, Mcm1 and α2 bind cooperatively to these sites and function to repress transcription and prevent the expression of the ***a**sg*. The P box therefore serves as an Mcm1-mediated activator site in the **a** cell type and as a repressor site in the α and diploid cell types. To study the in vivo DNA-binding specificity of Mcm1 alone and in complex with α2, we have engineered a yeast transcriptional reporter plasmid, pTBA23, which contains a heterologous *CYC1* promoter fused to the *lacZ* gene. A DNA fragment containing a symmetric consensus α2/Mcm1 recognition sequence, termed the AMSC, which is based on the natural α2/Mcm1-binding sites found in the promoters of **a**sg, was synthesized and cloned into the *CYC1* promoter between the UAS and TATA box (55) (Fig. 2A). This construct was introduced into an α cell type, and *lacZ* expression was repressed 130-fold when compared to the expression in a construct without an α2/Mcm1 recognition site (Fig. 2B). To examine the Mcm1-mediated activation from the α2/Mcm1 site in the absence of α2, the *CYC1* UAS sequences were removed from the reporter construct used in the repression experiments. This allows for the measurement of Mcm1-dependent activation of the *CYC1-lacZ* fusion when transformed into **a** cells (Fig. 2C). In this assay, the presence of the AMSC results in eightfold-higher *lacZ* expression than from a construct without an α2/Mcm1-binding site (Fig. 2D). To examine the base-specific requirements at each position in the site, symmetric base pair substitutions (one in each Mcm1 half-site) were constructed and assayed for their effect on Mcm1-mediated repression and activation (Fig. 2B and D). All three possible mutations were made at each position. The relative contribution of each base pair can then be surmised by the decrease in repression or activation caused by each substitution when compared with the wild-type consensus site (AMSC).

MADS box proteins recognize a conserved DNA sequence termed the CArG box, a partially symmetric 10-bp site which



B

	T_8	T_9	A_{10}	C_{11}	C_{12}	T_{13}	A_{14}	A_{15}
A	18X	14X	**130X**	29X	6X	168X	**130X**	**130X**
C	7X	8X	6X	**130X**	**130X**	148X	60X	9X
G	5X	3X	94X	5X	7X	136X	141X	103X
T	**130X**	**130X**	9X	13X	107X	**130X**	126X	119X

D

	T_8	T_9	A_{10}	C_{11}	C_{12}	T_{13}	A_{14}	A_{15}
A	2X	1X	**8X**	1X	1X	4X	**8X**	**8X**
C	4X	1X	3X	**8X**	**8X**	7X	3X	2X
G	1X	1X	7X	3X	2X	8X	4X	3X
T	**8X**	**8X**	4X	1X	5X	**8X**	3X	4X

FIG. 2. In vivo assays to determine the DNA-binding specificity of Mcm1. (A) Assay for α2/Mcm1-dependent repression of the symmetric base pair substitution operator mutants. The reporter construct pTBA24 or its derivatives were transformed into a *MAT*α strain in which the Mcm1 and α2 proteins were expressed from their endogenous promoters. The α2/Mcm1-mediated repression was measured from the β-galactosidase activity produced from the *CYC1-lacZ* reporter gene under the control of the wild-type or mutant α2/Mcm1 operator sites. (B) Results of the saturation mutagenesis of the Mcm1 DNA-binding site on α2/Mcm1-dependent repression. The sequence of one Mcm1 half-site in the wild-type AMSC is shown in the top row. Each single substitution identified in the half-site has the corresponding symmetric substitution in the other half-site. In the presence of the wild-type α2/Mcm1 operator site (AMSC), *lacZ* expression is repressed 130-fold (1.9 ± 0.2 U of β-galactosidase activity) when compared with a construct without an α2/Mcm1 operator site (250 ± 12.9 U). The fold repression for the mutant substitutions was derived in the same manner, and the numbers are the mean of three independent transformants. (C) Assay for Mcm1-dependent activation of the symmetric base pair substitution mutants. The reporter construct pTBA43 and its derivatives were constructed by removing the *CYC1* UAS sequences from the plasmids used in the repression assays. This allows for measurement of Mcm1-dependent activation when the reporter constructs are transformed into a *MAT***a** strain, which does not express the α2 repressor protein. (D) Results of the saturation mutagenesis of the Mcm1 DNA-binding site on Mcm1-dependent activation. The sequence of one Mcm1 half-site in the wild-type AMSC is shown in the top row. Each single substitution identified in the half-site has the corresponding symmetric substitution in the other half-site. The AMSC activates *lacZ* expression eightfold (6 ± 0.9 U of β-galactosidase activity) when compared with a construct without an Mcm1-binding site (0.75 ± 0.1 U of β-galactosidase activity). The fold activation values for the mutant substitutions are derived in the same manner and are the mean of three independent transformants.

contains a strongly conserved CC and symmetrically related GG bases that flank 6 bp that are predominantly A or T. In the SRF cocrystal structure, both guanines in each half-site are contacted by residue K163 (30). The outer guanine is contacted at the O-6 position, while the inner guanine is contacted at the N-7 position. In Mcm1, the homologous residue is K38, and would be predicted to contact the O-6 and N-7 atoms of the guanines at positions 11 and 12 in the AMSC site, respectively (Fig. 1B). The results of the in vivo assays at position 11 strongly agree with this prediction as substitution with any other base results in significant decreases in activation and repression. Substitutions at position 12 also agree with predictions based on the SRF structure. Transversions from CG to AT or GC result in significant reduction in the level of repression and activation. However, the substitution from CG to TA, which retains the position of the purine N-7 hydrogen bond acceptor, results in near-wild-type levels of transcriptional activation and repression.

The next position in the AMSC site, bp 13, is a thymine and is only partially conserved (50%) in the natural Mcm1-binding sites found in the promoters of ***a****sg*. If Mcm1 and SRF make similar contacts to this position, the backbone amide of Mcm1 residue R18 would make a base-specific contact with the O-2 atom of the thymine in the minor groove and residues T35 and R39 would be involved in phosphate contacts with this base pair in the major groove (Fig. 1B) (30). We have found that all of the substitutions at this position have little or no effect on either repression or activation, and therefore a specific base pair is not required at this position for Mcm1 function.

In the SRF cocrystal structure, the R143 side chain lies in the minor groove between the deoxyribose rings and the C-2 atoms of the bases corresponding to A_{14} and A_{15} in the AMSC site (30). This side chain is involved in base-specific contacts with the N-3 of the center adenine and the O-2 of the thymine at the adjacent position. If the homologous residue of Mcm1, R18, is making similar contacts with the DNA at these positions, any other base pair at position 14 in the AMSC site would cause steric interference with this side chain, and the expected result would be a large decrease in Mcm1-mediated activation and repression. However, we observed that the effects of substitutions at position 14 result in less than a twofold effect on repression and activation, suggesting that the base-specific requirements at position 14 are not stringent for Mcm1 function in vivo. The only significant reduction in repression and activation found with base pairs in the center of the DNA site was the transversion of the position 15 AT to CG.

Substitutions were also made outside of the conserved CArG box to investigate whether these positions are important for Mcm1 function in vivo. In the SRF cocrystal structure, there are no contacts to the base pair corresponding to position 10 in the AMSC site. We would therefore not expect substitutions at this position to greatly affect Mcm1 activity. However, substitution with either a TA or CG results in a greater than a 10-fold decrease in the level of repression. Interestingly, the same mutations cause only a twofold reduction in activation. The discrepancy between the repression and activation data for this position is the largest observed in the study and was unexpected. Based on both the α2 and SRF crystal structures, it would not be predicted that this position would be important for either activation or repression. One possibility is that in

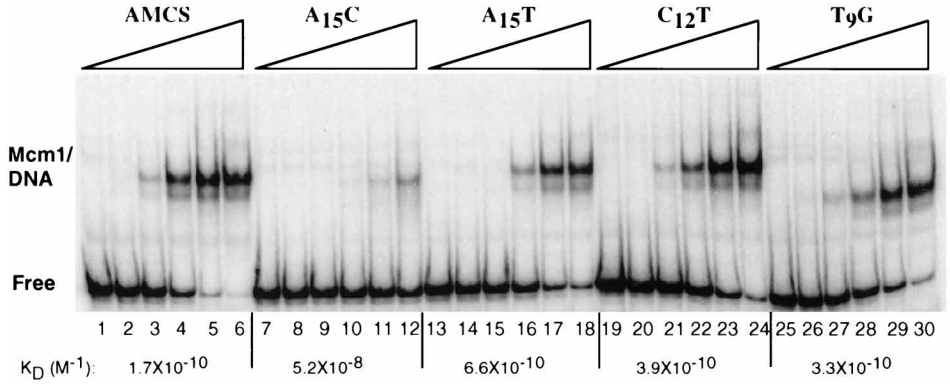


FIG. 3. EMSA analysis of mutant operator sites. EMSA analysis of Mcm1 binding to the AMSC (lanes 1 to 6) and the mutant sites $A_{15}C$ (lanes 7 to 12), $A_{15}T$ (lanes 13 to 18), $C_{12}T$ (lanes 19 to 24), and T_9G (lanes 25 to 30). Lanes 1, 7, 13, 19, and 25 contain labeled DNA sites in the absence of Mcm1 protein. Threefold titrations of Mcm1 were performed starting with a concentration of 2.2×10^{-11} M (lanes 2, 8, 14, 20, and 26) and ending with 1.8×10^{-9} M (lanes 6, 12, 18, 24, and 30). The observed dissociation constants for each binding site are listed in the figure under the corresponding lanes.

complex with Mcm1, α2 makes a contact to this base pair which would explain the lower levels of repression.

Substitutions at position T_9 produced the most severe effect on Mcm1-dependent transcriptional activation observed in the data set. The level of β-galactosidase expression found with these mutations is indistinguishable from that in the reporter construct without an Mcm1 recognition site. In addition, this level of activity was found in both the *nrp2* mutant and the wild-type strains. These substitutions also result in a very large decrease in the level of repression. The effect of this substitution on repression was expected because the α2 protein makes a base-specific contact to this position in the DNA minor groove and substitutions of this base pair affect α2 DNA binding alone (39, 50, 55). However, the effect of these substitutions on activation indicates that this position is also important for proper Mcm1 function in vivo. This result was unexpected because the crystal structure of SRF bound to DNA shows neither a base-specific nor a phosphate contact to this position (30). In addition, in vitro selection studies did not suggest that a specific base at this position was required for Mcm1 DNA binding or for other MADS box proteins (12, 25, 31, 52).

In the SRF cocrystal structure, the residues T159 and S162 are involved in a hydrophobic interaction with the thymine methyl group of the base pair corresponding to position 8 in the AMSC site. In Mcm1, S37 is conserved with the homologous S162 of SRF while V34 is substituted for the T159 residue. Substitutions of the base pair at position 8 result in a reduction in the levels of activation, with a transversion to GC having the largest effect. The remaining substitutions result in a two- to fourfold reduction in Mcm1-dependent activation. In addition, the levels of repression resulting from these substitutions were dramatically reduced, which was expected since α2 is also involved in a minor groove contact with the thymine at this position (50). The results of the substitutions at this position indicate that this base pair is important for Mcm1-dependent transcriptional regulation and also suggest that the base-specific requirements of Mcm1 and SRF may be conserved at this position.

Substitutions were also made at positions 6 and 7, as the SRF crystal structure indicates phosphate backbone contacts at these positions. Position 6 is contacted by K154 of the binding helix, and position 7 is contacted by T191 and H193 of the β-loop. In Mcm1, the lysine and threonine are conserved and correspond to K29 and T66, respectively. In contrast, a leucine residue is found at position 68 of Mcm1, which corresponds to H193 of SRF. Substitutions at positions 6 and 7 did not affect Mcm1-mediated activation (data not shown), indicating that the identity of these base pairs does not have a large effect on the position of the phosphate backbone or that Mcm1 does not form these contacts.

In summary, the results of the reporter assays indicate that in the context of a symmetric DNA-binding site, Mcm1 prefers the recognition sequence 5′-TTACCNAATTNGGTAA-3′ in vivo. These results show that the sequence specificity of Mcm1 extends well outside the edges of the CArG box and that base pairs in this region are important for transcriptional regulation. The effects of the specific substitutions on activation are similar to the effects on repression, indicating that Mcm1 has similar sequence specificity when binding alone or in complex with α2.

Analysis of Mcm1 DNA-binding affinity in vitro. The results shown above indicate that many of the substitutions have significant effects on Mcm1-mediated transcriptional repression and activation in vivo. To determine if the defects in transcriptional regulation were caused by decreases in the DNA-binding affinity of the Mcm1 protein for the mutant sites, EMSA were performed (Fig. 3). In general, the results of the gel shift analysis are in good agreement with the in vivo repression and activation data. Substitutions in the recognition sequence which result in large decreases in repression and activation also have decreased Mcm1 DNA-binding affinity in vitro. For example, the $A_{15}C$ substitution results in a 14-fold decrease in repression in vivo and a 33-fold decrease in DNA-binding affinity when compared with the wild-type site. On the other hand, the $A_{15}T$ substitution decreases Mcm1-binding affinity only fourfold in vitro, which compares favorably with the small effects seen with this mutation in vivo. Another example is the $C_{12}T$ mutation, which has a binding affinity which is decreased only slightly greater than twofold, while the in vivo repression and activation levels are only slightly decreased. We have performed EMSA analysis of the DNA-binding affinity of substitutions at other positions, and with the exception of substitutions at position 9, which will be discussed below, we have found a strong correlation between the in vitro and in vivo data. The results of the EMSA analysis therefore provide strong evidence that the decreases in repression and activation caused by these substitutions are most probably the result of decreased Mcm1 DNA-binding affinity for the mutant recognition sites.

BrU-mediated photo-cross-linking shows major groove contacts in the center of the site. As a complementary approach to the mutational analysis described above, we used protein-DNA cross-linking experiments to determine which positions in the recognition site are in close proximity to the Mcm1 protein. The photoactivated cross-linking reagent BrU, an isosteric analog of thymine, has been widely used to identify positions in a recognition site that are in close contact with a DNA-binding protein (2, 4, 8). The formation of a BrU-mediated covalently linked protein-DNA complex indicates that the protein is in van der Waals contact with the methyl group of a substituted thymine. Oligonucleotides that contain an Mcm1-binding site with individual BrU substitutions at each thymine in the half-site, positions 8, 9, 10, 13, 14, and 15, were synthesized. The singly substituted BrU-containing sites were labeled and allowed to incubate with the Mcm1 protein in the absence of light. The reaction mixtures were then subjected to UV radiation, and the results were visualized through denaturing polyacrylamide gel electrophoresis PAGE to separate the covalently linked Mcm1-DNA complex from free DNA, free protein, and noncovalently linked protein-DNA complexes. The results indicate that no BrU-mediated photo-cross-linking occurred at positions 8, 9, 10, and 13 (Fig. 4). Predictions based on the SRF crystal structure would suggest that a BrU substitution at position 8 may result in the formation of a cross-linked product since T159 and S162 were shown to be involved in interactions with the methyl group of this thymine. However, the failure to detect a cross-link does not necessarily indicate that a protein is not in direct contact with a thymine. For example, SRF also did not form a BrU-mediated cross-linked band with the BrU substitution at position 8 (data not shown). Mcm1-DNA cross-linked products, however, were observed at positions 14 and 15, indicating that the Mcm1 protein is in close contact with the thymine methyl groups at the center of the recognition site (Fig. 4). The controls show that the cross-linked complexes were BrU mediated, Mcm1 and UV dependent, and specific for the Mcm1 DNA recognition site (Fig. 4). The cross-linking efficiency is ~4% at position 15 but falls to <1% at position 14. The higher efficiency of cross-linking found at position 15 may indicate the close proximity of an aromatic amino acid (His, Phe, Trp, or Tyr) (2, 8, 49).

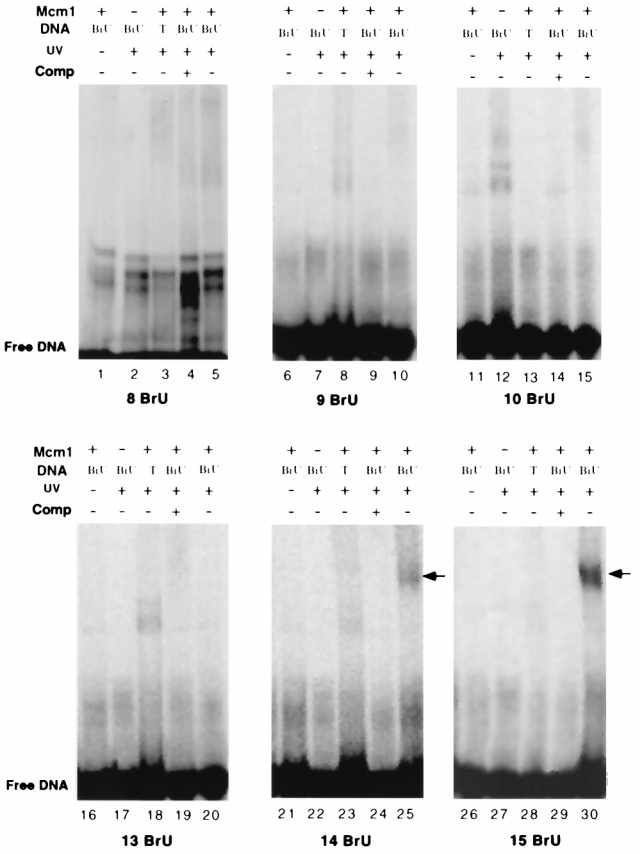


FIG. 4. Formation of cross-linked Mcm1-DNA complexes. Urea denaturing PAGE of the BrU-mediated photo-cross-linking reactions at positions 8 (lanes 1 to 5), 9 (lanes 6 to 10), 10 (lanes 11 to 15), 13 (lanes 16 to 20), 14 (lanes 21 to 25), and 15 (lanes 26 to 30). Lanes 1, 6, 11, 16, 21, and 26 are control reactions omitting UV. Lanes 2, 7, 12, 17, 22, and 27 are control reactions omitting the Mcm1 protein. Lanes 3, 8, 13, 18, 23, and 28 are control reactions with labeled MCMCS, the Mcm1-binding site without BrU, denoted by T in the figure. Lanes 4, 9, 14, 19, 24, and 29 are control reactions in which a 10-fold excess of unlabeled MCMCS competitor was added. Lanes 5, 10, 15, 20, 25, and 30 are the cross-linking reactions. Arrows indicate the position of the cross-linked Mcm1-DNA products.

Differences in DNA bending and binding by Mcm1 and SRF. As mentioned above, one of the discrepancies between the in vivo and in vitro analyses was that substitutions at position 9 result in relatively minor decreases (two- to fourfold) in DNA-binding affinity but produced severe effects on Mcm1-mediated repression and activation. Interestingly, we noticed that the protein-DNA complexes with substitutions at this position appeared to migrate at a higher rate in EMSA than did the wild-type AMSC or complexes with substitutions at other positions. The migration of a protein-DNA complex in an EMSA is dependent on many factors such as the size and charge of a protein, as well as the length of the DNA fragment (6). Another important factor that influences the migration of a protein-DNA complex is the amount of DNA bending induced by the protein (51). In the SRF cocrystal structure, the DNA recognition site is bent at an angle of 72° (30). In addition, EMSA experiments have shown that binding by Mcm1 causes significant DNA bending (38). Therefore, one possible explanation for the faster-migrating complex was that substitutions at position 9 alter the degree of Mcm1-dependent DNA bending. To further examine this phenomenon, circular permutation assays were performed on the wild-type consensus symmetric and T_9G mutant sites (51) (Fig. 5). The results indicate that the T_9G mutant site does not undergo the same degree of bending as the wild-type site. We have calculated an apparent bend angle of 95°C for the wild-type site, while the mutant operator site was bent at an apparent angle of only 54°. Clearly, Mcm1 is not able to bend the mutant site to the same degree as the wild-type site, suggesting that this position is important for maximum Mcm1-dependent bending of the recognition site.

In the SRF cocrystal structure, there are no apparent protein-DNA contacts with the base pair analogous to position 9 in the AMSC site (30), and therefore the in vivo activity and in vitro binding results of substitutions at this position in the Mcm1 site were unexpected. However, the crystal structure shows that the adjacent base pair, T_8, is contacted by residues from α-helix I of SRF, and it was suggested that this interaction may play a role in the protein-dependent DNA bending (30). One possible explanation for our results is that Mcm1 is involved in a base-specific contact with position 9 in the AMSC and that this interaction plays an analogous role in producing DNA bending to that of the SRF base-specific contact with position 8. An alternative explanation is that neither SRF nor Mcm1 directly contacts the recognition site at position 9 but that a specific base pair is required for allowing maximal DNA bending. If the latter possibility is correct, it would be pre-

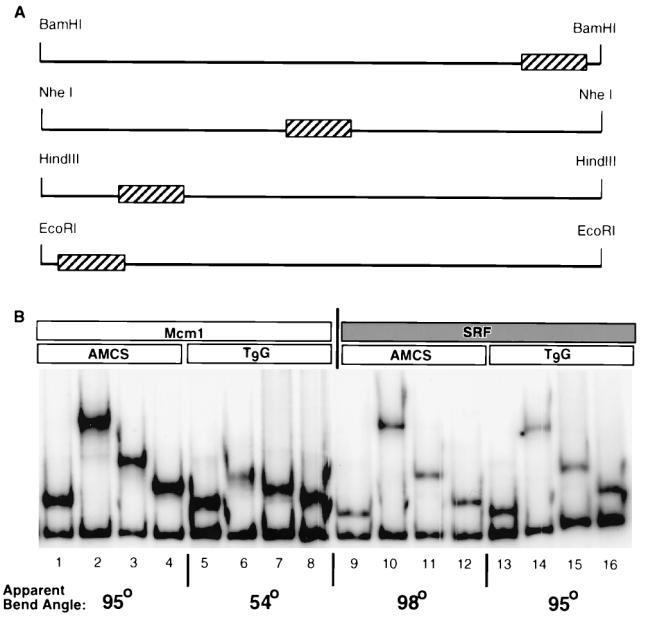


FIG. 5. Mutations at position 9 reduce the DNA bending of Mcm1 without affecting SRF. (A) Diagram of the relative position of the α2/Mcm1 recognition site in the 417-bp DNA fragments used as probes in the position permutation assay. (B) EMSA utilizing position permutation. EMSA analysis showing the Mcm1-dependent bending of the AMSC (lanes 1 to 4) and the T_9G mutant (lanes 5 to 8) and EMSA analysis showing the SRF-dependent bending of the AMSC (lanes 9 to 12) and the T_9G mutant operator (lanes 13 to 16) are shown. Lanes 1, 5, 9, and 13 contain the *Bam*HI fragment. Lanes 2, 6, 10, and 14 contain the *Nhe*I fragment. Lanes 3, 7, 11, and 15 contain the *Hin*dIII fragment. Lanes 4, 8, 12, and 16 contain the *Eco*RI fragment. The concentration of Mcm1 used for the AMSC was 3.0×10^{-10} M, while the concentration for the T_9G mutant was 9.4×10^{-10} M. The concentration of the SRF protein used for both the AMSC and the T_9G mutant was 3.2×10^{-10} M.

dicted that SRF also exhibits altered DNA bending in response to the base pair substitutions at this position. Conversely, if SRF DNA bending were not affected by substitutions at this position, it would suggest that the proteins are involved in different DNA interactions in this region and furthermore that the sequence requirements for bending differ between Mcm1 and SRF. We therefore performed the circular permutation assay with purified SRF protein, using the consensus symmetric and the T_9G mutant operator sites (Fig. 5). The results show that the SRF protein exhibits essentially the same apparent degree of bending for the mutant (95°) and the wild-type site (98°). The apparent degree of bending by SRF recovered from this assay is greater than the angle observed in the SRF cocrystal structure and in other biochemical studies (11, 30, 34). One possible explanation for this difference is that in the previous studies, the sequences adjacent to the CArG box are CG rich, which may affect the bend angle, while the site used in this study is AT rich throughout the region. The observation that the DNA-bending activity of Mcm1 is severely affected by substitutions at position T_9 while SRF is not suggests that the two proteins use different mechanisms to bend their recognition sites and that the sequence requirements for DNA bending differ between the two proteins.

In comparison with changes at position 9, we find that substitutions at other positions produce significantly larger decreases in DNA-binding affinity but not in Mcm1-mediated activation. For example, the $A_{15}C$ mutation resulted in a greater than 30-fold reduction in DNA-binding affinity (Fig. 3, lanes 7 to 12) while Mcm1-mediated activation was not as severely affected (Fig. 2D). In addition, the $A_{15}T$ mutation reduced the binding affinity by fourfold (Fig. 3, lanes 13 to 18), while transcriptional activation was reduced only twofold (Fig. 2D). In contrast, the T_9G substitution produced only a twofold decrease in the DNA-binding affinity (Fig. 3, lanes 25 to 30) but caused a large decrease in Mcm1-dependent activation (Fig. 2D). These results suggest that DNA bending may be important for proper transcriptional regulation by Mcm1.

DISCUSSION

To examine the DNA-binding activity of Mcm1, we have constructed a set of mutations in the Mcm1 recognition sequence and examined the effects of these mutations on Mcm1-dependent transcriptional regulation in vivo. The results of this study identify the base-specific requirements for Mcm1 DNA recognition when binding alone and with α2 in the context of a symmetric binding site. Our data indicate that Mcm1 prefers the recognition site 5′-TTACCNAATTNGGTAA-3′ in vivo. These results compare favorably with in vitro binding-site selection studies which determined the Mcm1 consensus binding site as (NotC)CCY(A/T)(A/T)(T/A)NN(A/G)G (52) and with previous in vivo studies involving single point mutations in an Mcm1-binding site (29, 39). However, our results not only show the importance of the bases within the CArG box but also indicate that Mcm1 has strong base-specific requirements that extend at least 3 bp before and after the conserved CArG box. The requirement for these extended contacts are supported by the crystal structure of SRF, which shows that it also makes base-specific contacts with these outer bases. Our observation that the specificity of these bases, while important for transcriptional activation, do not greatly affect the binding affinity of the protein suggests one possible explanation for why these positions were not identified through in vitro selection techniques.

The results of this study also provide insight into the mechanism of MADS box protein-DNA interactions. It appears that some of the base-specific requirements are conserved between Mcm1 and SRF. Many of the base pairs in the SRF structure which are involved in base-specific contacts in the major groove are also important for Mcm1-dependent transcriptional regulation and DNA binding. For example, K163 of SRF contacts the guanine bases in the recognition site, and mutation of the analogous positions in the Mcm1 site reduces Mcm1-mediated transcriptional regulation and DNA-binding activity. This result is consistent with the model that the homologous residue in Mcm1, K38, is involved in similar contacts with the DNA recognition site at these positions.

Although some of the base-specific requirements of Mcm1 and SRF are very similar, there appear to be some significant differences. For example, based on the SRF crystal structure, Pelligrini et al. (30) predicted that Mcm1 would not be able to make the same contacts in the minor groove as SRF at positions corresponding to bp 13 and 14 in the AMSC site (30). Our results on the in vivo sequence requirements of Mcm1, along with the results obtained from the in vitro selection studies on Mcm1 DNA-binding specificity (12, 25, 52), support this prediction and indicate that specific base pairs at these positions do not play an important role in Mcm1-mediated transcriptional regulation or DNA binding.

The only position in the center of the recognition site in which there appears to be a strong base-specific requirement is at position 15. In the SRF cocrystal structure, this position is contacted in the minor groove by residue R143 from the N-terminal extension (30). However, carboxyethylation interference experiments have suggested that Mcm1 may make con-

tact with the center of the site in the major groove (52). The BrU-cross-linking experiments presented here further support the model that there are base-specific contacts in the major groove at this position. The SRF protein, on the other hand, fails to cross-link at this position, which could imply that residues that are different in the two proteins may be involved in this major-groove thymine contact. The Mcm1 protein used in the cross-linking experiments does not contain any amino acids outside the MADS box region of homology, eliminating the possibility that regions external to the Mcm1 MADS box are involved in this cross-link. One possible explanation of our results is that the two proteins differ at the position corresponding to residue H41 in Mcm1. This position in SRF is T166, which lies on the binding face of the α-helix near the center of the recognition site. It is possible that the larger histidine residue of Mcm1 is closer to the thymine methyl group and is able to make the cross-link with the DNA. Histidine residues are very efficient at BrU-mediated photo-cross-linking (2), which may explain the relatively high level of cross-linking efficiency (4%) that we have observed at position 15. Mutational analysis of Mcm1 has also shown that H41 is important for Mcm1 DNA binding and transcriptional regulation (5). An alternative explanation is that identical residues of Mcm1 and SRF may form different contacts with the DNA such that only the Mcm1 residue is in direct contact with the thymine methyl group and allows the formation of the cross-link.

In general, the results of in vitro DNA-binding assays with the mutant operator sites correlate well with the in vivo data. There was, however, one base pair, position 9 in the AMSC site, in which there was a significant discrepancy between the in vivo and in vitro assays. Substitutions at this position had severe effects on Mcm1-dependent transcriptional regulation but had relatively modest effects on DNA-binding affinity. Further investigation into the effects of these substitutions revealed that this base pair is important for Mcm1-dependent DNA bending. This altered bending activity by Mcm1 was not expected, as no apparent base-specific contacts have been identified in the SRF cocrystal structure at this position (30). Our data support the crystal structure model since substitutions at this position did not affect SRF DNA binding or bending. These results also suggest that the sequence dependence and the mechanisms responsible for DNA bending by Mcm1 and SRF are different, even though the degrees of DNA bending induced by the two proteins are comparable. In addition, these results rule out the possibility that a TA base pair is required at this position to allow maximal DNA bending, even though it is not involved in protein-DNA contacts. It is likely that differences in the sequence of amino acids in this region of SRF and Mcm1 are responsible for the different contacts with these base pairs. However, it is also possible that Mcm1 uses the conserved amino acids in a different manner to form an alternate set of contacts.

An additional observation about the substitutions at position 9 is that in comparison with Mcm1 sites containing mutations at other positions, the DNA-binding affinity of Mcm1 is not appreciably decreased (Fig. 3, lanes 25 to 30) whereas the transcriptional regulation of Mcm1 is severely affected. For example, although the binding affinity of the $A_{15}C$ mutant is considerably lower than that of any of the T_9 substitutions (lanes 7 to 12), this mutant still retains considerable transcriptional activity, whereas the $C_{12}T$ mutation has a similar binding affinity (lanes 19 to 24) but has near-wild-type levels of activation. This suggests that proper DNA bending is important for Mcm1 activity in vivo. Protease sensitivity experiments have indicated that the conformation of Mcm1 changes upon binding to the P box of **a**sg, and it was suggested that this altered conformation is important for transcriptional activation (42). It is possible that this conformational change observed with Mcm1 DNA binding is directly linked to the protein-dependent DNA bending. Failure to produce wild-type levels of DNA bending may not allow the appropriate conformational change to occur in Mcm1. Therefore, the reduced transcriptional activity of the operator mutants that alter DNA bending may be the direct result of the protein not undergoing the proper conformational change which is required for Mcm1-dependent activation. Alternatively, the decrease in activation obtained with substitutions at this position may be a direct result of the reduced DNA bending. Similar correlations between DNA bending and transcriptional activation have been previously described for integration host factor in prokaryotes and for YY1 in mammalian systems (23, 27).

An alternative explanation for our results is that in vivo, another protein interacts with the DNA at position 9 and that loss of this interaction may account for the decrease in activation we have observed. Although this is a formal possibility, we have shown that the substitutions at position 9 have a direct effect on Mcm1 in vitro, which is consistent with the idea that DNA bending may have an effect on Mcm1-mediated transcriptional activity. Many of the MADS box proteins appear to bend their DNA recognition sites, although little is known about the function of bending (11, 30, 32, 34). One study has shown that the phosphorylation state of an SRF cofactor affects the gel shift mobility of the complex, which may be a result of altered bending (34). If changes in bending occur, this altered bending may play a role in SRF transcriptional activation. Further studies taking advantage of this characteristic of Mcm1 may provide a deeper understanding of the link between DNA bending and transcriptional regulation within the MADS box family of proteins and also in other systems.

ACKNOWLEDGMENTS

We thank T. Richmond for providing the SRF coordinates and S. Soisson and C. Wolberger for the SRF protein. We thank Yisheng Jin, Janet Mead Parent, Valérie Gailus-Durner, and Richard Ebright for helpful comments and for reading the manuscript.

T.B.A. and H.Z. are the recipients of a Charles and Joanna Busch Predoctoral Training Fellowship and a New Jersey Cell and Molecular Biology Fellowship, respectively. This work was supported by a grant from the National Institute of Health to A.K.V. (GM49265).

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