Architecture of the Bacteriophage T4 Activator MotA/Promoter DNA Interaction during Sigma Appropriation*

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Background: No physical exists of the bacteriophage T4 activator MotA with DNA.

Results: Using FeBABE, physical models, and ICM Molsoft, we determined how MotA interacts with DNA within the transcription complex.

Conclusion: The unusual “double-wing” motif in MotA CTD sits within the DNA major groove.

Significance: FeBABE analyses together with structures can be used to determine protein-DNA architecture within multiprotein complexes.

Gene expression can be regulated through factors that direct RNA polymerase to the correct promoter sequence at the correct time. Bacteriophage T4 controls its development in this way using phage proteins that interact with host RNA polymerase. Using a process called σ appropriation, the T4 co-activator AsiA structurally remodels the σ70 subunit of host RNA polymerase, while a T4 activator, MotA, engages the C terminus of σ70 and binds to a DNA promoter element, the MotA box. Structures for the N-terminal (NTD) and C-terminal (CTD) domains of MotA are available, but no structure exists for MotA with or without DNA. We report the first molecular map of the MotA/DNA interaction within the σ-appropriated complex, which we obtained by using the cleaving reagent, iron bromoacetamidobenzyl-EDTA (FeBABE). We conjugated surface-exposed, single cysteines in MotA with FeBABE and performed cleavage reactions in the context of stable transcription complexes. The DNA cleavage sites were analyzed using ICM Molsoft software and three-dimensional physical models of MotA−NTD, MotA−CTD, and the DNA to investigate shape complementarity between the protein and the DNA and to position MotA on the DNA. We found that the unusual “double wing” motif present within MotA−CTD resides in the major groove of the MotA box. In addition, we have used surface plasmon resonance to show that MotA alone is in a very dynamic equilibrium with the MotA box. Our results demonstrate the utility of fine resolution FeBABE mapping to determine the architecture of protein-DNA complexes that have been recalcitrant to traditional structure analyses.

Activation of transcription plays a vital role in the regulation of gene expression, allowing organisms to control the timing and location of gene products. Typically, activators bind to specific sites on the DNA and interact with components within the transcriptional machinery. These interactions then promote transcription from promoter sequences that would not normally be recognized by RNA polymerase in the absence of the regulator, allowing diversity in the selection of transcription start sites (for review, see Refs. 1 and 2).

Activation of the middle class of bacteriophage T4 promoters provides an excellent model system for investigating how protein-protein and protein-DNA contacts can redirect polymerase to a different promoter sequence. In this system two small T4 factors, the MotA activator and AsiA co-activator, divert the host Escherichia coli RNA polymerase (RNAP) from host promoters to the T4 middle class (for review, see Ref. 3). At most host promoters the primary specificity factor of RNAP, σ70, interacts with DNA elements in the −35, −15/−14, and −10 regions of the DNA using the σ70 regions 4, 3, and 2, respectively (for review, see Refs. 4–6). T4 middle promoters contain the −10 element, but they lack the −35 recognition sequence. Instead they have another element, the MotA box, that is located in the −30 region of the promoter and is recognized by the MotA protein (for review, see Ref. 3).

The ability of MotA to activate middle promoter transcription requires the T4 co-activator AsiA. AsiA binds tightly to σ70 Region 4 and structurally remodels this portion of σ (7). Consequently, σ70 Region 4 no longer binds to the −35 element or to its protein partner within the β subunit of core polymerase, the β-flap. This disruption helps MotA in two ways. First, it

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prevents the $\sigma^{70}$ Region 4/–35 DNA contact (8–11), allowing MotA unfettered access to the MotA box, which overlaps the –35 region of the DNA. Second, it allows MotA to interact with H5, the C-terminal residues of $\sigma^{70}$ that are normally fully secured within the $\beta$-flap (12). Because MotA targets the very same residues within H5 that are engaged by the $\beta$-flap (13), AsiA-induced rearrangement of $\sigma^{70}$ Region 4 is required to reveal this target for MotA binding. Other evidence suggests that AsiA also interacts with the $\beta$-flap (14). The hijacking of $\sigma^{70}$ Region 4 by AsiA and MotA engagement of H5 is called $\sigma$ appropriation.

MotA is a 2-domain protein consisting of a N-terminal domain (NTD, residues 1–96), and a C-terminal domain (CTD, residues 105–211) connected by a highly basic linker (15). There is no structure for full-length MotA; only structures for the NTD (16) and CTD (17) are known. MotA<sup>NTD</sup> is composed of five $\alpha$-helices, with helices 1, 3, 4, and 5 packing around the central helix 2 (Fig. 1A). The basic, hydrophobic cleft formed from this arrangement interacts with $\sigma^{70}$ H5 (12), and MotA with mutations within the NTD bind DNA but fail to activate transcription (12, 18, 19). MotA<sup>CTD</sup> consists of a saddle-shaped, double-wing motif generated by three $\alpha$-helices interspersed with six $\beta$-strands (17) (Fig. 1B). MotA<sup>CTD</sup> alone will bind MotA box DNA (20). The double-wing motif is unique among DNA-binding proteins, and its mode of interaction with DNA is, therefore, difficult to predict. The clearly defined domain structures have suggested that MotA is a bifunctional protein with different domains involved in protein–protein versus protein–DNA interactions. However, exactly how MotA engages the DNA has not been determined.

Given the lack of a MotA/DNA structure, we reasoned that we could use the chemical cleaving reagent iron bromoacetamidobenzyl-EDTA (FeBABE) to generate a map of the positions of various MotA residues relative to the DNA within the $\sigma$-appropriated transcription complex. FeBABE is a biochemical tool that can be covalently attached to the free sulfhydryl group of a cysteine residue in a protein (21). Induction of the Fenton reaction by the addition of ascorbate and hydrogen peroxide then generates hydroxyl radicals that cleave polypeptide or nucleic acid bonds proximal to the chelate. The resulting DNA cleavage patterns allow one to biochemically map the position of the protein relative to the DNA. This technique has proven useful for several applications, including orienting the domains of $\sigma^{70}$ relative to an <i>E. coli</i> promoter (22), confirming the position of $\sigma^{70}$ Region 4 relative to the –35 DNA (23–27), and determining the orientation of activators relative to the binding site (28–30). Because we wanted to generate a fine resolution map, we conjugated single surface-exposed residues throughout MotA to obtain extensive cleavage data. We then used the generated cut sites together with ICM Molssoft and three-dimensional physical models of MotA<sup>NTD</sup>, MotA<sup>CTD</sup>, and DNA structures to guide the position of MotA as we evaluated the shape complementarity between protein and DNA. Our results have allowed us to position the full-length MotA on the DNA within the transcription complex and demonstrate the utility of this approach to map a protein–DNA complex that has been recalcitrant to traditional structure analyses. In addition, we have used surface plasmon resonance to show that MotA alone is in a very dynamic equilibrium with the MotA element, dissociating from the DNA rapidly. Our results are consistent with a model in which MotA “samples” the DNA and is able to activate only when both its protein and DNA partners are available.

**EXPERIMENTAL PROCEDURES**

DNA—pDKT90 (31), which contains the T4 middle promoter $P_{uvx}$, was digested with BsaI to generate the linear template for <i>in vitro</i> transcription. 5'-32P-end-labeled $P_{uvx}$ DNA (a 200-bp fragment containing $P_{uvx}$ sequences from –94 to +83 relative to the start of transcription) that was used for the FeBABE cleavage reactions was generated by PCR and purified as described (31) except that Pfu polymerase (Agilent Technologies) was used. Oligodeoxyribonucleotides used for the surface plasmon resonance (SPR) analyses were obtained from Operon. The top strand of each double-strand oligomer also contained the sequence 5’-biotin-ATACATTATG to allow attachment of the DNA to the neutravidin present on the SPR chip.

pNW143, a araC/jar<sup>1</sup>/pACYC-based plasmid, contains WT motA cloned between the arabinose-inducible $P_{BAD}$ promoter and the rrnB transcription terminator (12). pMotA<sup>(cys<sup>–</sup>)</sup> is identical to pNW143 except that the single cysteine residue in MotA (Cys-182) has been mutated to an alanine. The cys<sup>–</sup> motA gene was generated by a modification of Bsl site-directed mutagenesis (32). Briefly, PCR was performed using pNW143, Pfu polymerase, and two pairs of oligomers; set 1 contained an oligomer with an XbaI site that is upstream of <i>motA</i> and the mutagenic oligomer with the BsaI cleavage site at the location of the Cys-182 codon; set 2 contained the mutagenic oligomer with the BsaI site and an oligomer with a SphI site that is downstream of <i>motA</i> and the mutagenic oligomer with the BsaI cleavage site at the location of the Cys-182 codon; set 2 contained the mutagenic oligomer with the BsaI site and an oligomer with a Spht site that is downstream of <i>motA</i> (sequences of primers are available upon request). The resulting PCR products were digested with BsaI and XbaI or with BsaI and SphI and then ligated with pNW143 that had been previously digested with XbaI and Spht.

Plasmids encoding motAC182A with the desired cysteine substitutions at specific locations were either derived from pMotAcys<sup>–</sup> again using BsaI site-directed mutagenesis or were obtained from Bioinnovatise (the plasmids encoding the E93C, E112C, T100C, E132C, K96C, and E105C substitutions), again in the pMotAcys<sup>–</sup> background. DNA sequence analyses, performed either by the Facility for Biotechnology Resources of the FDA or Macrogen, confirmed the sequence of the constructs throughout the cloned regions.

**Proteins**—<i>E. coli</i> core polymerase was obtained from Epicenter Technologies, and AsiA-His<sub>6</sub>-tagged was purified as described (33, 34). Purification of $\sigma^{70}$ was done as described (18, 35) using <i>E. coli</i> BL21(DE3)/pLysS (36) containing the plasmid pHN12 (35), which expresses $rpoD$. Wt MotA was purified as described (Ref. 12 or below).

Synthesis of MotA proteins was induced in BL21(DE3) cells (36) containing pNW143, pMotA(cys<sup>–</sup>), or the pMotA(cys<sup>–</sup>) derivatives with the specific cysteine mutations by growth at 37 °C in LB + 40 µg/ml kanamycin and 0.2% arabinose. After harvesting by centrifugation, cells were resuspended in sonication buffer (20 mM Tris-Cl (pH 7.9), 50 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, 1 mM PMSF) and broken by sonication. Cell fractions were obtained after centrifugation at 8750 × g.
FeBABE Analysis of MotA Binding to DNA

The MotA proteins were first isolated by batch or column phosphocellulose chromatography as described (31, 38) except that in some cases of batch chromatography the column was first washed extensively with sonication buffer containing a salt concentration (0.3 M NaCl) that did not elute the protein, and then the protein was eluted with small volumes of sonication buffer containing the eluting salt concentration (0.4 M NaCl). In this procedure, the last elution fraction was highly purified and essentially free of a contaminating nuclease (see below) and, thus, was used without further purification. In the other cases, protein fractions eluting from the phosphocellulose column were dialyzed versus AKTA binding buffer (50 mM potassium phosphate (pH 6.5), 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF), loaded onto a HiTrap SP cation exchange column (1 ml, GE Healthcare), and eluted with a gradient of 0–500 mM NaCl in AKTA binding buffer (40 ml total). When necessary, the MotA proteins were concentrated using Amicon Ultra Centrifugal Filters (3000 molecular weight cutoff) before being buffer-exchanged into MotA GC buffer (200 mM potassium phosphate (pH 6.5), 1 mM DTT, 1 mM benzamidine HCl, 1 mM EDTA, 1 mM EGTA, and 50% glycerol). Protein concentrations were determined by comparison to known amounts of WT MotA after SDS-PAGE and gel staining with Colloidal Coomassie Blue (Invitrogen).

Because of a DNA nuclease that co-purifies with MotA (31), fractions were tested for nuclease contamination by incubation with either double-strand plasmid or with 32P-labeled DNA before determining which fractions to pool. In addition, MotA proteins D43C and N187C were further purified by single-strand DNA cellulose chromatography using a 0.5-ml column equilibrated in sonication buffer. The column was washed with 2-ml steps of sonication buffer containing 100, 200, and 500 mM NaCl in sonication buffer. This further purification of the MotA D43C and N187C proteins, which eluted in the 500 mM NaCl steps, reduced but did not eliminate the DNase. Some of the other MotA mutants also contained low levels of nuclease, which yielded cutting outside of the PuvX promoter region (Fig. 3, A and B). This cutting was not dependent on sodium ascorbate/H2O2 that is needed to generate hydroxyl radicals from the conjugate FeBABE.

In Vitro Transcription Assays—Single round transcription assays in which the proteins were incubated with the DNA for only 20 s at 37 °C before the addition of rifampicin (Fig. 2A) were performed as described (12). A similar protocol was used for the assays in which the proteins were incubated for 8 min at 37 °C, except that no NTPs were present during the preincubation; NTPs plus 500 ng heparin were then added to start transcription (Fig. 2B). Gels were imaged by autoradiography followed by scanning with a Powerlook 2100XL densitometer.

Conjugating MotA Proteins with FeBABE—MotA protein (2 nmol), which had been dialyzed into either sonication buffer without 2-mercaptoethanol or GC buffer without DTT, was incubated with FeBABE (Dojindo Laboratories) (10 nmol in 0.5 μl of DMSO) for 1 h at 37 °C in a final volume of 40–50 μl. The protein was then dialyzed into GC buffer, which also served to remove excess FeBABE. Conjugation efficiencies, as determined by mass spectrometry (NIDDK Advanced Mass Spectrometry Facility), were: D43C, 79%; E93C, 95%; K96C, 85%; T100C, 95%, E105C, 93%; E112C, 60%; E132C, 54%; S136C, 27%; E143C, 27%; N157C, 40%; H170C, 11%; N187C, 44%. As expected, FeBABE treatment of MotA, which lacks a cysteine residue, resulted in no conjugation. In addition, conjugation of the natural cysteine of WT MotA (Cys-182) was low (8%), and conjugated WT MotA did not result in any specific FeBABE cleavages. Consequently, we could not use Cys-182 in this analysis.

Cleavage of DNA Using FeBABE-conjugated Proteins—Stable transcription complexes were formed by incubating PuvsX DNA (0.2 pmol, 5'-32P end-labeled as indicated on either the template or non-template strand) for 10 min at 37 °C with MotA (3.6 pmol) and AsiA-associated RNAP (24 pmol of AsiA and 1.7-pmol core with a ratio of σ70 from 1:1 to 1:2) in a 10-μl solution containing 40 mM Tris acetate (pH 7.9), 10 mM Tris-Cl (pH 7.9), 20 mM potassium phosphate (pH 6.5), 35 mM NaCl, 150 mM potassium glutamate, 14% glycerol (v/v), 4 mM magnesium acetate, 0.4 mM EDTA, 0.1 mM EGTA, 0.3 mM DTT, 0.02 mM 2-mercaptoethanol, and 0.0005% Triton X-100. (AsiA and σ70 were preincubated together first at 37 °C for 10 min to ensure formation of the AsiA/σ70 complex, and then this complex was incubated with core at 37 °C for 10 min to form the AsiA-associated RNAP.) Unstable complexes were challenged by the addition of 0.5 μl of 1 μg/μl heparin for 1 min at 37 °C.

FeBABE cleavage was performed essentially as described (23, 39). Reactions were initiated by the addition of 2 μl of 100 mM sodium ascorbate and 2 μl of 0.6% H2O2. After incubation for 10 min at 37 °C, the reactions were quenched by the addition of 30 μl of TE buffer (10 mM Tris (pH 8.0), 1 mM EDTA), 80 μl of 100 mM thiourea, and 0.5 μl of 1 μg/μl calf thymus, collected on ice, and phenol-extracted. The DNA was ethanol-precipitated, and DNA products were separated on 5% acrylamide, 7 M urea denaturing gels run in 0.5 × Tris borate-EDTA. Gels were imaged by autoradiography followed by scanning with a Powerlook 2100XL densitometer.

Orienting MotA on MotA Box DNA—The MotANTD and MotACTD structures were positioned independently on the DNA based on the FeBABE cleavage sites as detailed under “Results” using three-dimensional molecular models of the structures of MotACTD, MotANTD, and B-form DNA (generated by a Z-450 three-dimensional printer) and using the ICM Molsoft program.

The range for hydroxyl radicals generated by FeBABE has been reported to be ~18 Å (14 Å plus ~4 Å for the FeBABE moiety) (22, 40). Thus, the predicted cut sites given in Figs. 4 and 5 were determined using distances of 18 Å or less for all the residues except Asp-43 and His-170, where it was necessary to extend the distances to 25 and 24 Å, respectively, to predict any cuts. However, modest distortion or bending of the DNA could bring the cut site closer to the residue for these cases.

SPR Experiments—SPR experiments were conducted at 25 °C using a Biacore 3000 optical biosensor (GE Healthcare). Neu travidin (Thermo Scientific) was immobilized on carboxylated gold chips (C1 chips; GE Healthcare) by routine amine coupling chemistry in immobilization buffer (10 mM Hepes (pH 7.4), 150 mM NaCl, 0.005% Tween 20). Carboxyl groups on the chip were
activated with $N$-ethyl-$N'$-(3-dimethylaminopropyl) carbodiimide and $N$-hydroxysuccinimide, and neutravidin was injected in 10 mM sodium acetate (pH 5.0) until immobilization levels of 1300–1800 resonance units were achieved. Any remaining active sites were blocked with ethanolamine. The neutravidin surfaces were preconditioned with three 15-s injections of 10 mM NaOH before DNA capture.

DNA oligonucleotides were dissolved in TE buffer and then diluted into immobilization buffer. The biotinylated “top” strand was injected over a neutravidin surface until 30–40 resonance units were captured. The double-strand DNA surface was completed by injecting the complementary “bottom” strand until saturation was observed. A neutravidin-coated flow cell without DNA was used as a reference cell.

MotA protein was prepared in binding buffer (20 mM Tris (pH 8.0), 150 mM NaCl, 1 mM Tris(2-carboxyethyl)phosphine, 0.005% Tween 20, 5% glycerol) as a 3-fold dilution series starting at 1000 nM and was injected in triplicate at each concentration at a flow rate of 75 µl/min. MotA dissociated completely from the DNA surfaces, eliminating the need for a regeneration step. A series of buffer-only (blank) injections was included throughout the experiment to account for instrumental noise. The data were processed, double-referenced, and analyzed (41) using the software package Scrubber2 (Version 2.0c, BioLogic Software). Equilibrium dissociation constants ($K_d$) were determined by fitting the steady-state binding data to a 1:1 interaction model.

RESULTS

Selection of MotA Residues for Conjugation with FeBABE—In the presence of $\text{H}_2\text{O}_2$ and sodium ascorbate, FeBABE generates hydroxyl radicals through the Fenton reaction (40). Thus, conjugation of FeBABE at a specific cysteine residue positions a potential burst of hydroxyl radicals that can destroy chemical bonds within nearby DNA or protein. The resolution of FeBABE cleavage has been reported to be within ~3–5 bp of the DNA (22, 40). Our strategy was to position the FeBABE reagent at various sites on the surface of MotA as a way to generate a fine resolution map of the MotA/DNA architecture during σ appropriaition.

Although the structure of full-length MotA protein is not yet available, an NMR solution structure of MotA$^\text{NTD}$ (16) (residues 1–96, Fig. 1A) and an x-ray crystal structure of MotA$^\text{CTD}$ (17) (residues 105–211; Fig. 1B) have been determined. Within MotA, these two domains are connected by a highly basic, eight-residue linker. Using the structures, we selected the following residues as suitable for conjugation: Asp-43, Glu-93, and Glu-112 within MotA$^\text{NTD}$ (Fig. 1A) and Glu-105, Glu-112, Glu-132, Glu-143, Asn-157, His-170, and Asn-187 within MotA$^\text{CTD}$ (Fig. 1B). We also selected Thr-100 within the linker. Single cysteines were introduced at the selected sites using a MotA mutant whose WT cysteine (Cys-182) had been previously converted to an alanine (Fig. 1B).

The MotA Mutants with or without Conjugation Retain Significant Transcriptional Activity—To assess whether the MotA mutations or conjugation impaired activity, we assayed each mutant protein and its conjugated partner for its ability to activate transcription from the T4 middle promoter, $\text{RNAP}_{\text{eu}}$. We deliberately chose to incubate the DNA, MotA, AsiA-associated RNAP, and NTPs for only 20 s before the addition of rifampicin, which would limit transcription to a single round; WT MotA is active in such an assay (12). Thus, this protocol is a stringent test for the activity for each MotA mutant because it requires that the mutant, like WT MotA, rapidly forms a competent transcription complex.

The starting C182A mutant retained full activity relative to WT MotA (Fig. 2A), and except for T100C, E112C, and S136C, the MotA mutants and their conjugated partners retained a minimum of ~50% activity relative to wt. In most cases the activity of the conjugated protein was close to that of its unconjugated partner, indicating that the cysteine substitution rather than the conjugation caused the decrease in transcriptional activity.

The T100C, E112C, and S136C proteins were also tested in an assay that allowed more time for the formation of active complexes. In this case, the proteins and DNA were incubated for 8 min at 37 °C before the initiation of a single round of transcription. Under these conditions, the activity of the conjugated E112C and T110C increased, whereas conjugated Ser-136 remained at ~30% (Fig. 2B).
PuvsX with RNAP alone would be formed. Finally, we challenged was associated with AsiA, and consequently, no complexes of excess of AsiA relative to RNAP to ensure that all of the RNAP binding of MotA alone to the DNA (31). In addition, we used an ratio of MotA:DNA (34:1) that is insufficient to yield significant analyses only observe.

See “Experimental Procedures” and “Results” for assay details.

Cleavage Sites within the PuvsX Transcription Complex

Moter, PuvsX DNA, that had been 5’-32P-end-labeled on either template or non-template strand. For these analyses we performed FeBABE cleavage reactions using FeBABE-conjugated residue. The major cut sites ranged from 3, A and B) or when using the DNA alone (Fig. 3, A, lane 9 and 25; B, lane 8). Furthermore, as expected, no specific cleavages were observed using complexes formed with MotA C182A, which cannot be conjugated (Fig. 3, A, lane 25, B, lane 30).

Cleavage at position +1 was observed when using some of the conjugated mutants (transparent, red arrowheads in Fig. 3, A, lanes 11, 15, and 21 and B, lanes 10, 14, 16, and 30; dotted boxes within Fig. 3C). This phenomenon has been observed before when using FeBABE-conjugated D581C (22, 25) and reflects the position of the residue relative to the major and minor grooves of the DNA (40). Finally, not all of the conjugated mutants generated specific cleavages. FeBABE positioned at D43C (Fig. 3A, lane 29), E112C (Fig. 3A, lane 33), and N187C (Fig. 3A, lane 31) did not yield specific non-template strand cutting.

Positioning MotACTD on the DNA Using the FeBABE Cleavage Sites—MotA binds the MotA box element (31, 44) consensus sequence of (t/a)(t/a)(t/a)GCTT(c)(t/a) (for review, see Ref. 3) that within Puvx is located from positions −35 to −26 (Fig. 3C). In addition, EMSA has revealed that MotACTD alone retains DNA binding activity (20). Consequently, the range of cutting sites generated by the MotA mutants conjugated at residues within the CTD would need to be consistent with the position of the MotA box in Puvx. As seen in Fig. 3C, the major cut sites obtained with the CTD-conjugated mutants ranged from positions −20 to −39. This provided confidence that the FeBABE analyses were reporting the relevant position of MotA within the α-appropriated complex. In addition, the positions of the cleavages were consistent with the binding of a monomer of MotA (rather than a dimer or other multimers) within the transcription complex; previous work has indicated that MotA alone binds as a monomer to the MotA box (45).

To orient the MotACTD relative to the MotA box of Puvx, we used the FeBABE cleavage sites generated from conjugated residues present on the MotACTD surface (Fig. 1B). Using three-dimensional molecular models of MotACTD and B-form DNA, we first manually moved the MotACTD about DNA to determine the position that was consistent with the cleavage sites. We chose B-form DNA because previous footprinting analyses have been consistent with the idea that there is no severe distortion of MotA box DNA when MotA binds (31, 42, 44, 46).

The structure of MotACTD resembles a saddle whose under-side contains tyrosines, a phenylalanine, and multiple lysines and arginines (Fig. 1B). This is the only such surface on MotACTD and, thus, is consistent with an interaction between this part of the protein and the DNA (17). As discussed below, our model does place this surface toward the DNA (Fig. 4, A and B). However, we considered all possible positions before arriving at this orientation. Our search revealed only one orientation that was consistent with the cleavage sites.

![Image](Image.png)
FeBABE Analysis of MotA Binding to DNA

A Non-template Strand

B Template Strand

C

P

**P**

NTD

E93

K96

E105

E132

S136

E143

N157

H170

**Mota Box**

**P**

NTD

E93

K96

linker

T100

E105

E112

E132

S136

E143

N157

H170

N187

ACGAAAAAGGACTTTCTTAATATCCATATGGTTATATAGAAATTAAAC

TGCTTTTCTTCGAATTATATTAGGTATACCCATATTCTTTATTG
FIGURE 3. Positions of cleavage sites on PuvsX DNA by FeBABE-conjugated MotA proteins within the H9268-appropriated transcription complex. A and B, representative denaturing gels are shown. Acrylamide gels show the migration of 5'-32P PuvsX DNA end-labeled on the non-template or template strand after the FeBABE cleavage; reactions, performed 2–7 times for each conjugated protein, gave similar results to those seen here. The MotA residue substituted with cysteine and conjugated with FeBABE is indicated. Reactions were performed in the presence or absence of hydrogen peroxide and sodium ascorbate (NaAsc), which generate hydroxyl radicals from the FeBABE moiety. Major cleavage products specific for the conjugated residues are denoted with opaque, red arrowheads, which are centered within the range of cut sites. Weaker cut sites seen with some conjugated mutants are denoted with transparent, red arrowheads. Cleavage at position 1, marked with a red horizontal line and seen with some of the conjugated mutants in panel B, is discussed under “Experimental Procedures.” In some other cases, cleavages that were not specific to the presence of H2O2 and sodium ascorbate stemmed from a DNase contaminant present in some of the protein preparations (see “Results” for details). Ladder lanes show the products of a G+A reaction using the labeled PuvsX DNA, which were used to determine the position of cleavage. In A the DNA is oriented as indicated, and the following cut sites for conjugated residues are shown: red, Glu-105, NT 34 to 32, T 39 to 37 and 35; purple, Asn-157, NT 31, T 34 to 33; beige, Glu-112, T 36; yellow, Glu-132, T 29 to 28; orange, Glu-143, NT 25 to 23, T, 27 to 26; blue, His-170, NT 22 to 20, T 24 to 23. In B the DNA is oriented with position −41 at the left, and the conjugated residues (shown as spheres) and their major cleavage sites (given in Fig. 3C; shown as sticks) are color-coordinated. C, observed versus predicted cleavage sites for each of the conjugated MotACTD mutants. Cleavage sites were predicted using the ICM Molsoft program.

FIGURE 4. Position of MotA<sup>CTD</sup> on DNA determined from FeBABE cleavage sites. A, physical models of the MotA<sup>CTD</sup> structure (Ref. 17; PDB code 1KAF) and B-form DNA used to determine the orientation of MotA<sup>CTD</sup> on the DNA. B, PyMOL representations show the major cut sites and position of each of the cysteine-substituted, conjugated residues within MotA<sup>CTD</sup>. In both A and B the DNA is from positions −41 to −14 of PuvsX with the non-template (NT) strand in dark gray and the template (T) strand in light gray; the location of the MotA box is shown as the teal box. In A the DNA is oriented as indicated, and the following cut sites for conjugated residues are shown: red, Glu-105, NT 34 to 32, T 39 to 37 and 35; purple, Asn-157, NT 31, T 34 to 33; beige, Glu-112, T 36; yellow, Glu-132, T 29 to 28; orange, Glu-143, NT 25 to 23, T, 27 to 26; blue, His-170, NT 22 to 20, T 24 to 23. In B the DNA is oriented with position −41 at the left, and the conjugated residues (shown as spheres) and their major cleavage sites (given in Fig. 3C; shown as sticks) are color-coordinated. C, observed versus predicted cleavage sites for each of the conjugated MotA<sup>CTD</sup> mutants. Cleavage sites were predicted using the ICM Molsoft program.
FeBABE Analysis of MotA Binding to DNA

To optimize the orientation, we recreated the MotA\textsuperscript{CTD}/DNA complex found with the physical models within the ICM Molsoft program and then moved MotA\textsuperscript{CTD} slightly as we determined the distances between the conjugated residues and their closest base-pair neighbors. This resulted in the orientation seen in Fig. 4A and B and the predicted versus observed cleavage sites listed in Fig. 4C. Observed cut sites were generally within 1–2 bp. Given the resolution of the reagent and our gels, these results are consistent with the determined orientation. Although in a few cases predicted cut sites were not observed, it must be noted that the cuts were obtained in the presence of the full transcription complex, and the presence of other proteins can shield sites from reactivity.

By using three-dimensional physical models we were also able to investigate the shape complementarity between protein and the DNA. As seen in Fig. 4A, this analysis indicated that our determined position allows the MotA\textsuperscript{CTD} saddle to easily “sit” within the major groove of the MotA box DNA without clashes.

Positioning MotA\textsuperscript{NTD} Relative to the DNA—The position of MotA\textsuperscript{NTD} was determined using a similar process. In this case the orientation was dependent on the cleavages by the conjugated mutants E93C and K96C, which are located at the very C terminus of MotA\textsuperscript{NTD}. For these mutants, cleavage sites were centered just upstream of the MotA box (Fig. 3, A, lanes 11 and 21, B, lanes 10, and 14, and C). The overall orientation of the MotA\textsuperscript{NTD} was then positioned to be consistent with these cleavages together with the cut sites observed with the conjugated mutant D43C at positions −38 to −40 on the template strand (Fig. 3B, lane 21). This gave the final position of MotA\textsuperscript{NTD} (Fig. 5A); a comparison of the predicted versus the observed cut sites with the conjugated MotA\textsuperscript{NTD} mutants is shown in Fig. 5B.

Because we positioned the NTD and CTD domains of MotA independently, a crucial test of our determined positions was whether the space between the locations of the two domains was reasonable for the eight residues that connect the two domains. In our model (Fig. 5A), the C-terminal residue of MotA\textsuperscript{NTD} (Lys-96) is ~20 Å from Glu-105, the N-terminal residue of MotA\textsuperscript{CTD}. This is within the range of a peptide of 8 residues, which would have a maximum estimated length of 29 Å if fully extended (47) or a minimum estimated length of 12 Å, assuming 1.5 Å per residue, if present as a compact α helix.

Finally, additional evidence that is consistent with our position of MotA on the DNA stems from the cleavage results obtained with the conjugated T100C mutant, which is located in the linker. Conjugated T100C yielded weak, but reproducible, cleavage sites at positions −40 and −41 on the template strand of the DNA (Fig. 3, B, lane 26, and C). As seen in Fig. 5A, the position of these T100C cut sites are reasonable with our position of a linker that would connect MotA\textsuperscript{NTD} with MotA\textsuperscript{CTD}.

Taken together, the multiple cleavages seen with the conjugated mutants comprise a gratifyingly consistent picture of the position of the MotA\textsuperscript{CTD} and MotA\textsuperscript{NTD} relative to the DNA. This provides an overall confidence that we are observing the relevant position of MotA relative to the DNA within the σ-appropriated complex.

Surface Plasmon Resonance (Biacore) Analyses Are Consistent with MotA Interaction with Sequences Upstream and Downstream of the MotA Box Element—The FeBABE analyses yielded cleavage sites both upstream and downstream of the MotA box element itself, which could be indicative of MotA/DNA interactions outside of the MotA box sequence. However, given the resolution of FeBABE cleavage, it was not possible to conclude that MotA binding is actually affected by the DNA that borders its element. To investigate this possibility, we performed SPR analyses using various oligodeoxyribonucleotides (Fig. 6A). The full-length 22-bp oligomer contained a centered MotA box, whereas in the upstream and downstream versions the MotA box was shifted toward the 3’ or 5’ portions of the fragment, respectively. The half MotA box was an 18-bp fragment that lacks the upstream half of the MotA box sequence. Versions of these DNAs that contain a mutant MotA box, which fails to bind MotA (46), were also tested. In each case sensorgrams (resonance versus time) and binding isotherms (resonance versus concentration) were generated.

The apparent equilibrium dissociation constant determined by SPR for the full-length MotA box was 137 nM (Fig. 6, A and B1), in good agreement with the previously determined value of 130 nM obtained by EMSA using a 19-bp DNA oligomer (46). As expected, binding of MotA was dependent on the MotA box sequence; the full-length oligomer with mutations within the MotA box and the half MotA box bound poorly (Fig. 6, A and B, S and D). When using either the upstream or downstream oligo-
**Figure 6.** SPR analyses of MotA binding to double-strand oligodeoxyribonucleotides. 

**A**, sequences of DNA fragments used for study. Apparent dissociation constants ($K_{d(app)}$) were determined for MotA binding to the indicated DNAs. The top strand of each DNA also contained the sequence 5′/H11032 biotin-ATACATTATG before the sequence shown. The consensus sequence for the MotA box element (48) is indicated at the top, and the MotA box present in the DNAs is boxed and in green. Mutations within the MotA box element are in red. 

**B**, sensorgrams (response in resonance units (RU) versus time; left graph) and binding isotherms (response versus concentration; right graph) for each of the DNA fragments.
mers, the measured dissociation constants increased 3–4-fold (Fig. 6, A and B2, and Fig. 5B3). These results are consistent with the idea that the presence of DNA both upstream and downstream of the MotA box is needed for optimal MotA binding even though previous analyses of T4 middle promoter sequences indicate that conserved bases are not present in these regions (48).

MotA Rapidly Dissociates from MotA Box DNA—The SPR analyses revealed two other important features of MotA binding. First, this analysis provided the first kinetic analysis of the MotA/DNA interaction and indicated that it is very dynamic, with an off rate faster than 0.5 s⁻¹, the limit of the instrument (Fig. 6B1). Second, the sensogram indicated that saturation is difficult to achieve even at concentrations of MotA greater than 1 μM (Fig. 6B1). This has been observed previously when analyzing MotA binding by EMSA (46) or by fluorescence anisotropy (45). Although MotA binds to the MotA element as a monomer (45), EMSAs suggest that multimers of MotA associate with the DNA/DNA complex as the concentration of MotA increases. This phenomenon could account for the difficulty to saturate the system.

**DISCUSSION**

Mechanisms that change the promoter specificity of RNA polymerase are used by all organisms to control the temporal and spatial expression of genes. During infection, bacteriophage T4 uses σ appropriation, a novel and elegant strategy, to usurp E. coli RNAP, shifting host expression to viral gene expression (for review, see Ref. 3). In this process the T4 proteins AsiA and MotA interact with the σ^70 subunit of RNAP to switch the specificity of polymerase from recognition of the σ^70-dependent −35 element, present in host promoters, to the recognition of the MotA box, which is centered at position −30 in T4 middle promoters. AsiA binds tightly to σ^70 Region 4, remodeling this region and preventing its interaction with the −35 element (7). This remodeling also prevents the interaction of Region 4 and the C terminus of σ^70, H5, with the β-flap of core RNAP. Instead, in the σ-appropriate complex, H5 interacts with MotA^NTD (12, 13) (Fig. 7).

Understanding how the association of factors with RNAP can change core promoter recognition requires a molecular understanding of the protein–protein and protein–DNA contacts within the transcription complex. However, as yet there are no determined structures for any activator–RNAP–DNA complexes. Modeled structures for a CRP (c-AMP receptor protein)–RNAP–DNA complex (49) and for a λ CII–RNAP–DNA complex (50, 51) have relied on the determined structures of the activators with the DNA. In the case of the σ-appropriate complex, attempts to obtain structures for full-length MotA or any portion of MotA with DNA have been unsuccessful. Our work here demonstrates how FeBABE can be used to generate a fine resolution map of an activator relative to the DNA within the context of a multisubunit complex. We have generated the first molecular map of the MotA/DNA architecture and shown the orientation of MotA relative to the DNA within the transcription complex.

Because we have separate structures of the NTD and CTD domains of MotA, we modeled each separately on the DNA. The MotA^CTD structure is composed of a saddle of β-sheets with two distinct wings (Ref. 17 and Fig. 1B). This structural motif has not been observed outside of MotA so its mode of binding cannot be determined by analogy. From our analysis we see that the underside of the MotA^CTD double-wing helix “saddle,” with its basic and hydrophobic residues, aligns with the major groove of the downstream, half of the MotA box DNA. Thus, both “wings” of the double-winged helix motif are positioned within the major groove (Figs. 4A and 5A). On the other hand, the C-terminal end of MotA^NTD, the arginine-rich MotA linker, and another surface of MotA^CTD that includes residues Glu-105, Glu-112, Ser-136, and Asn-157 are located near the minor groove of the upstream portion of the MotA box (Fig. 4A and B). The placement of the MotA arginine-rich linker near the minor groove surface is consistent with multiple reports, indicating that arginine residues target minor grooves of DNA (52–54). Our modeled position is also consistent with previous work demonstrating that MotA recognizes major groove determinants that are in the downstream region (the TT at positions −29 and −28 of P_{uvx} (Fig. 3C)) and minor groove determinants that are in the upstream region (positions −33 and −32 of P_{uvx} (Fig. 3C)) of the MotA box (46).

Given that MotA^CTD alone can interact with the MotA box, a speculative MotA^CTD/DNA model was previously proposed in which the MotA^CTD alone contacted both the minor and major grooves of the DNA (17). Although this model was reasonable given the available information, our work indicates that the position of MotA^CTD is different; from our analysis, MotA^CTD is rotated about 90 degrees relative to its previously proposed position.

We determined the positions of MotA^CTD and MotA^NTD independently. Consequently, the eight-residue linker had to “fit” between the two domains for the model to be viable.
Indeed, we find that the distance between the modeled NTD and CTD is reasonable for this linker. In addition, the cleavage sites obtained with the conjugated linker mutant T100C are consistent with the positions of the two domains. We conclude that our FeBABE-derived “map” provides us with the orientation of the entire MotA protein on the DNA. We have previously found that the basic, hydrophobic cleft within MotA interacts with the C-terminal portion of $\sigma^{70}$ (Ref. 12; Fig. 1A). Thus, this work has provided us with the starting point for modeling the interaction of H5 with MotA. In other work$^5$ we used structure-based modeling together with the MotA/DNA architecture reported here to generate a model of the entire MotA-AsiA-associated RNAP-DNA complex.

AsiA-associated RNAP binds poorly if at all to middle promoter DNA as the binding of AsiA to free $\sigma^{70}$ Region 4/H5 from interacting with the $\beta$-flap of core. However, in the AsiA-associated RNAP, H5 is now free to be engaged by MotA (Fig. 7, A and B). Thus, the $\sigma$-appropriated complex might be assembled through MotA engagement of H5, which would then bring the entire transcriptional complex to the DNA, or by MotA binding to the MotA element, which would recruit the AsiA-associated complex. However, it seems likely that MotA must engage both its protein and DNA partners together. Our SPR analyses indicate that MotA alone is in a very dynamic equilibrium with the MotA element, dissociating from the DNA quite rapidly; previous work has determined relative high-equilibrium dissociation constants for the interaction of MotA alone with either H5 (16 $\mu$M; Ref. 12) or with the DNA (100–200 nM; Refs. 45 and 46). We conclude that MotA is an activator that samples the DNA and then activates when both its protein and DNA partners are available (Fig. 7C).

Although bacteriophage T4 (and presumably other related T4-like phages with AsiA and MotA analogs) are the only entities known to use $\sigma$-appropriation, elucidating the mechanisms of this process has applications outside of phage biology. This is because it is now known that within eukaryotic polymerase II, the basal transcription factor TFIIB together with TATA-binding protein (TBP) constitute the specificity subunit that is functionally similar to $\sigma$; domains within TFIIB correspond to $\sigma$ Regions 2 and 3, whereas TFIIB together with TBP corresponds to $\sigma$ Region 4 (55, 56) (for review, see Ref. 6). It is also known that TBP-related factors can exchange with TBP for expression from different promoters during development (57). Thus, in both TBP switching and MotA/AsiA activation, only a portion of the specificity factor, in both cases “Region 4,” is altered to change promoter specificity. The phage system of $\sigma$ appropriation then provides a simple system for investigating how modulation of a portion of the RNAP specificity factor can result in an overall switch in promoter recognition.

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