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The Bacteriophage T4 Transcription Activator MotA Interacts with the Far-C-Terminal Region of the σ^{70} Subunit of *Escherichia coli* RNA Polymerase

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Transcription from bacteriophage T4 middle promoters uses *Escherichia coli* RNA polymerase together with the T4 transcriptional activator MotA and the T4 coactivator AsiA. AsiA binds tightly within the C-terminal portion of the σ^{70} subunit of RNA polymerase, while MotA binds to the 9-bp MotA box motif, which is centered at −30, and also interacts with σ^{70}. We show here that the N-terminal half of MotA ($MotA^{NTD}$), which is thought to include the activation domain, interacts with the C-terminal region of σ^{70} in an *E. coli* two-hybrid assay. Replacement of the C-terminal 17 residues of σ^{70} with comparable σ^{38} residues abolishes the interaction with $MotA^{NTD}$ in this assay, as does the introduction of the amino acid substitution R608C. Furthermore, in vitro transcription experiments indicate that a polymerase reconstituted with a σ^{70} that lacks C-terminal amino acids 604 to 613 or 608 to 613 is defective for MotA-dependent activation. We also show that a proteolyzed fragment of MotA that contains the C-terminal half ($MotA^{CTD}$) binds DNA with a $K_{D(app)}$ that is similar to that of full-length MotA. Our results support a model for MotA-dependent activation in which protein-protein contact between DNA-bound MotA and the far-C-terminal region of σ^{70} helps to substitute functionally for an interaction between σ^{70} and a promoter −35 element.

A programmed cascade of transcriptional events is initiated when bacteriophage T4 infects its host *Escherichia coli* (reviewed in reference 57). T4 early genes are transcribed immediately after infection by using the existing host RNA polymerase holoenzyme comprising the core ($\alpha_2\beta\beta'$) and the σ^{70} subunit. Early T4 promoters do not require T4-encoded transcription factors, since they contain excellent matches to the ideal σ^{70} sequences in their −10 and −35 regions (61; reviewed in reference 60). In contrast, transcription from T4 middle promoters uses two T4 early gene products, the transcriptional activator MotA and the coactivator AsiA (23, 39, 43, 44; reviewed in reference 57). Late promoter utilization requires the replacement of σ^{70} by the T4 sigma factor, gp55, as well as other phage-encoded activators and coactivators (reviewed in reference 62).

The MotA protein binds as a monomer (6, 33) to a 9-bp element (MotA box) centered at position −30 of middle promoter DNA (3, 19, 23). In addition, MotA forms a complex with σ^{70} (18). Nuclear magnetic resonance and crystallographic studies indicate that the 211 amino acids of MotA are organized into an N-terminal domain (NTD) and a C-terminal domain (CTD) separated by a small flexible linker (15, 33, 34). Mutations within the NTD of MotA eliminate transcriptional activation and the formation of the MotA-σ^{70} complex (14, 18), suggesting that the NTD is the activation domain of MotA. Transcriptional activation of middle promoters also requires the T4 coactivator AsiA, which binds very tightly to σ^{70} (44, 55, 56). Binding sites for AsiA have been mapped within C-terminal amino acids (regions 4.1 and 4.2) of σ^{70} (8, 49, 50, 53, 59). Residues within region 4.2 normally contact the −35 element of host promoter DNA (5, 9, 17, 29, 54). In the absence of MotA, AsiA binding to σ^{70} inhibits transcription by polymerase from promoters that require recognition of the −35 canonical sequences (8, 45, 51), suggesting that the presence of AsiA inhibits the σ^{70} region 4.2-DNA interaction.

In this paper we show that the interaction of a MotA N-terminal peptide (amino acids 1 to 97) with σ^{70}, like the interaction of AsiA with σ^{70}, involves the C-terminal region of σ^{70}. In addition, deletions of the amino acids within the far-C-terminal region of σ^{70} (amino acids 604 to 613) impair the ability of RNA polymerase to perform MotA-dependent activation in vitro. We also show that a MotA C-terminal peptide, beginning at amino acid 102, binds DNA with an apparent dissociation constant like that of wild-type MotA. Our results support a model for MotA-dependent activation in which the interaction between the DNA-bound MotA and the C-terminal region of σ^{70} helps to substitute functionally for an interaction between σ^{70} and a promoter −35 element.

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MATERIALS AND METHODS

Strains. *E. coli* KS1 (12) contains a chromosomal *lacZ* reporter gene under the control of a derivative of the *lac* promoter P_{lac} that carries a lambda operator (O_R2) centered at position −62 in place of the binding site for the catabolite receptor protein normally associated with P_{lac}. KS1 also contains an F′ episome

bearing *lacI*^{q} and a gene for kanamycin resistance. *E. coli* XL1-Blue (Stratagene) was used for transformations during plasmid constructions.

DNA. Oligonucleotide primers were obtained from Gene Probe Technologies and Cruachem Inc. (Primer sequences are available upon request.) The 5′-^{32}P-labeled 74-bp P_{uvsX} DNA, containing the P_{uvsX} sequences from positions −56 to +18, was obtained as described previously (23). pDKT90, which contains the T4 middle promoter P_{uvsX}, has been described elsewhere (37). Linear templates for transcription, obtained by *Bsa*AI restriction of pDKT90, were purified by phenol extraction followed by ethanol precipitation.

The pBRα-σ^{70} chimera plasmid (11) contains a ColE1 replication origin, confers carbenicillin resistance, and directs transcription of an α-σ^{70} chimera gene under the control of the tandem promoters P_{lpp} and P_{lacUV5}. The resulting α-σ^{70} chimera protein is composed of the NTD of α (amino acids 1 to 248) fused in frame to the C-terminal region of σ^{70} (amino acids 528 to 613). The pBRα-σ^{70} derivative encoding α-σ^{70}(R596H) has been described previously (11), and the pBRα-σ^{70} derivatives encoding α-σ^{70} chimeras bearing substitutions H600A, H600R, and R608C were constructed similarly. All of these derivatives are identical to pBRα-σ^{70} except for the indicated changes. The α-σ^{38} chimera plasmid, pBRα-σ^{38} (11), is identical to pBRα-σ^{70} except that all of the σ^{70} sequences have been replaced with the comparable σ^{38} sequences (encoding amino acids 243 to 330). The σ^{70}/σ^{38} hybrid plasmid, pBRα-σ^{70}/σ^{38}, was constructed by replacing the σ^{70} sequences encoding amino acids 597 to 613 with the comparable σ^{38} sequences (encoding amino acids 312 to 330) by PCR and standard cloning techniques.

The pACλ*c*I32 plasmid (27), which was used to construct *c*I-bait fusion proteins, contains a chloramphenicol resistance marker, a p15A replication origin, and a short alanine linker engineered into the λ *c*I protein gene such that the gene of interest can be fused to the 3′ end of the *c*I protein by using *Not*I, *Asc*I, *Bst*YI, or *Bgl*II restriction sites. In this construction, the *c*I fusion is under the control of P_{lacUV5}. Fragments to be cloned into pACλ*c*I32 were obtained as PCR products of pMot58 (to construct pcI-MotA^{fl}, pcI-MotA^{NTD}, and pcI-MotA^{-}) (23), pMot21 (to construct pcI-Mot21^{NTD}) (18), or pAsiA (to construct pcI-AsiA) (25) by using PfuTurbo DNA polymerase (Stratagene) and appropriate primers. Primers contained the necessary *Not*I or *Bgl*II sites to allow ligation with pACλ*c*I32 that had been previously cleaved with *Not*I and *Bgl*II. PCR products were purified by using a Wizard PCR purification system (Promega) and cloned into pACλ*c*I32 by using standard techniques. The σ$^{1\text{-}570}$ gene was obtained as a PCR product of pJH62 (gift of V. J. Hernandez, State University of New York at Buffalo), a plasmid that contains the entire *rpoD* gene (encoding σ^{70}). PCR was performed with Amplitaq polymerase (Perkin-Elmer), a primer that annealed to the start of the σ^{70} gene and began with an *Xba*I recognition sequence, and a primer that annealed to sequences surrounding codon no. 571, introduced a stop codon of TAA at that position, and ended with a *Sac*I recognition sequence. After cleavage with *Xba*I and *Sac*I, the PCR product was ligated into pet21a(+) (Novagen) that had been previously cleaved with *Xba*I and *Sac*I. The *Cla*I/*Sac*I fragment, which contained the C-terminal region of the σ^{70} gene, was then obtained from the resulting plasmid. This fragment was used to replace the corresponding fragment in pLHN12 (22, 42), a plasmid that contains the wild-type σ^{70} gene downstream of a T7 promoter. The resulting plasmid was designated pσ$^{1\text{-}570}$.

pσ^{fl}, which contains an N-terminal His-tagged σ^{70} gene, has been described previously (63). This plasmid produces full-length σ^{70} with the amino acid sequence MRGSHHHHHHGSSGLVPRGSGLGTRL at its N terminus (σ^{fl}). PCR products encompassing the C-terminal region of σ^{R596H} and σ^{R608C} were obtained from pBRα-σ^{R596H} and pBRα-σ^{R608C}, respectively, by using a primer that annealed at the *Cla*I site within the σ^{70} sequence and another primer that annealed just downstream of the σ^{70} gene and introduced a *Hin*dIII site. PCR products encompassing the C-terminal region of σ^{70} with a stop codon at amino acid position 608 or 604 were obtained by using pBRα-σ^{70}, the same upstream primer, and a primer that introduced a TAA at amino acid position 608 or 604, respectively, and introduced a *Hin*dIII site. In each case, the products were digested with *Cla*I and *Hin*dIII and then ligated into pσ^{fl} that had been previously digested with *Cla*I and *Hin*dIII, resulting in pσ^{R596H}, pσ^{R608C}, pσ$^{\Delta 608\text{-}613}$, and pσ$^{\Delta 604\text{-}613}$.

DNA sequence analyses (47) of the inserted sequences in each mutant σ^{70} plasmid confirmed that only the intended changes were present. In some cases, this sequencing was done at the Center for Agricultural Biotechnology, University of Maryland.

Proteins. AsiA and MotA were purified as described previously (25). Wild-type σ^{70} and σ$^{1\text{-}570}$ were purified from cultures of pLHN12/pLysS/BL21(DE3) and pσ$^{1\text{-}570}$/pLysS/BL21(DE3), respectively, as described previously (22). σ^{fl}, σ^{R596H}, σ^{R608C}, σ$^{\Delta 608\text{-}613}$, and σ$^{\Delta 604\text{-}613}$ were purified as described previously (63), except that cells were broken by sonication. *E. coli* core polymerase was purchased from Epicentre Technologies.

The C-terminal MotA fragment (MotA$^{cloned\ CTD}$), which contained MotA amino acids 105 to 211, was obtained as follows. BL21(DE3) cells containing a plasmid that expresses the MotA$^{cloned\ CTD}$ gene under the control of the T7 promoter Φ10 (15) (plasmid was the gift of M. Finnin, S. Porter, and S. White, St. Jude's Children's Hospital, Memphis, Tenn.) were grown in L broth plus 25 μg of kanamycin/ml at 37°C to mid-log phase. The synthesis of MotA$^{cloned\ CTD}$ was induced by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside) to 1 mM. Cells were harvested after 2 h and broken by sonication, and highly purified MotA$^{cloned\ CTD}$ was obtained after phosphocellulose chromatography as described previously (25), except that proteins were eluted from the column by steps of 0.2, 0.5, and 1 M NaCl in sonication buffer. The 0.5 M NaCl eluate, which contained the bulk of the C-terminal MotA protein, was used for subsequent experiments. A control fraction was obtained by the same procedure, except that the cells used were not induced.

Proteolyzed MotA protein (MotA$^{proteolyzed\ CTD}$) generated with endogenous proteases as a partially purified fraction of the wild-type protein (0.8 M phosphocellulose fraction) (23) was loaded onto a phosphocellulose column. Fractions containing the 13.5-kDa MotA$^{proteolyzed\ CTD}$ eluted with a higher salt concentration (peak fractions eluting with 0.47 M NaCl) than that of the wild-type protein (peak fraction eluting with 0.4 M NaCl). To obtain an N-terminal analysis of the proteolyzed fragment, the 13.5-kDa peptide was purified by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (31) and then transferred to a polyvinylidene difluoride membrane (Novex) by using a Novex Western transfer apparatus and a buffer of 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (pH 11), 1 mM EDTA, and 10% methanol. N-terminal sequence analysis (16 cycles performed at the W. M. Keck Foundation Biotechnology Resource Laboratory, Boyer Center for Molecular Medicine, Yale University) indicated that the 13.5-kDa gel band was composed of approximately equal amounts of two proteins. One protein matched the amino acid sequence of MotA starting at amino acid 102. The other matched the amino acid sequence of *E. coli* 50S ribosomal protein L24.

The concentrations of MotA$^{proteolyzed\ CTD}$ and MotA$^{cloned\ CTD}$ were estimated after SDS-polyacrylamide gel electrophoresis by comparing these proteins with a known amount of wild-type MotA. Levels were corrected for the fact that the MotA fragments were one-half the size of the wild type and that in the case of MotA$^{proteolyzed\ CTD}$, only one-half of the band seen on the gel corresponded to the MotA peptide.

β-Galactosidase assays. β-Galactosidase assays were performed by a modification of the procedure of Jain (28). Because the synthesis of either AsiA or MotA is toxic for *E. coli* (25), overnight cultures were always started from single colonies obtained by streaking the −80°C culture stock onto Luria-Bertani plates containing 50 μg of kanamycin/ml, 50 μg of carbenicillin/ml, and 25 μg of chloramphenicol/ml. The plates were then incubated at 37°C, and single colonies were used to inoculate liquid cultures of M9 plus Casamino Acids (Quality Biological) supplemented with the same antibiotics. Cultures were grown overnight at 37°C with aeration, diluted 1:100 in fresh media plus antibiotics, and then grown to mid-log phase. IPTG either was present throughout growth (for the assays in Fig. 3) or was added at the indicated concentration once the cells reached mid-log phase (for the assays in Fig. 4 and 5), and then the cells were grown for another 60 min. A final optical density at 600 nm ($OD_{600\text{-final}}$) of the cells was determined, and the cell pellets from 5 ml of culture were harvested by centrifugation at 1,880 × *g* at 4°C and then resuspended on ice in 0.2 ml of *lacZ* buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM 2-mercaptoethanol). Cell extracts, obtained after the addition of 20 μl each of 0.1% (wt/vol) SDS and $CHCl_3$ and vigorous vortexing of the cell suspension for 10 s, were kept on ice until needed. A 20-μl aliquot of the extract was then added to 0.98 ml of the *lacZ* buffer, and the mixture was incubated for 5 min at 28°C. The reaction was started by the addition of 0.2 ml of a solution containing 4 mg of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) per ml of *lacZ* buffer. β-Galactosidase activity was measured by the hydrolysis of ONPG. After the solution turned yellow, the reaction was stopped by the addition of 0.5 ml of 1 M Na_2CO_3, and the time needed for the reaction (ΔT) was noted. Reaction mixtures were briefly vortexed and then centrifuged at 830 × *g*. The OD_{420} of the clear supernatant was then measured. The β-galactosidase activity in Miller units (40) was determined as $(2{,}000 \times OD_{420})/(\Delta T \times OD_{600\text{-final}})$.

Relative β-galactosidase activities at a 100 μM IPTG concentration (Fig. 5) were calculated as follows:

$$\frac{\text{units with KS1/pcI-AsiA/pBR}\alpha\text{-}\sigma^{(\text{mutant})} - \text{control}}{\text{units with KS1/pcI-AsiA/pBR}\alpha\text{-}\sigma^{70} - \text{control}}$$

where the control was the units obtained with KS1/pACλ*c*I32/pBRα-σ^{70}, and

$$\frac{\text{units with KS1/pcI-MotA}^{\text{NTD}}\text{/pBR}\alpha\text{-}\sigma^{(\text{mutant})} - \text{control}}{\text{units with KS1/pcI-MotA}^{\text{NTD}}\text{/pBR}\alpha\text{-}\sigma^{70} - \text{control}}$$

where the control was the units obtained with KS1/pcI-Mot21$^{\text{NTD}}$/pBRα-σ^{70}.

Native protein gels. Protein complexes were assayed by electrophoresis on native polyacrylamide gels as described previously (18).

In vitro transcriptions. Incubation buffer I contained 44 mM Tris-Cl (pH 8), 50 mM NaCl, 42% glycerol, 1 mM EDTA, 0.18 mM dithiothreitol, 0.008% Triton X-100, and 0.2 mM 2-mercaptoethanol. Incubation buffer II contained 5 mM Tris-Cl (pH 7.5), 69 mM Tris-acetate (pH 7.9), 25 mM NaCl, 5% glycerol, 0.18 mM EDTA, 0.27 mM dithiothreitol, 260 mM potassium glutamate, 6.9 mM magnesium acetate, and 173 μg of bovine serum albumin/ml. DNA buffer contained 7.4 mM Tris-Cl (pH 7.9), 51 mM Tris-acetate (pH 7.9), 57 mM NaCl, 2.1% glycerol, 0.66 mM EDTA, 0.13 mM dithiothreitol, 0.21 mM 2-mercaptoethanol, 190 mM potassium glutamate, 5.1 mM magnesium acetate, 130 μg of bovine serum albumin/ml, 220 μM ATP, 220 μM GTP, 220 μM CTP, and 11 μM [α-^{32}P]UTP (6.5×10^5 dpm/pmol). Finally, 1× Tris-borate-EDTA (TBE) contained 2.5 mM EDTA and 89 mM Tris-borate (pH 8.3).

Proteins were preincubated and mixed with the DNA template and other transcription components as indicated in the figure legend. Reaction mixtures were then placed at 37°C for 20 s before the addition of 0.5 μl of rifampin at 300 μg/ml. After an additional 7.5 min at 37°C, reaction mixtures were collected on dry ice. Twenty-five microliters of gel loading solution (1× TBE containing 7 M urea, 0.1% bromophenol blue, and 0.1% xylene cyanol FF) was added, and the samples were heated at 95°C for 2 min. An 8-μl sample was then subjected to electrophoresis in a 4% polyacrylamide, 7 M urea denaturing gel run in 0.5× TBE.

Protein-DNA gel retardation assays. Gel retardation assays were performed by using the procedure of Hinton (23). $K_{D(app)}$ was determined as the protein concentration needed to retard 50% of a 5′-^{32}P-labeled 74-bp fragment containing P_{uvsX} in a 10-μl reaction mixture that contained 0.05 pmol of P_{uvsX} DNA and 1.25 pmol of P_{tac} competitor DNA.

Quantitation of autoradiograms. After autoradiography, films were scanned with a Scanmaster III Plus from Howtek, Inc. Quantification was performed with Diversity One software from Protein-Databases, Inc.

RESULTS

A σ^{70} lacking the last 43 amino acids ($\sigma^{1\text{-}570}$) fails to make a discrete complex with MotA. The T4 MotA and AsiA proteins each form a complex with σ^{70} that can be distinguished from the free proteins on native protein gels (18) (Fig. 1, lanes 4 and 5). σ^{70} contains 613 amino acids, which are divided into regions 1 through 4 based on sequence similarity among the various members of the sigma protein family (35). Binding sites for AsiA have been found within the C-terminal region of σ^{70} in both region 4.1 and region 4.2 (amino acids 567 to 600) (8, 49, 50, 53, 59). To test whether the last 43 residues of σ^{70}, which include region 4.2, were also important for MotA-σ^{70} complex formation, we assayed the formation of complexes by using $\sigma^{1\text{-}570}$, a truncated σ^{70} that contains amino acids 1 through 570. As expected, AsiA did not form a complex with $\sigma^{1\text{-}570}$ (Fig. 1, lane 8). MotA also did not form the discrete complex with $\sigma^{1\text{-}570}$ that was seen with σ^{70} (Fig. 1, compare lanes 9 and 5). Instead, a very diffuse band migrating behind the position of free AsiA was observed. These results indicate that a σ^{70} missing region 4.2 is not capable of forming a discrete complex with MotA that is stable under the conditions of electrophoresis and thus implicate region 4.2 in the MotA-σ^{70} interaction.

The C-terminal region of σ^{70} interacts with the N-terminal domain of MotA in an *E. coli* two-hybrid assay. To investigate further the possibility of an interaction between MotA and the C-terminal region of σ^{70}, we used an *E. coli*-based two-hybrid system (11, 27). In this system (Fig. 2), a chromosomal reporter *lacZ* gene lies downstream of a promoter that has a binding site for λ *c*I protein at position −62. The bait is created by the fusion of a protein or domain of interest to the 3′ end of *c*I. The prey consists of σ^{70} amino acids 528 to 613, which start within region 3.2 and then include all of region 4 plus the far-C-terminal region (35). This prey is fused in frame to the NTD of the α subunit of RNA polymerase. The addition of IPTG induces the synthesis of both the α-σ^{70} chimera and the *c*I-bait protein. An interaction between the bait protein positioned at −62 and the C-terminal region of σ^{70}, which is available in the pool of RNA polymerase that contains the α-σ^{70} chimera, then increases *lacZ* transcription.

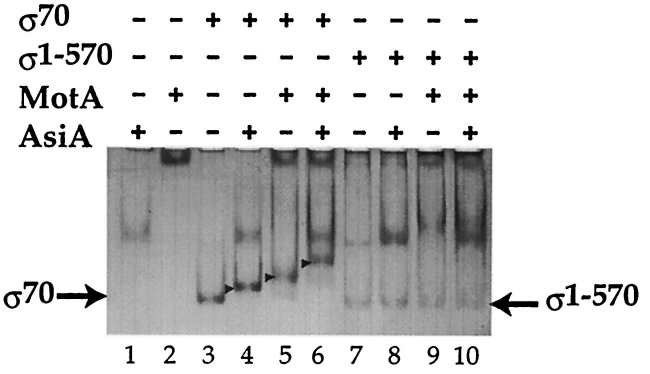

FIG. 1. Formation of discrete complexes of AsiA-σ^{70} and MotA-σ^{70} requires the last 43 amino acids of σ^{70}. A 7.25-μl mixture containing 17 mM Tris-Cl (pH 7.9), 280 mM NaCl, 22% glycerol, 0.7 mM EDTA, 0.7 mM 2-mercaptoethanol, 0.04 mM dithiothreitol, and (as indicated by + or −) 80 pmol of AsiA, 50 pmol of MotA, 14 pmol of σ^{70}, and 14 pmol of $\sigma^{1\text{-}570}$ was incubated at 37°C for 5 min. Samples were then subjected to electrophoresis in a 6% polyacrylamide native protein gel, and proteins were detected after staining with colloidal Coomassie blue (Invitrogen). The positions of free σ^{70} and free $\sigma^{1\text{-}570}$ are indicated. (Both the σ^{70} and the $\sigma^{1\text{-}570}$ preparations contain a slow-moving band that is consistent with the presence of σ dimer.) The locations of the AsiA-σ^{70} complex, the MotA-σ^{70} complex, and the AsiA-σ^{70}-MotA complex are indicated by arrowheads.

As has been previously reported (10), there was a large increase in β-galactosidase activity upon the addition of IPTG to cells containing p*c*I-AsiA and pBRα-σ^{70} (Fig. 3). To assay an interaction between MotA and the σ^{70} prey, we tested a bait consisting of *c*I fused to the entire MotA gene (*c*I-MotA$^{\text{fl}}$) and a bait in which the *motA* gene was positioned out of frame with *c*I (*c*I-MotA^{-}). The addition of IPTG resulted in an increase in

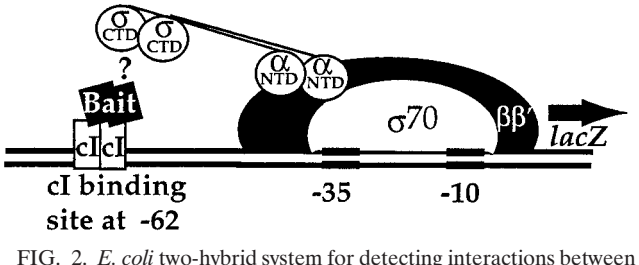

FIG. 2. *E. coli* two-hybrid system for detecting interactions between the C-terminal region of σ^{70} and other proteins. The cartoon depicts the positions of RNA polymerase subunits β, β′, and σ^{70} and the α-σ^{70} chimera, which consists of the N-terminal domain of α fused to the C terminus of σ^{70}, at a promoter upstream of the reporter *lacZ* gene. The *c*I-bait fusion protein is located at position −62. (See text for details.)

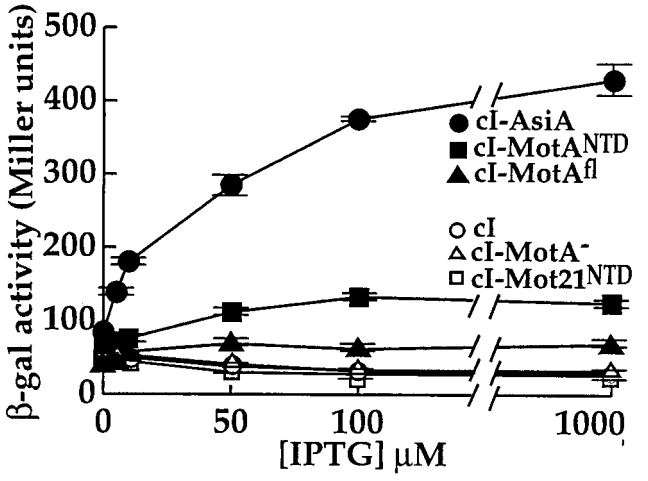

FIG. 3. AsiA, MotA, and MotA^{NTD} each interact with the C-terminal region of σ^{70} in the *E. coli* two-hybrid assay. β-Galactosidase (β-gal) activity is plotted versus IPTG concentration for KS1 cells containing pBRα-σ^{70} and p*c*I-AsiA (●), p*c*I-MotA^{NTD} (■), p*c*I-MotA^{fl} (▲), p*c*I-Mot21^{NTD} (□), p*c*I-MotA^{-} (△), or pACλ*c*I32 (○). Cultures were grown continuously in the presence of the indicated concentrations of IPTG. Points and standard deviations (indicated by error bars) represent the averages of the results of three assays. In this assay, in which cultures were grown continuously in the presence of IPTG, MotA^{NTD} gave higher levels of β-galactosidase activity than did MotA^{fl}. However, in assays in which cultures were grown for only 1 h with IPTG, the activities seen with MotA^{NTD} and MotA^{fl} were similar (data not shown).

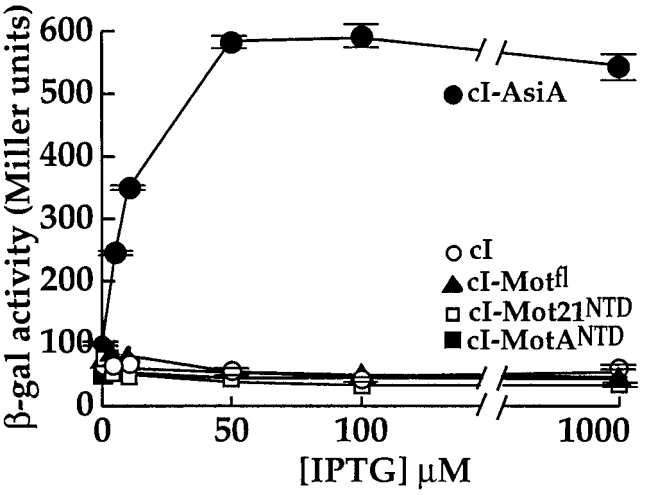

FIG. 4. MotA^{NTD} does not interact with the C-terminal region of σ^{38}. β-Galactosidase (β-gal) activity is plotted versus IPTG concentration for KS1 cells containing pBRα-σ^{38} and p*c*I-AsiA (●), p*c*I-MotA^{NTD} (■), p*c*I-MotA^{fl} (▲), p*c*I-Mot21^{NTD} (□), or pACλ*c*I32 (○). Cultures were grown to mid-log phase in the absence of IPTG and then grown in the presence of the indicated IPTG concentrations for 1 h. Points and standard deviations (indicated by error bars) represent the averages of the results of two assays.

the level of β-galactosidase activity in cells containing the *c*I-MotA^{fl} fusion compared to the activity seen in the presence of *c*I-MotA^{-} or *c*I alone (Fig. 3). Although this was only a twofold increase over background, it was highly reproducible. In addition, in control assays, cells containing p*c*I-MotA but lacking pBRα-σ^{70} expressed background levels of *lacZ* (data not shown), indicating that by itself p*c*I-MotA was not responsible for the increase in β-galactosidase activity.

Residues within the NTD of MotA are required for MotA activation (14, 18). Induced synthesis of a *c*I-MotA^{NTD} fusion, which contained MotA amino acids 1 to 97, in cells containing the α-σ^{70} chimera resulted in a significant increase in β-galactosidase activity (Fig. 3). As a control, we constructed a plasmid containing a fusion of *c*I with the comparable NTD of Mot21, a mutant of MotA in which the first 8 amino acids of MotA have been replaced with 11 different amino acids. Mot21 is a positive control mutant of MotA (18). Full-length Mot21 binds DNA like wild-type MotA but fails to activate transcription or form a complex with σ^{70} in a native protein gel (18). Production of *c*I-Mot21^{NTD} in cells containing the α-σ^{70} chimera resulted in a β-galactosidase activity curve that was coincident with that observed with *c*I alone (Fig. 3). In addition, the presence of p*c*I-MotA^{NTD} alone (in the absence of pBRα-σ^{70}) resulted only in background levels of β-galactosidase activity (data not shown). Taken together, these results suggest that MotA interacts with the C-terminal region of σ^{70} and that the NTD of MotA is sufficient for this interaction.

The MotA^{NTD}-σ^{70} interaction in the two-hybrid assay involves the far-C-terminal region of σ^{70}. σ^{38}, an alternative sigma factor for *E. coli* RNA polymerase that is used during stationary phase and under certain conditions of stress (58; reviewed in references 20 and 21), has a region 4 which is similar in amino acid sequence to that of σ^{70} (35). Replacement of the σ^{70} residues in the α-σ^{70} chimera with the comparable region of σ^{38} (amino acids 243 to 330) resulted in background levels of β-galactosidase activity when tested with either *c*I-MotA^{NTD} or *c*I-MotA^{fl} but resulted in nearly 600 Miller units of β-galactosidase activity when tested with *c*I-AsiA (Fig. 4). Furthermore, an α-σ^{70}/σ^{38} hybrid chimera, in which only the last 17 amino acids of σ^{70} (597 to 613) were replaced with comparable σ^{38} residues (amino acids 312 to 330), also gave background levels of β-galactosidase activity with *c*I-MotA^{NTD} (Fig. 5). In contrast, this chimera gave only twofold less activity with the *c*I-AsiA fusion than did the α-σ^{70} chimera (Fig. 5). These results suggest that the far-C-terminal region of σ^{70} is involved in the σ^{70}-MotA interaction.

To investigate the need for specific σ^{70} C-terminal residues, we tested α-σ^{70} chimeras containing the single amino acid substitutions R596H, H600A, H600R, or R608C in the σ^{70} moiety (Fig. 5). The R596H, H600A, and H600R substitutions had little effect on the level of β-galactosidase activity observed with either *c*I-MotA^{NTD} or *c*I-AsiA. In addition, the R608C substitution had no significant effect on the level of β-galactosidase activity observed with *c*I-AsiA. However, this substitution caused a significant defect when the mutant chimera was tested with *c*I-MotA^{NTD}. Varying the IPTG concentration from 5 μM to 1 mM gave results similar to those obtained with 100 mM IPTG (data not shown), indicating that the low level of β-galactosidase activity observed with *c*I-MotA^{NTD} and α-σ^{R608C} could not be improved by increasing the levels of these proteins. In summary, these two-hybrid assays suggested that a residue(s) within the far-C-terminal region of σ^{70} is important for an interaction with MotA but is relatively unimportant for the σ^{70}-AsiA interaction.

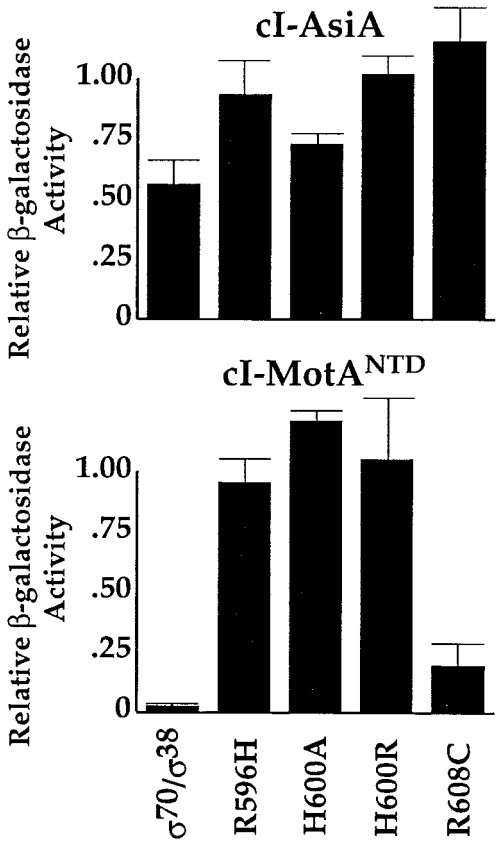

FIG. 5. The MotA-σ^{70} interaction in the *E. coli* two-hybrid assay requires the last 17 amino acids of σ^{70}. Relative β-galactosidase activity is shown for assays with *c*I-AsiA (top panel) or *c*I-MotA^{NTD} (bottom panel) with α-σ^{70}/σ^{38}, α-σ^{70}(R596H), α-σ^{70}(H600A), α-σ^{70}(H600R), or α-σ^{70}(R608C). (See Materials and Methods for the determination of relative β-galactosidase activity.) Points and standard deviations (indicated by error bars) represent the averages of the results of two to eight assays.

Polymerase reconstituted with a σ^{70} lacking either amino acids 608 to 613 or amino acids 604 to 613 is defective for MotA-dependent transcription in vitro. To investigate the involvement of the far-C-terminal amino acids of σ^{70} in MotA-dependent transcription, we tested σ^{70} mutant proteins in single-round in vitro transcription reactions by using a DNA template containing the T4 middle promoter P_{uvsX}. Control reactions contained either wild-type σ^{70} (σ^{fl}) or σ^{R596H}, a σ^{70} whose mutation had no effect in the two-hybrid assays. With both of these σ^{70}s, transcription from P_{uvsX} was observed in the presence of polymerase alone, was inhibited by the addition of AsiA, and was greatly activated by MotA/AsiA (Fig. 6 and data not shown). MotA alone resulted in a small (approximately twofold) increase (Fig. 6 and data not shown). Previous work has indicated that MotA alone has no effect on P_{uvsX} transcription in multiple-round transcription reactions (24) but gives this small effect in single-round reactions (D. M. Hinton, unpublished data).

Despite the fact that in the two-hybrid assays the σ^{70} R608C substitution behaved as if it impaired the σ^{70}-MotA interaction, polymerase containing this mutation was fully active in the in vitro transcription reactions (data not shown). This result suggested either that the MotA-σ^{70} interaction inferred from the two-hybrid assays was not relevant or that in the context of the transcription complex, this single mutation was not sufficient to impair MotA activity. Thus, we investigated whether σ^{70} C-terminal deletions of 6 ($\sigma^{\Delta 608\text{-}613}$) or 10 ($\sigma^{\Delta 604\text{-}613}$) amino acids affected transcription from P_{uvsX}. Although the level of transcription was lower than that of the wild type, polymerase containing either deletion was able to transcribe from P_{uvsX} in the absence of MotA/AsiA. Thus, the deletions did not destroy polymerase activity (Fig. 6A). When polymerase with $\sigma^{\Delta 608\text{-}613}$ or $\sigma^{\Delta 604\text{-}613}$ was used, AsiA alone inhibited transcription significantly or partially, respectively (Fig. 6). Thus, polymerases with these deletions were still susceptible to AsiA inhibition, although the larger deletion was more resistant to AsiA action. In contrast, polymerase with either σ^{70} deletion was significantly impaired in MotA/AsiA activation at P_{uvsX} (Fig. 6). The transcription results support the idea that the far-C-terminal region of σ^{70} is important for MotA to function effectively as an activator. We speculate that the single R608C substitution was not sufficient to interfere with MotA activation in vitro because other stabilizing contacts within the transcription complex compensated for this mutation.

The CTD of MotA binds DNA. We tested MotA$^{cloned\ CTD}$, a protein containing amino acids 105 to 211, and MotA$^{proteolyzed\ CTD}$, a proteolyzed fraction of MotA starting at amino acid 102, for their abilities to bind a DNA fragment containing the T4 middle promoter P_{uvsX} in a gel retardation assay. Both peptides bound P_{uvsX} DNA (Fig. 7 and data not shown). The $K_{D(app)}$ determined for MotA$^{proteolyzed\ CTD}$ was 400 nM, a value that is similar to the previously reported values of 220 nM (6) and 130 nM (52) for wild-type MotA. For MotA$^{cloned\ CTD}$, the determined $K_{D(app)}$ was fourfold higher (2,000 nM). These results suggest that the CTD of MotA is sufficient to bind DNA. As expected, MotA$^{proteolyzed\ CTD}$, which lacks the MotA^{NTD} that is required for activation, did not support activated transcription from P_{uvsX} in vitro (data not shown).

DISCUSSION

Bacteriophage T4 middle promoters represent a hybrid of host and phage promoter sequences, having an excellent match to the σ^{70} −10 recognition sequence but lacking a good match to σ^{70} recognition sequences at −35 (3, 19, 38). It is common for a promoter that lacks canonical −35 sequences to require an activator(s) for transcription initiation by RNA polymerase. Such activators fall into two general categories (reviewed in references 4, 26, and 46). Class I activators bind to sites located upstream of the promoter sequences (at −61.5 or farther), while class II activators bind to sites centered near position −41.5, immediately adjacent to core promoter sequences. In both cases, however, these proteins appear to work by contacting polymerase directly and stabilizing the interaction of σ^{70} region 4.2 with noncanonical sequences within the −35 region of the promoter (2, 11, 30; reviewed in references 4, 26, and 46).

Evidence indicates that MotA/AsiA-dependent activation does not fit either class I or class II. The MotA binding site lies within the core promoter sequence rather than adjacent to it or

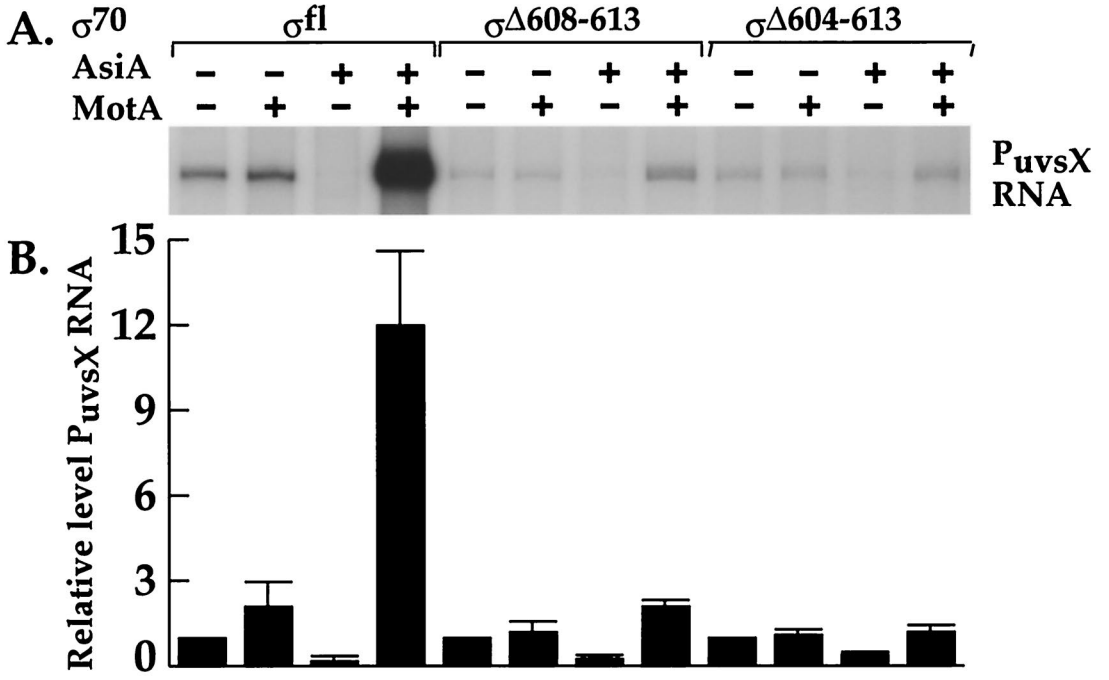

FIG. 6. Polymerase reconstituted with $\sigma^{\Delta 608\text{-}613}$ or $\sigma^{\Delta 604\text{-}613}$ is defective for MotA-dependent transcription at P_{uvsX}. AsiA (23 pmol) and the indicated σ^{70} (1.3 pmol) were incubated for 10 min at 37°C in 2.5 μl of incubation buffer I. The mixture was placed on ice, and then 0.5 pmol of core polymerase in 2.89 μl of incubation buffer II was added. Transcription was initiated by adding an aliquot (2.16 μl) of the resulting solution to 2.35 μl of DNA buffer containing 0.02 pmol of linearized pDKT90 DNA with or without 1.9 pmol of MotA. (A) The ^{32}P-labeled P_{uvsX} RNA obtained after a set of single-round transcription reactions; (B) quantitation of the results from three independent reactions. For each σ^{70}, the values shown for +AsiA, +MotA, and +AsiA/MotA were normalized relative to a value of 1 for that polymerase alone.

farther upstream (3, 19, 38). In addition, within the MotA-AsiA-RNA polymerase-middle promoter complex, σ^{70} retains its contacts with the −10 region of the DNA, but the upstream protein-promoter contacts are significantly rearranged (1, 24). We have previously proposed a model to explain this middle promoter architecture (52). In this model (Fig. 8), AsiA interacts with residues within region 4 of σ^{70} (8, 49, 50, 53, 59), MotA binds to the MotA box (23, 48) and interacts with σ^{70} (18), and σ^{70} region 2.4 retains its contacts with the −10 element of the promoter DNA (24). We speculated that positioning an interaction between MotA and the C-terminal region of σ^{70} would be reasonable, given that residues within region 4.2 of σ^{70} normally interact with the −35 sequences of the DNA and that the MotA binding site is centered at −30 (52). The work here demonstrates that the NTD of MotA, which contains residues needed for activation (14, 18), can

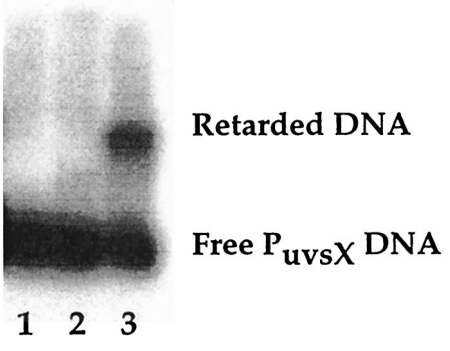

FIG. 7. A C-terminal peptide of MotA binds DNA. Gel retardation assays, which contained 0.5 pmol of the ^{32}P-labeled 74-bp P_{uvsX} DNA, 26 ng of poly(dI-dC) competitor DNA, and buffer (lane 1), protein fraction from uninduced BL21(DE3) cells containing the plasmid with $MotA^{cloned\ CTD}$ (lane 2), or 16 pmol of $MotA^{cloned\ CTD}$ protein in a purified fraction from induced BL21(DE3) cells containing the plasmid with $MotA^{cloned\ CTD}$ (lane 3), are shown. The fraction used in lane 2 was purified in a manner similar to that of the fraction used in lane 3.

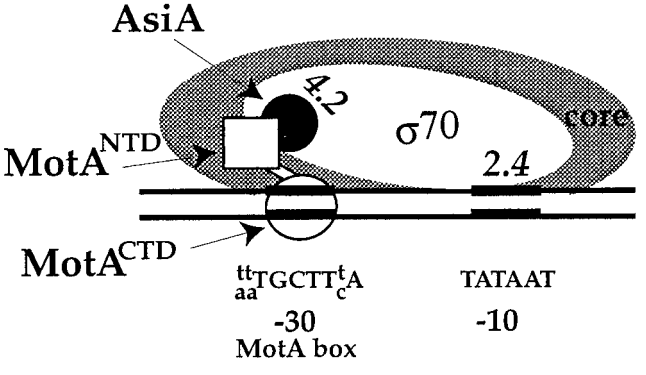

FIG. 8. Model of MotA/AsiA activation at a T4 middle promoter. The cartoon depicts the positions of σ^{70}, AsiA, and MotA at a T4 middle promoter. $MotA^{CTD}$ interacts with the MotA box motif (5′ [T/A][T/A]TGCTT[T/C]A 3′) centered at −30. Both AsiA and $MotA^{NTD}$ interact with the C-terminal region of σ^{70}. The positions of σ^{70} regions 2.4 and 4.2 are shown. (See text for details.)

interact with the C-terminal region of σ^{70}. In addition, we have shown that a C-terminal peptide of MotA starting at amino acid 102 can bind DNA with a binding constant similar to that of the full-length protein. These results are consistent with the idea that the two physical domains of MotA (15), an NTD that is formed by five α-helices and a short β-ribbon (34) and a CTD that is composed of three α-helices interspersed with six β-strands (33), represent two functionally distinct domains. We suggest that MotA belongs to a class of activators that is distinct from both class I and class II. Instead of stabilizing the typical contacts between region 4.2 of σ^{70} and the DNA, an activator in this third class functionally replaces such contacts by serving as a molecular bridge between σ^{70} and the DNA.

The amino acid sequence of region 4 of σ^{38} is similar to that of region 4 of σ^{70} (35) and recognizes the same −35 canonical promoter element (13, 16). We found that AsiA interacted with the C-terminal region of σ^{38} in the two-hybrid assay. AsiA binds to a broad surface of σ^{70} region 4.2 (8), and AsiA can tolerate amino acid changes throughout this binding surface (41). In addition, in the two-hybrid assay, the AsiA-σ^{70} interaction was not significantly affected by mutations at residues R596, H600, or R608 (reference 10 and this paper). Thus, the simple explanation for an AsiA-σ^{38} interaction is that this interaction occurs because region 4.2 of σ^{38} is very similar to that of σ^{70}. However, this result is surprising, since AsiA neither inhibits transcription by polymerase containing σ^{38} nor forms a complex with full-length σ^{38} in a native protein gel (8). Our results suggest that AsiA is indeed able to interact with the C-terminal region of σ^{38} but that some feature of the full-length protein prevents this interaction.

In contrast to the results seen with AsiA, replacement of even the last 17 amino acids of σ^{70} with comparable σ^{38} residues eliminated the stimulation of β-galactosidase activity observed with *c*I-MotA^{NTD}. This effect is specific for MotA, since the σ^{70}/σ^{38} chimera worked both with *c*I-AsiA (this paper) and with a *c*I that had been fused to the *E. coli* anti-sigma protein Rsd (S. L. Dove and A. Hochschild, unpublished data). In addition, a substitution of R608C in α-σ^{70} also reduced the interaction with *c*I-MotA^{NTD} but had no effect on the interaction with *c*I-AsiA. However, not all substitutions within the C-terminal region of σ^{70} weakened the interaction of σ^{70} with *c*I-MotA^{NTD}. In particular, a substitution at R596 or H600 had no significant effects. In contrast, the substitution R596H significantly reduced the interaction between the fused σ^{70} moiety and either Rsd from *E. coli* or the related regulator, AlgQ, from *Pseudomonas aeruginosa* (10). The specific effects of these various substitutions argue that the defects seen with *c*I-MotA^{NTD} and the mutant α-σ^{70} chimeras arise from a loss of the MotA-σ^{70} interaction rather than a misfolding of the mutant chimeras. Furthermore, our in vitro transcription experiments indicated that a σ^{70} lacking 6 or 10 C-terminal amino acids is defective for MotA activation of transcription. Taken together, our results are consistent with the idea that the far-C-terminal region of σ^{70} contacts MotA and that this contact is necessary for MotA to work as an activator. Previous work has indicated that an amino acid substitution at position 604 can partially suppress the growth defect of a T4 *motA*-positive control mutant in vivo (7), a result that is compatible with this conclusion.

The far-C-terminal region of σ^{70} lies within an alpha helix (amino acids 603 to 613) (5, 36) at the very end of the protein. This region is just C-terminal of residues that interact with the −35 region of DNA (residues 584, 585, and 588) (5, 9, 17, 29, 54) and of residues that have been implicated in the interactions of σ^{70} with *E. coli* class II activators (residues 590 to 603) (32, 36). Thus, the C terminus of σ^{70} appears to be involved both in class II activation and in the architecturally different activation achieved by MotA/AsiA. Further studies will be needed to determine exactly how the region is configured in class II- versus MotA/AsiA-dependent activation.

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