The MotA Transcriptional Activator of Bacteriophage T4 Binds to Its Specific DNA Site as a Monomer†

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ABSTRACT: During bacteriophage T4 middle mode gene expression, the MotA transcription factor binds to T4 middle promoters at a −30 mot box consensus sequence to allow activation. Previous binding studies showed that MotA forms multiple gel-shifted complexes with DNA, and structural evidence suggested that MotA dimerizes upon DNA binding. We have shown that a short (13 bp) mot box DNA substrate binds MotA protein but fails to form slower migrating complexes. Therefore, the slower migrating complexes in gel shift assays are caused by DNA-mediated binding events. Competition experiments indicate that the slower migrating complexes are formed by nonspecific binding events, while the first-shifted complex is caused by specific binding to the mot box. Saturation binding experiments revealed that the stoichiometry of MotA binding to DNA is 1:1 in the first-shifted complex, while the slower complexes apparently contain MotA multimers. Gel shift assays using mixtures of MotA and a GST−MotA fusion protein supported the conclusion that the first-shifted complex contains one protein molecule bound to DNA. Furthermore, MotA monomers were cross-linked by glutaraldehyde under conditions where slower complexes exist, but not under conditions that lead to only the first-shifted complex. We conclude that MotA binds specifically to the mot box as a monomer and that additional nonspecific binding events require flanking DNA.

Bacteriophage T4 controls gene expression by using different promoter elements and modifying the host RNA polymerase (for review, see refs *1*−*3*). During middle mode transcription, a T4-encoded transcription factor, MotA, is required for activation and binds to the mot box, a unique consensus sequence centered at −30 in middle promoters. A second T4 protein, AsiA, binds to the σ^{70} subunit of host RNA polymerase and restricts transcription to T4 middle promoters when MotA is also present (*4*−*6*). To date, little is known about the protein−protein interactions required for activation and open complex formation at middle promoters.

MotA was first implicated as a middle mode transcription factor when mutations in this gene were found to alter expression of several prereplicative genes. Progress on middle mode transcription accelerated once the *motA* gene was sequenced (*7*) and the protein was overproduced and purified (*8, 9*). Activation of middle promoters in vitro requires MotA with T4-modified RNA polymerase (*8, 9*). T4-modified polymerase contains the T4 AsiA protein (*4, 5*), and addition of purified AsiA to unmodified RNA polymerase was shown to be sufficient to direct MotA−dependent middle mode transcription (*6*).

The 23.5-kDa MotA protein consists of two approximately equal-sized domains (*10*). The C-terminal domain (MotCF) is involved in DNA binding, and its secondary structure was determined by NMR analysis (*11*). The structure shows homology to one-half of the eukaryotic TATA-binding protein, which is an intramolecular dimer (*12*). The N-terminal domain (MotNF) is important in transcriptional activation (*13, 14*). Its structure, which was solved by NMR and X-ray crystallography, consists of six α-helices and a short β-ribbon (*14*). Although the MotNF structure showed no homology to other known protein structures, the surface of this domain contains an acidic/hydrophobic patch reminiscent of eukaryotic acidic activation domains (AADs). These AADs are poorly understood but have been shown to be critical for activation by several transcription factors, including GAL4, VP16, and p53 (for review, see refs *15* and *16*). Mutations of an acidic and a hydrophobic residue within the MotA surface patch caused a defect in T4 growth and greatly decreased transcriptional activation without affecting DNA binding (*14*).

The structural studies of MotNF revealed a dimer in the crystal form (*14*). The dimerization interface consists of C-terminal α-helices that form an antiparallel coiled-coil motif, interacting primarily via hydrophobic residues. However, MotNF dimers failed to form in solution, as shown by the calculated Stokes radius obtained by gel filtration and dynamic light scattering (*14*). Finnin et al. (*11, 14*) proposed that MotA undergoes DNA-directed dimerization, with two monomers binding to DNA in close proximity and thus aligning properly for dimerization through conformational changes (*17, 18*).

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Binding studies using the gel shift method have shown that MotA binds specifically to the mot box sequence (*9, 19*). However, high concentrations of protein resulted in multiple bands, with as many as four shifted complexes generated with a long (>50 bp)[1] DNA substrate (*9, 19*). DNase I footprinting assays of MotA on DNA templates containing T4 middle promoters revealed protection within and surrounding the mot box at lower MotA concentrations. However, as additional protein was added, further protection occurred downstream of the mot box [within and around −10 for P_{uvsY} (*9*) and from +40 to +57 for P_{uvsX} (*19*)]. These two downstream regions share a related sequence, and March-Amegadzie and Hinton (*19*) suggested that MotA may recognize this alternative sequence in addition to the mot box.

Hinton et al. (*20*) analyzed MotA binding to DNA in the presence of RNA polymerase. While an apparent ternary complex of MotA, RNA polymerase, and DNA formed whether AsiA protein was present or not, activation occurred only in the presence of AsiA. The presence of AsiA also altered the protection patterns in DNase I footprinting assays. These results indicate that RNA polymerase with AsiA is necessary for the protein−protein and protein−DNA interactions involved in open complex formation and transcription initiation.

In this report, we present the results of protein binding assays that clarify the nature of the multiple shifted complexes seen in gel shift assays. The results demonstrate that the first binding event involves specific binding of MotA to the mot box, but that slower migrating complexes are caused by nonspecific binding to the DNA substrate. We also show that the first bound complex contains a MotA monomer bound to the DNA, while the larger complexes contain multimers of MotA.

EXPERIMENTAL PROCEDURES

Proteins and Oligonucleotides. Overexpressed MotA protein was purified as described earlier (*14*). The MotA concentration in one pool was determined directly by amino acid analysis (Harvard Microchemistry Facility). The MotA concentration in this pool was also measured relative to BSA using the Bio-Rad protein assay procedure. When compared to the value obtained by amino acid analysis, MotA was found to bind about 1.4-fold less dye than an equivalent amount of BSA. Therefore, for any pure MotA preparation, the protein concentration obtained from the Bio-Rad protein assay with a BSA standard curve is multiplied by a factor of 1.4 to obtain the final MotA concentration.

To construct a GST−MotA fusion, the 720-bp *Nde*I−*Bam*HI fragment with the *motA* gene was cleaved out of plasmid pRS31 (*9*) and ligated to *Bam*HI-linearized pGEX-3X (Pharmacia), which contains the GST coding sequence. The ligation mixture also contained a *Nde*I−*Bam*HI duplex oligonucleotide linker (5′-TAACGCGGTG-3′/5′-GATCCACCGCGT-3′), which aligned the 3′-end of the GST coding sequence in frame with the 5′-end of the *motA* gene.

[1] Abbreviations: ATP, adenosine 5′-triphosphate; bp, base pair(s); BSA, bovine serum albumin; cpm, counts per minute; DTT, dithiothreitol; EDTA, (ethylenedinitrilo)tetraacetic acid; GST, glutathione *S*-transferase; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

Table 1: DNA Substrates Used To Study MotA Binding[a]

DNA template	size	sequence (5′ to 3′)
MOTL30	30	GCCGGAGTAT **AATGCTTCA** CGAGCTCGGCG
MOTM21	21	GCGTAT **AATGCTTCA** CGTTGC
MOT21R	21	AT **AATGCTTCA** CGAGCTCGGC
MOT21L	21	GCCGGAGTAT **AATGCTTCA** CG
MOTS13	13	AT **AATGCTTCA** CG
MOTMX	30	GCCGGAGTAT CGCTAGGAT CGAGCTCGGCG

[a] The mot box is shown in bold. The complementary strands, which are not shown, were annealed to the indicated strand to form the appropriate duplex substrates. The oligonucleotide substrate MOT21F, used to determine the association of MotA to DNA (Figure 4), contains the same sequence as MOTM21. In MOT21F, one strand of the duplex was end-labeled with fluorescein phosphoramidite during synthesis.

The GST−MotA fusion was overproduced and purified as follows. *Escherichia coli* BL21 with pGEX-*motA* were grown at 37 °C in L broth with 2% glucose, 40 μg/mL ampicillin, and 10 μg/mL chloramphenicol; induced for 3 h with IPTG (0.2 mM); collected by centrifugation; washed with 20 mL of cold PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.3), collected again by centrifugation; and frozen at −80 °C. The cells were resuspended in cold PBS, homogenized in a pestle tissue grinder five times to break up cell clumps, treated with lysozyme (0.2 mg/mL for 1 h on ice), and then sonicated to complete lysis. Triton X-100 (0.8% v/v) was added, and the mixture was incubated for 30 min on ice and then centrifuged for 12 min at 12000*g*. The protein was isolated from the supernatant using a glutathione Sepharose 4B column as described by Pharmacia. Eluates were pooled and dialyzed against MotA sonication buffer and then frozen at −80 °C. Protein concentration was obtained using the Bio-Rad assay with BSA as the protein standard.

The various oligonucleotides for the gel shift and cross-linking experiments were synthesized either by the Duke University Botany Department Oligonucleotide Synthesis Facility or by Gibco BRL. Duplex DNA was formed by heating the oligonucleotides (in 10 mM Tris-HCl, pH 7.6; and 1 mM EDTA) to 90 °C for 2 min and then allowing the substrate to slowly cool (<1 °C/min) to 22 °C. The sequences of the DNA substrates used in this study are shown in Table 1. Each of the substrates contains the consensus mot box from the bacteriophage T4 *uvsY* promoter (5′-AATGCTTCA-3′), except MOTMX, which contains a randomized sequence replacing the mot box. Substrates for the gel shift assays had one strand end-labeled with ^{32}P by T4 polynucleotide kinase (New England Biolabs, Inc.). Unless otherwise indicated, nonspecific competitor DNA consisted of a 50-bp oligonucleotide with the following T4 *uvsW* gene sequence: 5′-CATGGATATTAAAGTACATTTT-CACGACTTCAGTCATGTACGCATCGATG-3′, and 5′-GATCCATCGATGCGTACATGACTGAAGTCGTGAA-AATGTACTTTAATATC-3′. Oligonucleotides were quantitated prior to annealing into duplexes by absorbance at 260 nm. The extinction coefficient of each oligonucleotide was determined by the formula $\epsilon = (\#A \times 15.3) + (\#G \times 11.8) + (\#C \times 7.4) + (\#T \times 9.3)$ (formula obtained from Gibco BRL). The amount of oligonucleotide (micrograms per milliliter) per optical density (OD) unit was calculated by dividing the molecular weight by ϵ.

Gel Shift and Saturation Binding Assays. Gel shift reaction mixtures contained 10 μL of MotA sonication buffer

[20 mM Tris-HCl, pH 7.9; 1 mM EDTA; 10% glycerol; and 1 mM β-mercaptoethanol (*21*)] with the indicated amount of MotA protein and 5 μL of binding buffer A (20 mM Tris-HCl, pH 7.9; 1 mM EDTA; 10% glycerol; 1 mM DTT; and 100 μg/mL BSA) with the indicated amount of end-labeled DNA substrate and unlabeled competitor DNA. Samples were incubated at 22 °C for 10 min and then placed on ice. A 2.5-μL aliquot of loading buffer (10% Ficoll-400 and 0.15% bromphenol blue) was added, and the samples were subjected to electrophoresis through a nondenaturing 8% polyacrylamide gel (29:1 total acrylamide:bisacrylamide) in TBE buffer (89 mM Tris base, 89 mM boric acid, and 2.5 mM Na_2EDTA) at 4 °C and 125 V for 3.5 h. Quantitation of the bands in Figure 6A was performed using an AMBIS direct radioisotope counting system.

Fluorescence Measurements. Binding reactions for fluorescence measurements were conducted in 300 μL of binding buffer B (20 mM Tris-HCl, pH 7.9; 1 mM EDTA; 2% glycerol; and 1 mM β-mercaptoethanol) containing 15 pmol of duplex DNA substrate. The fluorescein-labeled substrate, MOT21F, was synthesized and PAGE purified by the Genosys Corporation and has the sequence 5′-F-GCG-TATAATGCTTCACGTTGC-3′ and 5′-GCAACGTGAAG-CATTATACGC-3′. MotA protein (in binding buffer B) was titrated into the reaction mixture, and anisotropy measurements were taken at 25 °C.

The dissociation constant of the MotA−MOT21F complex was determined by steady-state anisotropy measurements of MOT21F as a function of added MotA. The fraction of MOT21F bound was calculated from the anisotropy limits of the titration, corresponding to free MOT21F (A_f) and MotA−MOT21F complex (A_b), and the observed anisotropy (A), using the formula

$$\text{fraction bound} = (A - A_f)/((A_b - A)Q + A - A_f)$$

Q is the ratio of the quantum yield of the bound and free forms, which takes into account any changes in the fluorescence signal from the fluorophore after protein binding (*22*). The value of Q averaged 0.90 ± 0.01 ($n = 3$). Calculations accounted for changes in the total reaction volume during the titration of protein.

Glutaraldehyde Cross-Linking. Reaction mixtures contained the indicated amount of protein and DNA substrate in 10 μL of MotA sonication buffer. Samples were preincubated at 22 °C for 5 min, 5 μL of cross-linking buffer (20 mM Tris-HCl, pH 7.9; 1 mM EDTA; 10% glycerol; 1 mM DTT; and 0.075% glutaraldehyde) was added, and incubation was continued for 30 min at 22 °C. The reactions were terminated by adding 15 μL of 2× loading buffer (0.1 M Tris-HCl, pH 6.8; 1.2 mM β-mercaptoethanol; 4.3% SDS; 0.002% bromphenol blue; and 10% glycerol), the samples were heated to 95 °C for 5 min, and the products were then separated by electrophoresis through a denaturing polyacrylamide gel (12% separating gel, pH 8.8, 27:1 acrylamide:bisacrylamide; 5.7% stacking gel, pH 6.8, 27:1 acrylamide:bisacrylamide) in glycine running buffer (0.1% SDS, 0.19 M glycine, and 25 mM Tris base) at 25 mA for 5 h. Protein bands were visualized using a copper staining technique (*23*).

RESULTS

Two Modes of DNA Binding. Previous gel shift assays revealed multiple bands when MotA binds to a DNA substrate containing a mot box (*9, 19*). The number of bands apparently depended on the length of the DNA substrate, with short substrates (17−30 bp) forming 2−3 shifted complexes and longer substrates (>50 bp) forming at least 4 shifted complexes. The correlation between DNA length and the number of shifted complexes suggested that the additional binding events after the primary shift are DNA-mediated (i.e., they involve MotA−DNA interaction as opposed to MotA−MotA interaction).

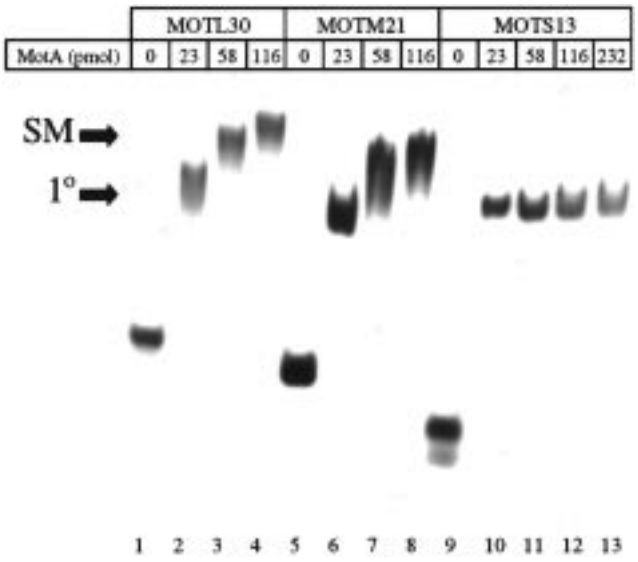

FIGURE 1: MotA binding to DNA substrates of varying lengths. Reaction mixtures (15 μL) contained 0.05 pmol of DNA substrates of 30 (lanes 1−4), 21 (lanes 5−8), or 13 bp (lanes 9−13) with the indicated amount of protein. Samples also contained nonspecific competitor DNA at 10 μg/mL. The primary (1°) and slower migrating (SM) complexes are indicated with arrows.

To test whether the additional binding events are DNA-mediated, we conducted binding assays using DNA substrates of various lengths. Our goal was to find a short substrate that allows the first protein binding event (primary complex) but not additional binding events (slower migrating complexes). The gel shift experiment used three DNA substrates of varying sizes (30, 21, and 13 bp), each of which contained the 9-bp mot box consensus sequence at the center. With MOTL30 and MOTM21, slower migrating complexes formed as the MotA concentration was increased, indicative of multiple binding events (Figure 1, lanes 1−8). However, with the short MOTS13 template, only the primary complex was detected (lanes 9−13), even when a large excess of protein was present (lane 13). These results argue that the slower migrating complexes require a protein−DNA interaction in the DNA region flanking the primary MotA−mot box complex. We did not observe evidence of cooperativity between the binding events with the longer substrates, as almost all the DNA shifted to the primary complex before any appeared in slower migrating complexes. The slower migrating species were not completely discrete bands, which may indicate that the complexes have a relatively low stability during electrophoresis.

Having proven that the slower migrating complexes require DNA flanking the mot box, we next asked whether the flanking DNA segment needed to be upstream or downstream of the mot box. Since the mot box is not palindromic, MotA binding to the mot box may have a distinct orientation. We

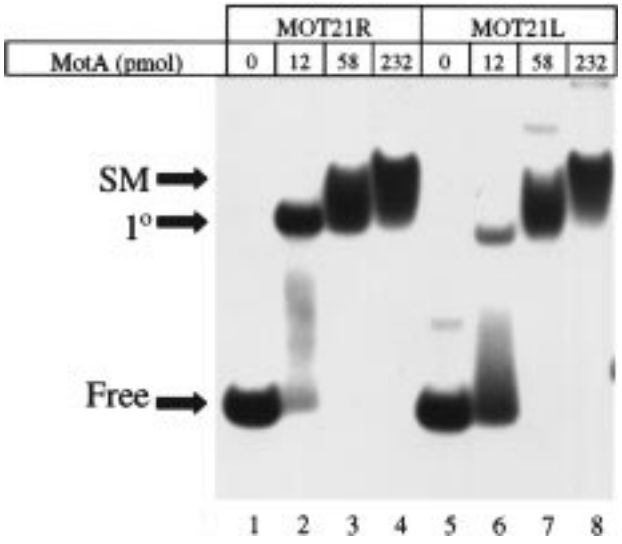

FIGURE 2: Gel shift assay to determine orientation requirement for slower migrating complexes. Reaction mixtures (15 μL) contained the indicated amount of MotA protein along with 0.05 pmol of substrate containing a flanking region either downstream (lanes 1−4) or upstream (lanes 5−8) of the mot box (see Table 1). Samples also contained nonspecific competitor DNA at 10 μg/mL. Free DNA and primary (1°) and slower migrating (SM) complexes are indicated with arrows.

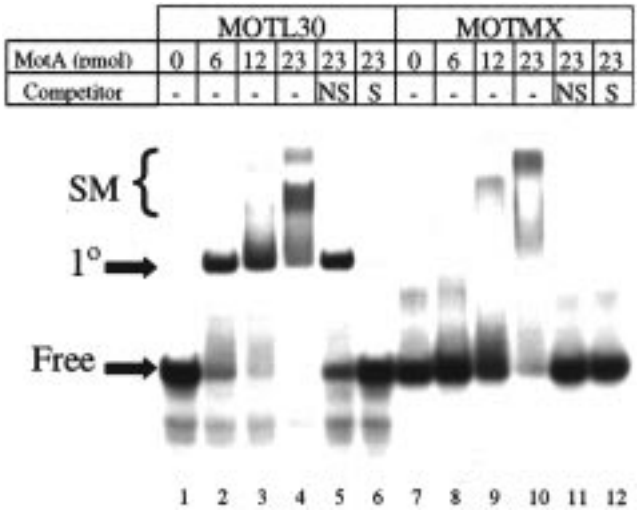

FIGURE 3: Specificity of MotA binding. Gel shift reaction mixtures (15 μL) contained the indicated amount of MotA protein and 1 pmol of labeled DNA substrate. The 30-bp DNA substrate contained either a mot box (MOTL30) (lanes 1−6) or a random sequence in place of the mot box (MOTMX) (lanes 7−12). The specific competitor (S) was unlabeled MOTL30 duplex DNA (200 pmol; lanes 6 and 12), and the nonspecific competitor (NS) was unlabeled MOTMX substrate (200 pmol; lanes 5 and 11).

used two DNA substrates (MOT21R and MOT21L) that are identical to MOTL30 except that 9 base pairs were eliminated from either end. This alteration positioned the mot box at one end of the substrate, with flanking DNA either upstream or downstream (see Table 1; compare to MOTS13). With both substrates, as the MotA concentration was increased, the primary complex appeared first and then slower migrating complexes formed (Figure 2). These results argue that the second binding event does not require flanking DNA in a specific orientation with respect to the mot box, consistent with nonspecific protein−DNA binding.

Specificity of Binding for Slower Migrating Complexes. To examine the specificity of the DNA binding events, we analyzed the effect of a large excess of specific or nonspecific competitor DNA. In this experiment, we also tested a nonspecific substrate, MOTMX, which is a 30-bp DNA containing a random sequence in place of the mot box. With the mot-box-containing substrate (MOTL30), the primary complex formed at low protein concentrations (Figure 3, lanes 2 and 3) and slower migrating complexes appeared at a higher MotA concentration (lane 4). With the nonspecific MOTMX substrate, the primary complex was absent but slower migrating complexes formed at the higher protein concentrations (Figure 3, lanes 8−10). Once again, the slower complexes formed diffuse bands during the gel shift, especially with the MOTMX substrate.

We next asked whether a specific or nonspecific competitor would compete away the various complexes by adding unlabeled competitor DNA at about 9-fold molar excess to MotA. When nonspecific competitor was added to the MOTL30 reaction mixture, all of the slower migrating complexes disappeared, and most of the labeled substrate was found in the primary complex (Figure 3, lane 5). As expected, almost all of the shifted complexes disappeared when excess specific competitor was added to the reaction (lane 6). When the same competition was performed with the MOTMX substrate, all bound forms disappeared with either competitor (lanes 11 and 12). We conclude from these results that MotA specifically binds to the mot box to form the primary complex, while the slower migrating species depend on nonspecific protein−DNA binding (see Discussion).

Affinity of MotA to MOT21F. Because MotNF is a dimer in the crystal (*14*), we asked whether the primary complex contains a MotA monomer or dimer. To conduct saturation binding experiments and determine the stoichiometry of MotA binding, we first needed to determine the dissociation constant (K_d).

Although gel shift assays such as those above are readily quantifiable, measurements of the fractions of free and bound DNA are not made at equilibrium. During the course of electrophoresis, the relative DNA and protein concentrations change, and the solution conditions used during the preapplication binding step differ from those of the electrophoresis buffer. Therefore, an alternative method for measuring the MotA−DNA affinity was explored.

Measurement of fluorescence anisotropy is a spectroscopic technique that is particularly well suited for the study of protein binding to small oligonucleotides (*22*, *24*), and it has been used to study a variety of protein−DNA interactions (for examples, see refs *25* and *26*). We measured the fluorescence anisotropy of a fluorescein-labeled 21-bp mot-box-containing oligonucleotide (MOT21F) as a function of MotA concentration (see Experimental Procedures). A plot of the fraction of protein bound as a function of the log of the concentration of free MotA revealed a K_d value of 2.2 $\times 10^{-7}$ M (Figure 4). One concern during this determination was the possible formation of the larger complexes that were observed during the gel shift assays, especially at higher protein concentrations. Formation of such complexes during anisotropy measurements would affect the calculated K_d value for the primary complex since we are assuming a single binding event. However, this does not appear to be a

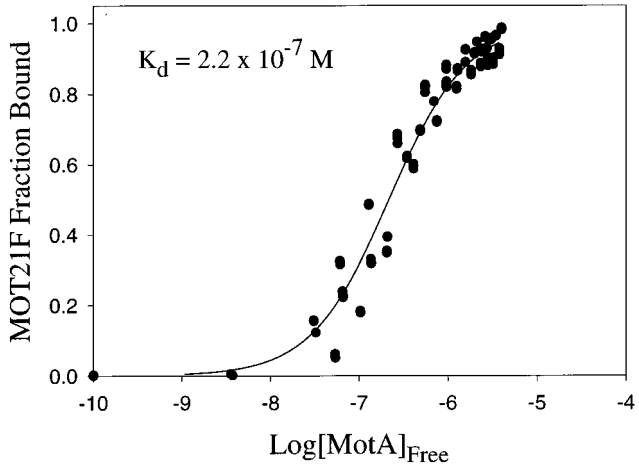

FIGURE 4: Association of MotA with MOT21F. The affinity of MotA to the fluorescein-labeled MOT21F duplex oligonucleotide was measured by fluorescence anisotropy of MOT21F as a function of MotA concentration under conditions described in Experimental Procedures. The anisotropy of free MOT21F was 0.054 ± 0.0005, and that of MotA−MOT21F complex was 0.191 ± 0.006 ($n = 3$). The fraction of substrate bound was calculated as described in Experimental Procedures and was plotted as a function of the log of the concentration of free MotA. The data were obtained from three separate experiments and were fit to the Michealis−Menten equation by nonlinear regression assuming a single class of noninteracting MotA binding sites. The binding curve revealed a K_d of 2.2×10^{-7} M. Residuals were randomly distributed throughout the titration.

problem since we obtained a similar K_d value using a fluorescein-labeled 13-bp duplex (data not shown). In addition, the covalently bound fluorescein does not seem to affect DNA binding because gel shift experiments showed that MotA has similar affinities for MOT21F and MOTM21 (data not shown).

Saturation Binding Assays of MotA to DNA. To complete the measurement of the K_d of MotA binding to DNA, saturation binding experiments could be conducted to determine the stoichiometry of binding. Reaction mixtures contained a DNA concentration at least 10-fold higher than the K_d to favor protein binding. We first attempted to use anisotropy to measure binding under stoichiometric conditions but were unable to distinguish a valid stoichiometric point. The binding experiment was then repeated using gel shift of labeled MOTS13, which only forms the primary complex in gel shifts (see above). As the [MotA]:[DNA] stoichiometry approached 1, nearly all of the substrate shifted into the primary complex (Figure 5A). The amounts of free and bound DNA were quantitated for accurate determination. The amount of bound form increased in a roughly linear manner, approximating the predicted ideal curve for monomer binding and clearly deviating from the predictions for a protein dimer binding event (Figure 5B). These results clearly indicate that MotA binds as a monomer in the primary complex.

Binding with Mixtures of MotA and GST−MotA. To further analyze whether a monomer or a dimer of MotA is bound to DNA in the primary complex, we performed gel shift assays containing mixtures of MotA and a GST−MotA fusion protein. Fusion proteins have been used previously to study stoichiometry of protein binding to DNA (for example, see ref *27*). Because fusion proteins are larger than the wild-type protein, heterodimers can be distinguished

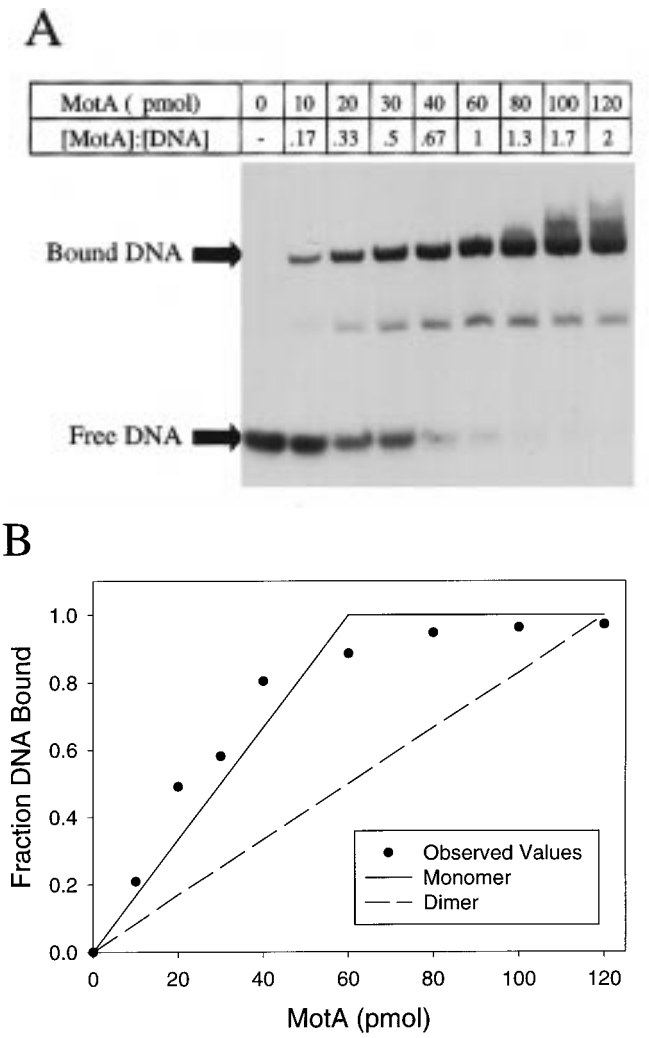

FIGURE 5: Saturation binding of MotA to MOTS13. Reaction mixtures contained 60 pmol of DNA substrate and increasing amounts of MotA protein. (A) Autoradiogram of the gel shift assay. Free DNA and bound complex are indicated with arrows. A very minor population of a larger complex formed at the highest protein concentrations. The total radioactivity from each band was quantitated using an AMBIS direct radioisotope counting system. (B) Plot of fraction of DNA bound versus MotA protein added. The observed values (●) are compared with the expected values if the primary complex contains a MotA monomer (solid line) or dimer (dashed line).

when a mixture of wild-type and fusion protein produces a shifted band that migrates between the shifted homodimer bands. If a MotA dimer binds to form the primary complex, then a unique heterodimer species should be present when wild-type MotA (23.5 kDa) and GST−MotA (50 kDa) are present in the reaction mixture. If, as suggested above, a monomer of MotA binds to form the primary complex, then no unique species should be detected with the protein mixture.

GST−MotA was overproduced and purified as described in Experimental Procedures. The purified fusion protein was able to bind the MOTL30 substrate, though at a lower affinity than wild-type MotA (Figure 6; compare lanes 5−7 with lanes 2−4). As expected from the larger mass of GST−MotA, the first shifted complex migrated much more slowly than the primary complex with the nonfusion protein. We failed to observe any additional, more slowly migrating complexes with GST−MotA. Most importantly, when the

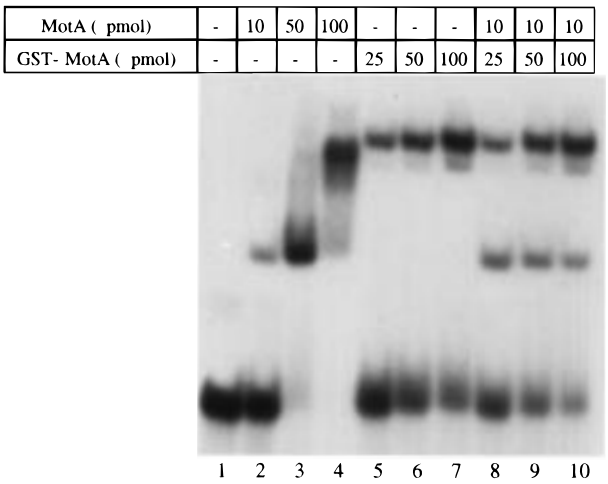

FIGURE 6: DNA binding by MotA and GST−MotA protein mixtures. Reaction mixtures contained 46 pmol of MOTL30 substrate and the indicated amount of proteins. The samples were preincubated at 22 °C for 10 min prior to the addition of substrate.

samples contained various mixtures of the two proteins, bands that comigrated with both MotA and GST−MotA primary complexes were readily observed, but no unique species consistent with a heterodimer were formed (lanes 8−10). The failure to observe the heterodimer complex supports the conclusion that the primary complex contains MotA monomers bound to DNA.

Glutaraldehyde Cross-Linking of MotA Monomers on DNA. As a final experiment to investigate the multimeric state of MotA, we analyzed protein cross-linking with glutaraldehyde. On the basis of the above stoichiometry, we predicted that cross-linking of two monomers would occur with the slower migrating complexes but not with the primary complex.

We looked for cross-linked MotA monomers by electrophoresis under protein denaturing conditions after glutaraldehyde treatment. With untreated MotA or MotA treated with cross-linker in the absence of DNA, only the monomer product was detected (Figure 7, lanes 1 and 2). The lack of cross-linked dimer indicates that dimerization does not occur in solution in the absence of DNA. A faster migrating smear was detected when MotA was treated with cross-linker in the absence of DNA, perhaps due to intramolecular cross-linking. A 30-bp DNA substrate was added to the samples at [MotA]:[DNA] ratios of 1:1, 2:1, and 3:1 (lanes 3−5, respectively). A unique band appeared near the predicted size of a MotA dimer when the [MotA]:[DNA] ratio was 2:1 or 3:1, where the bound complex should be predominantly the slower migrating form. However, cross-linked product was not detected at the stoichiometry of 1:1, where the primary complex would predominate. Furthermore, when we repeated the reactions using a 13-bp substrate (lanes 6−8), no dimeric band was detected at the [MotA]:[DNA] ratios of 1:1 and 2:1. A faint, smeared band did appear at the predicted dimer size at the ratio of 3:1 (lane 8), correlating with a very small amount of slower migrating complexes at high protein concentrations with MOTS13 substrate (see Figure 5A). We conclude that cross-linked dimers form when slower migrating complexes are present, but not under conditions that lead to only the primary shift.

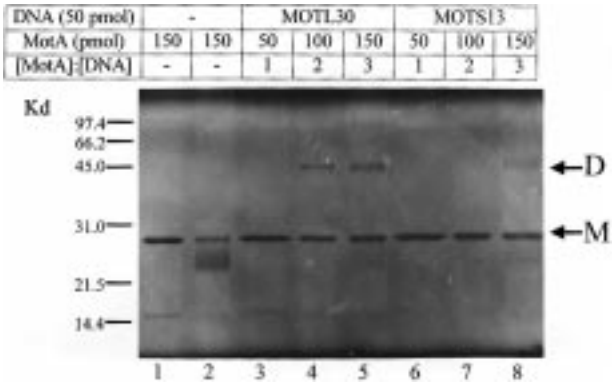

FIGURE 7: Protein cross-linking by glutaraldehyde. Reaction mixtures contained either 50 (lanes 3 and 6), 100 (lanes 2 and 7), or 150 pmol of MotA (lanes 1, 2, 5, and 8) with either no DNA (lanes 1 and 2), 50 pmol of the 30-bp substrate (MOTL30) (lanes 3−5), or 50 pmol of the 13-bp substrate (MOTS13) (lanes 6−8). All samples except the control (lane 1) were treated with 0.025% glutaraldehyde. Protein amounts were normalized prior to loading the gel. Predicted sizes of monomer (M) and dimer (D) bands are indicated with arrows. Bars to the left of the gel indicate migration of molecular weight protein markers (Bio-Rad broad-range SDS−PAGE standards).

DISCUSSION

The bacteriophage T4 transcription factor MotA activates phage middle mode transcription by binding to middle promoters at the mot box consensus sequence. We have studied the specificity and stoichiometry of MotA binding to DNA templates containing a mot box. In previous studies, gel shift assays revealed multiple shifted complexes (*9*, *19*) and footprinting assays revealed expanded regions of protection at higher MotA concentrations (*9*, *19*). We found that formation of multiple gel shift complexes is dependent on the size of the DNA substrate and that binding can be limited to the primary complex by using a short (13−bp) DNA substrate. Formation of the primary complex requires the mot box, and MotA binds specifically to that sequence, as shown by competition assays. The slower migrating complexes form by nonspecific protein−DNA binding since additional binding (after primary complex formation) can occur either upstream or downstream of the mot box. Furthermore, formation of the slower migrating complexes is blocked by the addition of excess nonspecific competitor DNA.

The ability to block formation of slower migrating species by using a short DNA substrate indicates that protein−DNA interactions are required for these complexes. However, protein−protein interactions may also be involved in formation of slower migrating complexes. Cross-linked MotA species are observed with glutaraldehyde treatment of the slower complexes, and thus the MotA monomers can be in close proximity when bound to DNA in this form. It is interesting that the primary complex is not detected by gel shift assays using a substrate missing the mot box. The formation of only slower migrating species on such nonspecific substrates suggests that MotA cannot bind stably to nonspecific DNA without additional protein−protein contacts that are formed by MotA multimers. Such protein−protein interactions are likely to be weak, however, since discrete slower migrating bands were not observed in the gel shift assay.

We obtained a dissociation constant of 2.2×10^{-7} M for MotA binding to DNA using fluorescence anisotropy measurements. Using the determined K_d value, saturation binding assays clearly indicated that the primary complex contains a MotA monomer bound to DNA. This conclusion was further supported by experiments using a MotA fusion protein, where we were unable to detect heterodimers when mixtures of MotA and GST−MotA were used to form primary complexes during gel shift analysis. Finally, the results of glutaraldehyde cross-linking experiments provided additional evidence that the primary complex contains a monomer of MotA. Cross-linking between MotA monomers failed to occur under conditions where only the primary complex was present, but cross-linking did occur when the slower migrating complexes existed.

The crystal structure of the MotA activation domain reveals a dimer, with strong interactions via an antiparallel coiled-coil motif at the C-terminal α-helices (*14*). However, both NMR studies and the measured Stokes radius indicate that the activation domain (MotNF) is a monomer in solution (*14*). Furthermore, MotA dimers were not cross-linked in the absence of DNA (Figure 7), and we have now demonstrated that MotA monomers bind specifically to DNA. These results clearly indicate that the antiparallel coiled-coil in the dimer crystal is not relevant for the primary binding event. Is this coiled-coil involved in formation of the slower migrating complexes? We detected protein cross-linking under conditions where slower migrating complexes form, indicating close contact between two monomers. However, we saw no evidence for cooperativity between monomers in the formation of the slower migrating forms, which might be expected if the coiled-coil was important. Our results do indicate that the slower migrating complexes require both protein−DNA and protein−protein contacts. The protein−protein contacts can occur either upstream or downstream of the oriented MotA at the mot box. Therefore, unless the MotNF domain is very flexibly attached to the MotCF (DNA binding) domain, it seems unlikely that the antiparallel coiled-coil from MotNF is involved in the formation of these MotA dimers.

Our results do not support a biological significance for MotA multimers. Although the results do not formally rule out the possibility that a dimer is required for activation and open complex formation, it seems much more likely that MotA activates transcription as a monomer. This would be an unusual case for prokaryotic transcription factors, which commonly form multimers to allow activation. Multimeric binding of transcription factors can result in a large increase in specificity and stability (for review, see ref *28*). In the case of MotA, there does not seem to be a large difference between the affinities of specific and nonspecific binding. This raises the question of how MotA can specifically recognize and bind to middle promoters during a T4 infection.

Several eukaryotic transcription factors (e.g., GATA-1 and TTF-1) are able to specifically bind to DNA as monomers (*29*, *30*). However, eukaryotic promoters commonly contain multiple binding sites for transcription factors, presumably helping to stabilize protein binding through protein−protein contacts. While some T4 middle promoters contain additional mot boxes in proximity to the primary mot box, there is no consistent alignment, and other middle promoters have only a single mot box. Thus, interactions of two or more specifically bound MotA monomers do not seem to be used by T4 to increase promoter recognition. Using an assay involving protection from restriction enzyme cleavage, March-Amegadzie and Hinton (*19*) obtained evidence that MotA affinity to DNA increases in the presence of T4-modified RNA polymerase. Consistent with this view, in vitro transcription assays require a much lower MotA concentration than MotA−DNA binding assays. The greater stability of the ternary complex (MotA−DNA−modified RNA polymerase) over either binary protein−DNA complex may explain how MotA efficiently activates middle promoters.

Activation at T4 middle promoters requires two T4 proteins (MotA and AsiA) and the host σ^{70}-containing RNA polymerase. AsiA and σ^{70} interact strongly (*4*); a segment of σ^{70} containing the 4.2 region has recently been shown to be involved in this interaction (*31*). The 4.2 region contains a proposed helix−turn−helix motif involved in recognizing and binding to the −35 sequence of *E. coli* promoters. Since the mot box overlaps the −35 region normally recognized by σ^{70}, it has been suggested that MotA interacts with σ^{70} (*13*). This association would be similar to the interaction between phage λ cI protein and σ^{70} (*32*), which also involves the 4.2 region. We have recently shown that mutations of the acidic/hydrophobic surface patch of MotNF decrease activation, suggesting that this region interacts with σ^{70} and/or AsiA protein (*14*). Experiments can now be directed at understanding the protein−protein interactions required for activation of T4 middle promoters.

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REFERENCES

1. Wilkens, K. P., and Ruger, W. (1994) in *Molecular Biology of Bacteriophage T4* (Karam, J. D., Ed.) pp 132−141, ASM Press, Washington, DC.
2. Stitt, B., and Hinton, D. (1994) in *Molecular Biology of Bacteriophage T4* (Karam, J. D., Ed.) pp 142−160, ASM Press, Washington, DC.
3. Williams, K. P., Kassavetis, G. A., Herendeen, D. R., and Geiduschek, E. P. (1994) in *Molecular Biology of Bacteriophage T4* (Karam, J. D., Ed.) pp 161−175, ASM Press, Washington, DC.
4. Stevens, A. (1976) in *RNA Polymerase* (Losick, R., and Chamberlin, M., Eds.) pp 617−627, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
5. Stevens, A. (1977) *Biochim. Biophys. Acta 475*, 193−196.
6. Ouhammouch, M., Adelman, K., Harvey, S. R., Orsini, G., and Brody, E. N. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 1451−1455.
7. Uzan, M., Brody, E., and Farve, R. (1990) *Mol. Microbiol. 4*, 1487−1496.
8. Hinton, D. M. (1991) *J. Biol. Chem. 266*, 18034−18044.
9. Schmidt, R. P., and Kreuzer, K. N. (1992) *J. Biol. Chem. 267*, 11399−11407.
10. Finnin, M. S., Hoffman, D. W., Kreuzer, K. N., Porter, S. J., Schmidt, R. P., and White, S. W. (1993) *J. Mol. Biol. 232*, 301−304.
11. Finnin, M. S., Hoffman, D. W., and White, S. W. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 10972−10976.

12. Nikolov, D. B., Hu, S.-H., Lin, J., Gasch, A., Hoffman, A., Horikoshi, M., Chua, N.-H., Roeder, R. G., and Burley, S. K. (1992) *Nature 360*, 40−46.
13. Gerber, J. S., and Hinton, D. M. (1996) *J. Bacteriol. 178*, 6133−6139.
14. Finnin, M. S., Cicero, M. P., Davies, C., Porter, S. J., White, S. W., and Kreuzer, K. N. (1997) *EMBO J. 16*, 1992−2003.
15. Hahn, S. (1993) *Cell 72*, 481−483.
16. Kodadek, T. J. (1994) in *Transcription Mechanisms and Regulation* (Conaway, R. C., and Conaway, J. W., Eds.) pp 493−513, Raven Press, New York.
17. Luisi, B. F., Xu, W. X., Otwinowski, Z., Freedman, L. P., Yamamoto, K. R., and Sigler, P. B. (1991) *Nature 352*, 497−505.
18. Pabo, C. O., and Sauer, R. T. (1992) *Annu. Rev. Biochem. 61*, 1053−1095.
19. March-Amegadzie, R., and Hinton, D. M. (1995) *Mol. Microbiol. 15*, 649−660.
20. Hinton, D. M., March-Amegadzie, R., Gerber, J. S., and Sharma, M. (1996) *J. Mol. Biol. 256*, 235−248.
21. Hinton, D. M., March-Amegadzie, R., Gerber, J. S., and Sharma, M. (1996) *Methods Enzymol. 274*, 43−57.
22. Lakowicz, J. R. (1983) in *Principles of Fluorescence Spectroscopy*, pp 111−153, Plenum Press, New York.
23. Lee, C., Levin, A., and Branton, D. (1987) *Anal. Biochem. 166*, 308−312.
24. Weber, G. (1975) *Adv. Protein Chem. 29*, 1−83.
25. LeTilley, V., and Royer, C. A. (1993) *Biochemistry 32*, 7753−7758.
26. Alexander, K. A., and Phelps, W. C. (1996) *Biochemistry 35*, 9864−9872.
27. Nelson, W. C., and Matson, S. W. (1996) *Mol. Microbiol. 20*, 1179−1187.
28. Ptashne, M. (1992) *A Genetic Switch*, Cell Press and Blackwell Scientific Publications, Cambridge, MA.
29. Omichinski, J. G., Trainor, C., Evans, T., Gronenborn, A. M., Clore, G. M., and Felsenfeld, G. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 1676−1680.
30. Damante, G., Fabbro, D., Pellizzari, L., Civitareale, D., Guazzi, S., Polycarpou-Schwartz, M., Cauci, S., Quadrifoglio, F., Formisano, S., and Di Lauro, R. (1994) *Nucleic Acids Res. 22*, 3075−3083.
31. Severinova, E., Severinov, K., Fenyo, D., Marr, M., Brody, E. N., Roberts, J. W., Chait, B. T., and Darst, S. A. (1996) *J. Mol. Biol. 263*, 637−647.
32. Li, M., Moyle, H., and Susskind, M. M. (1994) *Science 263*, 75−77.

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