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An N-Terminal Mutation in the Bacteriophage T4 *motA* Gene Yields a Protein That Binds DNA but Is Defective for Activation of Transcription

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The bacteriophage T4 MotA protein is a transcriptional activator of T4-modified host RNA polymerase and is required for activation of the middle class of T4 promoters. MotA alone binds to the −30 region of T4 middle promoters, a region that contains the MotA box consensus sequence [(t/a)(t/a)TGCTT(t/c)A]. We report the isolation and characterization of a protein designated Mot21, in which the first 8 codons of the wild-type *motA* sequence have been replaced with 11 different codons. In gel retardation assays, Mot21 and MotA bind DNA containing the T4 middle promoter P_{uvsX} similarly, and the proteins yield similar footprints on P_{uvsX}. However, Mot21 is severely defective in the activation of transcription. On native protein gels, a new protein species is seen after incubation of the σ^{70} subunit of RNA polymerase and wild-type MotA protein, suggesting a direct protein-protein contact between MotA and σ^{70}. Mot21 fails to form this complex, suggesting that this interaction is necessary for transcriptional activation and that the Mot21 defect arises because Mot21 cannot form this contact like the wild-type activator.

Transcription of the middle class of bacteriophage T4 genes requires T4-modified host RNA polymerase and a transcriptional activator, the product of the T4 *motA* gene (reviewed in reference 43). The modification to the polymerase required for MotA-dependent transcription has been identified as the T4 AsiA protein (13, 32), a factor that binds tightly to σ^{70} (41, 42). A mutation in the *motA* gene was discovered because the mutant phage fails to give normal patterns of T4 prereplicative protein synthesis (25). Subsequent work demonstrated that this abnormal protein pattern observed during a T4 *motA* mutant infection occurs because the middle (MotA-dependent) class of promoters does not become active (6). Middle promoters are characterized by an excellent match to the −10 consensus sequence for σ^{70}, the specificity subunit of RNA polymerase, but they lack a recognizable σ^{70} −35 sequence. Instead they share a 9-bp sequence, a MotA box, centered 30 bp upstream of the start of transcription (2, 6). The MotA protein binds to this sequence and is required for transcription initiating at T4 middle promoters by T4-modified polymerase in vitro (10, 24, 37). Recent results indicate that while T4-modified polymerase can bind a middle promoter in the absence of the activator, MotA protein is needed to form the open complex (12).

The *motA* gene encodes a protein of 211 amino acids (45). Although several T4 *motA* mutants have been isolated and characterized (7, 17, 18, 20, 25, 26, 39, 47), analyses of the sequence changes present in these mutations reveal that all retain at least the first 59 amino acids of the wild type-MotA sequence (33, 45, 47). We describe the isolation and characterization of an N-terminal mutation of *motA*, *mot21*, in which the first 8 codons of the wild-type *motA* gene are replaced with 11 different codons. The wild-type MotA and Mot21 proteins bind similarly to the T4 middle promoter P_{uvsX}, but Mot21 fails to activate transcription. We present evidence to suggest that wild-type MotA makes a direct contact with the σ^{70} subunit of RNA polymerase and that Mot21 is defective in this interaction.

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MATERIALS AND METHODS

Strains, phage, and complementation tests. *Escherichia coli* NapIV*supD* (29) and tabG (34) were from K. Kreuzer, Duke University, Durham, N.C. *E. coli* N99cI^{+} (from Pharmacia) and N4830 (from S. Adhya, National Institutes of Health, Bethesda, Md.) contain copies of the wild-type *c*I and the *c*I857 mutant λ repressor, respectively. Wild-type T4D^{+} phage was from B. Stitt, Temple University, Philadelphia, Pa. The temperature-sensitive T4 *motA* mutant *ts*G1 and the *motA* amber mutant *am*G1 (25, 26) were from K. Kreuzer. Complementation tests with these mutants were performed as described previously (10).

General methods. Procedures for plasmid constructions and transformations were as previously described (10, 14). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed according to the procedure of Laemmli (23), using 10 to 20% acrylamide gels from Integrated Separation Systems. Protein determinations were performed using the Bio-Rad protein assay and bovine serum albumin (BSA) as a standard. DNA sequencing of the *mot21* gene in pMOT21 was performed by the dideoxy sequencing method (35).

Isolation of *mot21* and plasmids. The ampicillin-resistant vector pMS114 contains a multiple cloning site between P_L, the leftward promoter of λ, and a strong Rho-dependent transcription terminator (38). A *motA* gene containing the *mot21* mutation was isolated as the result of an attempt to clone the wild-type *motA* gene into this vector. A 970-bp *Hae*II-to-*Kpn*I fragment (T4 map units 163.853 to 162.884) was isolated after digestion of an *Eco*RV fragment (map units 164.985 to 160.900) of bacteriophage T4 dC-containing DNA (Fig. 1; see reference 22 for T4 map unit designations). This DNA was ligated to pMS114, which had been linearized within the multiple cloning site by digestion with *Kpn*I. The ligation product was then incubated with T4 DNA polymerase in the presence of all four deoxynucleoside triphosphates (dNTPs) to create blunt ends at the *Hae*II site of the insert and the other end of the vector and religated. After transformation of *E. coli* N99cI^{+} cells, none of the ampicillin-resistant colonies contained the full-length 970-bp T4 insert, suggesting that the presence of this fragment was deleterious. Thus, we attempted to generate a *motA* clone starting just at the region encoding the *motA* ribosome binding site but lacking farther upstream sequences. DNA from the above-described ligation reaction was digested with *Sac*I to cleave the vector DNA upstream of the insert and with *Hin*dIII to cleave just within the T4 *motA* gene at T4 map unit 163.575. This DNA was then ligated in the presence of a double-stranded oligodeoxyribonucleotide which would recreate the ribosome binding site and the missing 5′ end of the *motA* coding region. After transformation of *E. coli* N99cI^{+}, 24 ampicillin-resistant colonies were isolated. Eighteen transformants lacked any T4 inserts, and five transformants, including pMOT8, contained the T4 DNA from the *Hin*dIII site within the *motA* gene to the *Kpn*I site, thus lacking the N-terminal portion of *motA*. One transformant, designated pMOT21, contained the T4

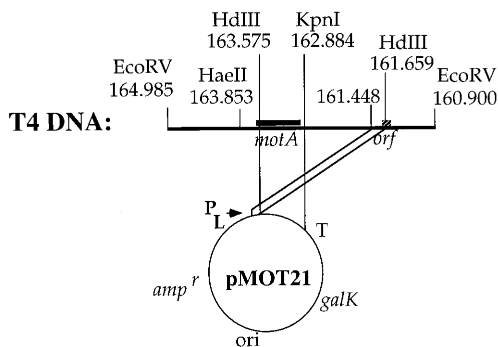

FIG. 1. Diagram of pMOT21. The top line shows the T4 *Eco*RV fragment from T4 map units 164.985 to 160.900 (see reference 22 for map unit designations) and the positions of restriction sites referred to in the text. pMOT21 contains the T4 DNA from map units 161.448 to the *Hin*dIII (HdIII) site at 161.659 inserted immediately upstream of T4 DNA from the *Hin*dIII site at 163.575 to the *Kpn*I site at 162.884. This construction replaces the start of the *motA* gene with the start of an unidentified open reading frame (*orf*) in T4. P_L, strong leftward promoter of lambda; T, transcription termination site from the *E. coli* insertion sequence IS*2*; *galK*, *E. coli* galactokinase gene; ori, origin of replication from pBR322; *amp*^{r}, gene encoding β-lactamase.

fragment from the *Hin*dIII site in the *motA* gene to the *Kpn*I site, preceded by 212 bp of extra DNA. Sequence analysis revealed that the additional sequences matched those of T4 DNA from map unit 161.448 to a *Hin*dIII site at map unit 161.659 (Fig. 1). The origin of the DNA is unclear. The most likely explanation is that it stemmed from a trace level of a contaminating fragment present even after gel purification of the wanted 970-bp *Hae*II-to-*Kpn*I fragment.

Plasmid pMOT21ΔP was derived from pMOT21 by removing 257 bp of vector DNA from the *Bgl*II site upstream of the λ P_L promoter sequences to the *Bgl*II within the multiple cloning site (just upstream of the T4 insert). Plasmid pMOT61 contains the same *Bgl*II deletion upstream of the wild-type *motA* gene (10).

pDH428 contains 4,432 bp of T4 DNA (from T4 map units 24.854 to 20.422) which includes the T4 middle promoters P_{segA} and P_{uvsX} (15). pDKT90 and pDKT90ΔHD contain T4 sequences from −94 to +83 and −38 to +83, respectively, relative to the start of transcription from P_{uvsX} (24).

Proteins. To obtain Mot21, a 1-liter culture of pMOT21/N4830 was grown to mid-log phase at 29°C in L broth containing 25 μg of ampicillin per ml. The synthesis of Mot21 was then induced by shifting the culture to 41°C for 1 h. Cells were harvested by centrifugation and then purified by using the purification protocol for MotA (13) except that 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF) was used as the protease inhibitor rather than phenylmethylsulfonyl fluoride (PMSF). Briefly, this protocol involves sonication to break the cells, the isolation of a low-speed (8,600 × *g*) supernatant, and purification of the Mot21 protein after phosphocellulose (PC) and double-stranded DNA-cellulose chromatography. This procedure yielded 2.5 mg of Mot21. Of this, a nearly homogeneous fraction (1.3 ml at 0.53 mg/ml) was used for the native protein gels. For other experiments, partially purified Mot21, obtained after low-speed centrifugation or PC chromatography, was used. The concentrations of Mot21 in these fractions were estimated at 6 pmol/μl (low-speed supernatant) and 12 pmol/μl (PC fraction) on the basis of a comparison of the level of Mot21 to a known amount of MotA protein as seen after SDS-PAGE.

Low-speed supernatant and PC fractions obtained from pMOT8/N4830 cells were used as control fractions for the Mot21 protein. Wild-type MotA was supplied as purified protein (13) or as a partially purified fraction after PC chromatography. T4-modified RNA polymerase and T4 AsiA protein were obtained as described previously (10, 13).

Plasmid pLHN12 (kind gift of Lam Nguyen and Richard Burgess) contains the *rpoD* gene of *E. coli* encoding the σ^{70} subunit of RNA polymerase downstream of a T7 promoter (30; described in reference 9). Sigma protein was purified from plysS/BL21(DE3) cells (44) containing pLHN12 by the procedure of Hernandez et al. (9).

Assays. Gel retardation experiments and DNase I protection experiments of 5′-end-labeled fragments containing P_{uvsX} and MotA or Mot21 were performed as described previously (24). Gel retardation experiments with a P_{uvsX} fragment, T4-modified RNA polymerase, and MotA or Mot21 were performed by the procedure of Hinton et al. (12) as follows. A 74-bp fragment (0.05 pmol), labeled on the 5′ end of the top strand and containing the P_{uvsX} sequence from −56 to +18, was incubated in a 5-μl reaction mixture with 0.4 pmol of T4-modified RNA polymerase, MotA or Mot21 (amounts indicated in figures), 20 mM Tris-HCl (pH 7.9), 120 mM KCl, 4.8 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM dithiothreitol, (DTT), and 50 μg of BSA per ml. After incubation on ice for 10 min and then at 37°C for 10 min, protein-DNA complexes were challenged by the addition of 1 μl of 250 ng of heparin in 30% sucrose and then incubated for an additional minute at 37°C. Protein-DNA complexes were then separated from free DNA by PAGE as described previously (12).

For the in vitro transcription in Fig. 6A, a 4-μl reaction mixture containing 0.04 pmol of a 1,792-bp *Aha*I-to-*Cla*I fragment from pDH428, 0.4 pmol of T4-modified RNA polymerase, MotA or Mot21 as indicated, 12.5 mM Tris-Cl (pH 7.9), 75 mM KCl, 3 mM $MgCl_2$, 0.06 mM EDTA, 0.3 mM DTT, and 30 μg of BSA per ml was incubated for 10 min on ice and then for 10 min at 37°C. Pretranscription complexes were challenged for 1.5 min at 37°C by the addition of 0.5 μl of 500 μg of heparin per ml. Transcription was started by the addition of 0.5 μl of a solution containing 0.7 mM each ATP, GTP, and CTP and 35 μM [α-^{32}P]UTP (2×10^5 dpm/pmol). For the reaction in Fig. 6B, 0.04 pmol of pDKT90 DNA, linearized with *Bsa*AI, was incubated for 10 min at 37°C with 0.2 pmol of T4-modified RNA polymerase, MotA or Mot21 as indicated, 44 mM Tris acetate (pH 7.9), 167 mM potassium glutamate, 4.4 mM magnesium acetate, 0.11 mM EDTA, 0.11 mM DTT, and 110 μg of BSA per ml in a reaction volume of 4.5 μl. Transcription was started by the addition of 0.5 μl of 1 mM each ATP, GTP, and CTP and 50 μM [α-^{32}P]UTP (4×10^5 dpm/pmol). Following the addition of triphosphates, all transcription reaction mixtures were incubated for 7.5 min at 37°C. A 0.5-μl solution of 6 mM each NTP was then added, and the reaction mixtures were incubated for an additional 7.5 min. Products were isolated and analyzed on 4% polyacrylamide–7 M urea denaturing gels as described previously (10).

Protein-protein complexes were assayed by electrophoresis on native polyacrylamide gels. Solutions containing 80 pmol of AsiA, 14 pmol of σ^{70}, 50 pmol of MotA, 50 pmol of Mot21, and/or the protein buffers were incubated at 37°C for 5 min. For complexes containing MotA, AsiA, and/or σ^{70}, the incubation buffer contained 17 mM Tris-HCl (pH 8.0), 21% glycerol, 0.7 mM EDTA, 0.28 M NaCl, 0.7 mM 2-mercaptoethanol, 0.03 mM DTT, 0.03 mM AEBSF, and 0.6 mM PMSF. The same buffer was used for complexes containing Mot21 except that the AEBSF and PMSF concentrations were 0.4 and 0.3 mM, respectively. (The slightly different conditions for Mot21 stemmed from the different protein storage buffer.) Proteins were separated by native PAGE for 1 h at 120 V. The 6% polyacrylamide gels, having an acrylamide-to-bisacrylamide ratio of 37.5:1, were formed in minicassettes (Novex) and stained after electrophoresis by using Novex colloidal Coomassie blue stain.

RESULTS

Isolation of *mot21* and induction of the Mot21 protein. Plasmid pMOT63, which contains the bacteriophage T4 *motA* gene downstream of the T7 promoter ϕ10, has been used to obtain high levels of the wild-type MotA protein (10, 24). However, our attempts to clone the *motA* gene downstream of P_L, the strong leftward promoter of phage lambda, were not successful. Instead these attempts yielded pMOT21 (detailed in Materials and Methods and shown schematically in Fig. 1), in which a fragment of T4 DNA (212 bp, map units 161.448 to 161.659) was inserted upstream of the *Hin*dIII site present at the start of the *motA* gene. This insertion replaces the first 8 codons of the wild-type *motA* sequence with 11 different codons of an unidentified T4 open reading frame (diagrammed in Fig. 2). DNA sequence analysis of the entire *mot21* gene confirmed that this N-terminal change is the only difference between the wild-type gene and *mot21* (data not shown).

Heat treatment of N4830 (*c*I857) cells containing pMOT21 induced the production of a protein, designated Mot21, that migrated just slightly behind wild-type MotA during SDS-PAGE (Fig. 3, lanes 1 and 3). pMOT8 is a plasmid that lacks the 212-bp insert of pMOT21 and thus lacks a ribosome binding site and N terminus for *motA*. Induction of cells containing pMOT8 failed to produce the protein seen with cells containing pMOT21 (Fig. 3, lane 2). After PC chromatography, the Mot21 extract was greatly enriched for Mot21, but no such protein was obtained after similar treatment of the pMOT8 extract (Fig. 3, lanes 4 and 5, respectively).

Mot21 and wild-type MotA bind P_{uvsX} DNA similarly. To determine whether Mot21 could bind DNA, the low-speed supernatant and PC fractions containing Mot21 were tested for the ability to retard a 74-bp ^{32}P-labeled fragment containing the MotA-dependent promoter P_{uvsX}. Previous work has shown that wild-type MotA retards this fragment with a distinctive pattern: at least four different species are seen as the

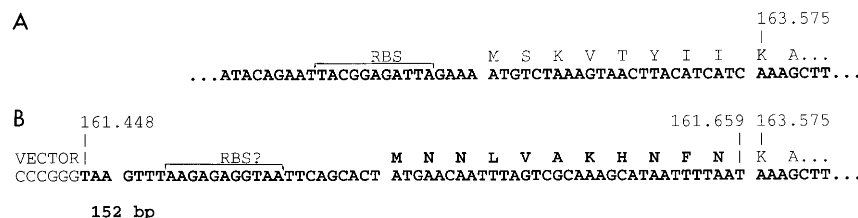

FIG. 2. Sequence of the *mot21* mutation in pMOT21. (A) DNA sequence of the start of the wild-type *motA* gene, with the protein sequence given above. The presumed ribosome binding site (RBS) is indicated. (B) DNA sequence of the start of *mot21*, with the protein sequence given above. Amino acids that differ from the wild type are shown in boldface. A possible ribosome binding site (RBS?) upstream of the ATG start is marked. T4 map units at the insert junctions are indicated.

concentration of MotA is increased (24). A similar pattern was seen for the Mot21 protein, indicating that in this assay, it is qualitatively similar to the wild-type protein (Fig. 4, lanes 3 to 6). Control fractions obtained from pMOT8-containing cells failed to retard the P_{uvsX} fragment (Fig. 4, lanes 2 and 7).

To analyze the binding of Mot21 to the DNA in more detail, DNase footprint analyses were performed with fragments that contained the P_{uvsX} promoter sequence from −94 to +83. As has been seen previously in studies using wild-type MotA, Mot21 protected the template strand from positions −25 to −59 and from positions +40 to +57 (Fig. 5A, lane 3). These regions include the MotA boxes present in P_{uvsX} at −30, −35, and −51 as well as a region downstream of the transcription start that lacks a recognizable MotA box. Protection by either wild-type or mutant MotA required similar but very high levels of protein relative to the DNA (Fig. 5A, lanes 3 and 5). Other evidence suggests this result may be due to the inability of MotA to bind tightly to the DNA in the absence of modified RNA polymerase (24).

Previous work had demonstrated that the presence of the MotA box centered at −51 has no effect on the binding of MotA to the downstream sequences (24). Using a P_{uvsX} template whose T4 sequences extend to −38, we found that the loss of this upstream MotA box is also unimportant for the downstream binding of Mot21 (Fig. 5B, lanes 3 and 5). Taken together, the results of the DNA binding experiments suggest that Mot21 is fully competent to bind this middle promoter DNA.

The presence of Mot21 fails to complement the T4 *motA* mutants *ts*G1 and *am*G1 for growth in *E. coli* tabG. While most T4 *motA* mutants grow upon infection of *E. coli*, they fail to plate on *E. coli* tabG (34; reviewed in reference 43). Complementation of *motA* mutants by the wild-type *motA* gene in tabG has been demonstrated by using the plasmid pMOT61 (10). This plasmid contains the wild-type *motA* gene cloned into the same vector used for pMOT21 except that it lacks the P_L promoter. Presumably the low level of read-through transcription from other promoters on the plasmid is sufficient to produce enough of the *motA* gene product for complementation. To test whether Mot21 could complement T4 *motA* mutants in tabG, plasmid pMOT21ΔP was constructed by deleting the λ P_L promoter by using the procedure that created pMOT61. (The P_L promoter must be removed to obtain stable transformants of tabG since no lambda *c*I repressor is present in this strain.) Unlike pMOT61, which contains the wild-type *motA* gene, pMOT21ΔP was unable to complement the *motA* amber mutant *am*G1 (data not shown) or to complement the *motA* temperature-sensitive mutant *ts*G1 at the nonpermissive temperature. Infection of pMOT21ΔP/tabG by *ts*G1 yielded an efficiency of plating of 0.01 at 40°C relative to 29°C, whereas infection of pMOT61/tabG yielded an efficiency of plating of 1. This result suggests that the *mot21* mutation results in a biologically inactive MotA protein.

Mot21 protein is defective for transcriptional activation from MotA-dependent promoters in vitro. Mot21 was assayed for its ability to activate transcription from middle promoters in vitro. Single-round transcription experiments were performed with a fragment from plasmid pDH428 that includes the T4 middle promoters P_{uvsX} and P_{segA} as well as a MotA-independent promoter, P_{580} (Fig. 6A). T4-modified polymerase alone generated transcripts from the MotA-independent promoter P_{580} but not the middle promoters (lane 1). Transcription from the middle promoters required the presence of

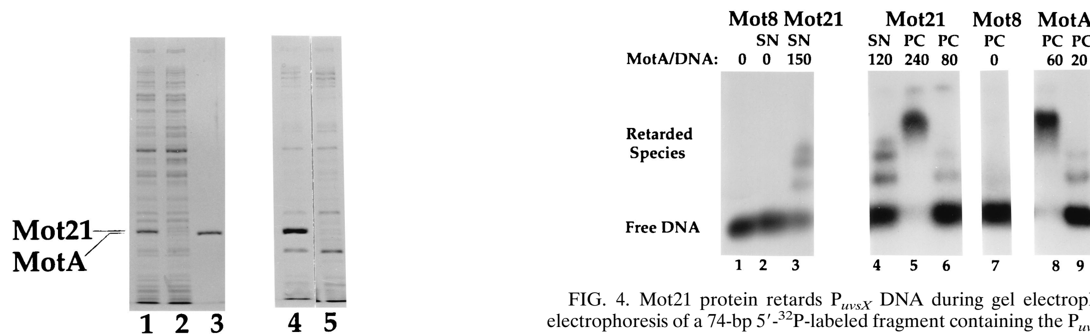

FIG. 3. SDS-PAGE of wild-type MotA and Mot21 proteins. Lanes 1 and 2, low-speed supernatant fractions from N4830 cells containing pMOT21 and pMOT8, respectively (lane 1, 1.8 μg of total protein; lane 2, 2.4 μg); lane 3, purified wild-type MotA protein (0.4 μg); lanes 4 and 5, 0.5 M NaCl wash of the PC columns for cells containing pMOT21 and pMOT8, respectively (lane 4, 1.3 μg of protein; lane 5, 1 μg). The positions of wild-type MotA and Mot21 proteins are indicated.

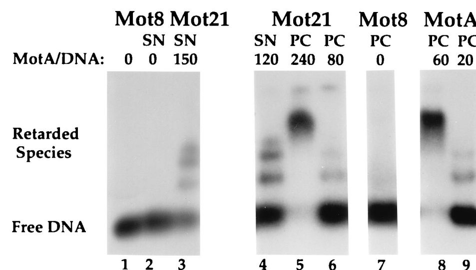

FIG. 4. Mot21 protein retards P_{uvsX} DNA during gel electrophoresis. Gel electrophoresis of a 74-bp 5′-^{32}P-labeled fragment containing the P_{uvsX} sequence from −56 to +18 (0.04 [lanes 1 to 3] or 0.05 [lanes 4 to 9] pmol of DNA) was performed with the indicated molar ratios of mutant or wild-type MotA protein to DNA. Lane 1, no added protein; lane 2, low-speed supernatant (SN) fraction from cells containing pMOT8; lanes 3 and 4, low-speed supernatant fraction from cells containing pMOT21; lanes 5 and 6, 0.5 M NaCl wash of PC column for cells containing pMOT21; lane 7, 0.5 M wash of PC column for cells containing pMOT8; lanes 8 and 9, wild-type MotA fraction similar to that of Mot21. The positions of the free DNA and retarded species are indicated.

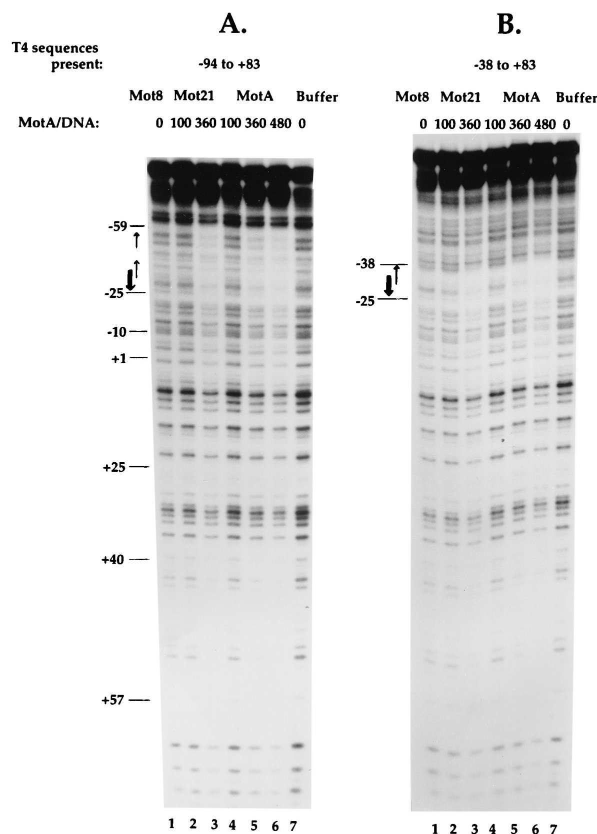

FIG. 5. Mot21 and wild-type MotA protein yield similar DNase I footprints on P_{uvsX} DNA. DNase I protection experiments were performed with the indicated proteins and a 200-bp fragment (0.05 pmol) containing P_{uvsX} sequences from −94 to +83 (A) or a 216-bp fragment containing P_{uvsX} sequences from −38 to +83 (B). In each case, the DNA was labeled on the 5′ end of the template (bottom) strand. Base positions, determined from a G+A sequencing ladder, are given. The dark arrow denotes the position of the MotA box at −30; the positions of other MotA boxes at −35 and −51 are indicated by the lighter arrows.

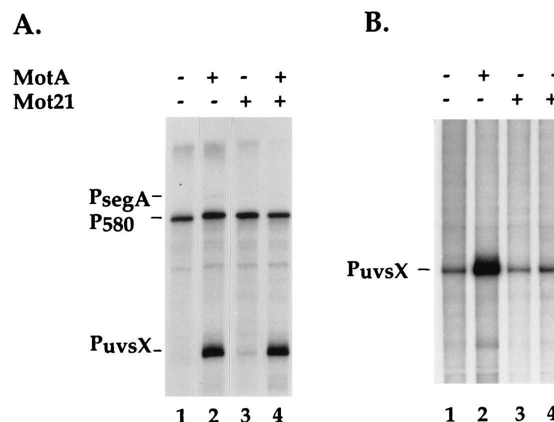

FIG. 6. Mot21 protein fails to activate transcription from P_{uvsX} in vitro. (A) Transcription of a 1,792-bp fragment (0.04 pmol) from plasmid pDH428 containing T4 sequences from the *Eco*RI site within the β*gt* gene (T4 map unit 24.854) to the *Cla*I site within the *uvsX* gene (map unit 23.137). The positions of transcripts arising from two MotA-dependent promoters, P_{segA} and P_{uvsX}, and a MotA-independent promoter (P_{580}) are marked. Lane 1, no MotA; lane 2 MotA (0.4 pmol); lanes 3, Mot21 (2 pmol); lane 4 Mot21 (2 pmol) and MotA (0.4 pmol). (B) Transcription from plasmid pDKT90 linearized with *Bsa*AI (0.04 pmol). This plasmid contains T4 sequences from −94 to +83 relative to the start of transcription from P_{uvsX}. The position of the transcription from P_{uvsX} is marked. Lane 1, no MotA; lane 2, MotA (1 pmol); lanes 3, Mot21 (1 pmol); lane 4, Mot21 (3 pmol).

MotA (lane 2). Addition of Mot21 yielded only a very low level of transcription from P_{uvsX} (lane 3). Addition of both Mot21 and MotA gave a result similar to that seen with MotA alone (lane 4). Thus, Mot21 activates poorly, if at all, under these conditions, but it does not interfere with the ability of wild type MotA to activate.

In the experiment shown in Fig. 6A, pretranscription complexes were challenged with heparin to obtain a single round of transcription. However, this challenge could also affect the ability of Mot21 to activate if its binding to the DNA were much less stable than that of the wild-type protein. The somewhat higher levels of Mot21 relative to the DNA needed for the gel retardation assays (Fig. 4) suggested that less stable binding by the mutant protein was a possibility. Therefore, activation by Mot21 was assayed without the heparin challenge. In addition, the transcription reactions were performed in a buffer containing potassium glutamate, which previous work has indicated is optimal for MotA activation of transcription from P_{uvsX} (24). Even under these conditions, very little transcription from P_{uvsX} was observed in the presence of Mot21 (Fig. 6B, lanes 3 and 4). Taken together, the results of these experiments indicate that Mot21 is severely defective in the activation of transcription by T4-modified polymerase.

MotA interacts with the σ^{70} subunit of RNA polymerase, and Mot21 is defective in this interaction. Gel retardation experiments have shown that a unique complex (designated M1) is formed in the presence of T4-modified RNA polymerase, MotA, and a DNA fragment containing P_{uvsX} (reference 12 and Fig. 7, lanes 3 to 5). In the absence of MotA, T4-modified polymerase forms a complex with P_{uvsX}, designated M2, but this species migrates slightly faster than that obtained with the activator (reference 12 and Fig. 7, lanes 2 and 8). Characterization of these complexes has revealed that M1 is a stable open complex and that MotA is needed to form this transcriptionally active species (12). As shown in lanes 6 and 7 of Fig. 7, Mot21 fails to generate the M1 complex. This result suggests that Mot21 is defective in protein-protein and/or protein-DNA contacts needed to form the open complex.

Formation of the M1 complex and MotA-dependent transcriptional activation require RNA polymerase that has been modified by the phage; although unmodified polymerase can use P_{uvsX}, it does not form the M1 species and it is not activated by MotA (10, 12). Previous work has shown that the association of the T4 AsiA protein with the host RNA polymerase is the modification required for MotA function (13, 32). AsiA binds tightly to the σ^{70} subunit of RNA polymerase (41, 42), suggesting that this protein-protein interaction may be important in the formation of the transcriptionally competent open complex. To investigate whether other protein-protein interactions are possible, we incubated solutions containing MotA, AsiA, and/or σ^{70} and then analyzed the proteins by native PAGE (Fig. 8). As expected, σ^{70} and AsiA together formed a protein complex that migrated in a different position from either AsiA or σ^{70} alone (lane 5 versus lanes 1 and 4). Incubation of AsiA and MotA failed to yield a new complex (lanes 3 versus lanes 1 and 2). However, a new protein band was seen after incubation of MotA and σ^{70} (lane 6), suggesting a direct interaction between the transcriptional activator and this RNA polymerase subunit. The appearance of a different species after incubation of MotA, AsiA, and σ^{70} (lane 7) suggested that both AsiA and MotA can bind to σ^{70} simultaneously. The protein band observed with MotA and sigma

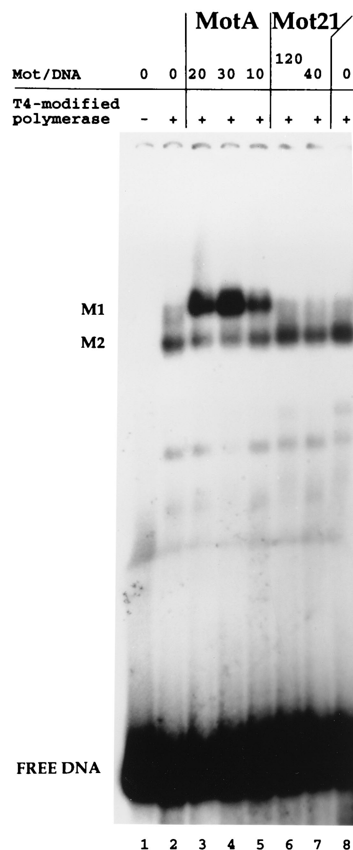

FIG. 7. Mot21 fails to generate the pretranscription complex formed by MotA, T4-modified polymerase, and P_{uvsX}. A 74-bp fragment containing P_{uvsX} (from −56 to +18, 0.05 pmol) was incubated with T4-modified RNA polymerase and the indicated amount of MotA or Mot21, and the protein-DNA complexes were separated from free DNA by PAGE as described in Materials and Methods. Lane 1, no protein; lane 2, T4-modified polymerase alone; lanes 3 to 5, T4-modified polymerase plus MotA (PC fraction); lanes 6 and 7, T4-modified polymerase plus Mot21 (PC fraction); lane 8, T4-modified polymerase plus fraction from pMOT8/N4830 cells corresponding to Mot21 in lane 6. The positions of the free DNA and the protein-DNA complexes made with T4-modified polymerase and MotA (M1) or T4-modified polymerase alone (M2) are indicated.

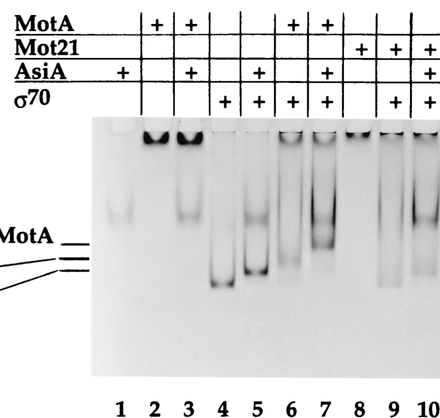

FIG. 8. MotA, but not Mot21, forms a protein-protein complex with σ^{70}. Solutions containing σ^{70}, AsiA, MotA, and/or Mot21 (as indicated) were incubated, and proteins were separated by native PAGE as described in Materials and Methods. The positions of new species seen formed in the presence of AsiA and σ^{70}, MotA and σ^{70}, or AsiA, MotA, and σ^{70} are indicated.

(lane 6) was less discrete than that seen with AsiA and sigma (lane 5), suggesting that this complex may be less stable than the AsiA-sigma complex. This could explain why other analyses, which have found a tight association between AsiA and sigma, have not detected a MotA-sigma interaction (31, 40).

Unlike the case for MotA, incubation of Mot21 with σ^{70} failed to give a discrete new complex, although the smearing of the σ^{70} and Mot21 bands suggested that an unstable complex may have been formed (Fig. 8, lane 9). Incubation of Mot21, AsiA, and σ^{70} yielded only the σ^{70}-AsiA complex (lane 10), indicating that the presence of Mot21 did not eliminate the association of AsiA and σ^{70}. Taken together, these results suggest that MotA activation involves a direct association of MotA with the σ^{70} subunit of RNA polymerase and that Mot21 is defective in this interaction.

DISCUSSION

The bacteriophage T4 MotA protein is a DNA-binding protein needed for transcriptional activation of phage middle promoters (reviewed in reference 43). While the −10 region of each of these promoters has the consensus sequence for the σ^{70} subunit of RNA polymerase, the −35 region lacks a σ^{70} consensus sequence. Instead a 9-bp motif (a MotA box) is centered 30 bp upstream of the start of transcription. MotA binds to this sequence (10, 37) and activates transcription in the presence of host RNA polymerase that has been modified by its association with the T4 AsiA protein (13, 32). Although T4-modified RNA polymerase is capable of binding to a middle promoter, open complex formation requires MotA (12). How the actions of MotA, AsiA, and RNA polymerase result in formation of the open complex is unclear.

The *mot21* mutation affects the N-terminal region of MotA, substituting 11 different amino acids for the first 8 of the wild-type protein. Mot21 chromatographs like the wild-type protein on PC and double-stranded DNA-cellulose, and the protein behaves similarly to the wild type in DNA binding assays. However, Mot21 fails to activate transcription. Thus, Mot21 has the phenotype of a positive control mutant, in which the DNA binding activity has been separated from the activating function. No other such mutants of *motA* have been reported.

How does the N-terminal change in *mot21* specifically affect transcriptional activation? We have shown that the stable pretranscription complex made by MotA and T4-modified polymerase at the middle promoter P_{uvsX} is not formed with Mot21, implying that the protein-protein or protein-DNA contacts needed to generate this complex are not possible with the mutant protein. Previous work has demonstrated that the coactivator for MotA, the T4 AsiA protein, binds tightly to σ^{70} (41, 42). On native gels, we observe this AsiA-σ^{70} complex, but in addition, we see new protein species in the presence of either MotA and σ^{70} or MotA, σ^{70}, and AsiA. These results suggest that MotA also contacts sigma. The fact that Mot21 fails to form this complex argues that the contact between MotA and sigma is important and that Mot21 fails to activate because it is defective in this interaction.

This idea that a MotA-sigma contact is needed for activation is attractive because it offers an explanation for other findings. First, in middle promoters, the position of the MotA box overlaps the region where σ^{70} would make contact with the −35 region of an *E. coli* promoter. From gel retardation and footprinting experiments, it appears that σ^{70} makes the usual contacts to the −10 consensus sequence of the middle promoter P_{uvsX}, but the presence of MotA and AsiA significantly alters

the protein-DNA contacts between positions −20 and −40 (12). Thus, a direct interaction between MotA, positioned at the MotA box, and σ^{70} could be needed to preclude the possibility of sigma trying to contact this region of the promoter also. In addition, we have previously found that very high levels of MotA relative to DNA are needed for gel retardation or footprinting by the activator alone (10, 24), but much lower levels are required in the presence of T4-modified RNA polymerase (12, 24). This result is understandable if MotA binding to the DNA must be stabilized by a contact between the activator and polymerase. It would also explain why the presence of Mot21, which binds DNA but lacks this contact, does not interfere with MotA activation.

Our results suggest that MotA joins a growing list of activators that make direct contact with *E. coli* polymerase through either the α or the σ subunit (reviewed in references 3 and 19). However, it appears that in MotA, the DNA binding and activating domains may be physically separable, a feature that is common for eukaryotic (reviewed in references 8 and 28) but not prokaryotic activators. Our characterization of *mot21* suggests that the far N-terminal region of the protein is needed for activation, while other work has demonstrated that the C-terminal half of the protein by itself retains DNA binding ability (11). Furthermore, the N-terminal and C-terminal halves of MotA have been isolated separately, resulting in structural data for each half (4, 5) and supporting the idea that MotA is a modular protein. In contrast, well-characterized *E. coli* activators, such as lambda repressor (16), *E. coli* CRP (cAMP) receptor protein) (1; reviewed in reference 21), and *E. coli* AraC (regulator of the L-arabinose operons) (27), have DNA binding and activating functions which are separable by mutation but are located very close to each other within the protein. The recent discovery of other activating domains on cAMP receptor protein that are distinct from its DNA binding region (36, 46) appears to represent a prokaryotic example similar to MotA. However, the data presented here do not definitively localize the activating domain of MotA to the N terminus, and these results are also consistent with other possibilities. The *mot21* mutation may inhibit an activating function that is located elsewhere on the protein rather than destroying the domain itself, or the N-terminal mutation may affect another function, such as oligomerization of the protein, which is a prerequisite for sigma contact. Further work will be needed to determine how the N-terminal portion of MotA affects the ability of MotA to contact σ^{70} and to activate transcription. The isolation of *mot21* provides a tool for dissecting the contribution of this region to transcriptional activation.

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ADDENDUM IN PROOF

Recent experiments indicate that a double mutation in MotA at amino acids 30 and 31 reduces transcriptional activation without affecting DNA binding (M. S. Finnin, M. P. Cicero, C. Davies, S. J. Porter, S. W. White, and K. N. Kreuzer, unpublished data).

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