The Interaction between the AsiA Protein of Bacteriophage T4 and the $\sigma^{70}$ Subunit of Escherichia coli RNA Polymerase*

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The AsiA protein of bacteriophage T4 binds to the $\sigma^{70}$ subunit of Escherichia coli RNA polymerase and plays a dual regulatory role during T4 development: (i) inhibition of host and phage early transcription, and (ii) coactivation of phage middle-mode transcription, which also requires the T4 DNA binding transcriptional activator, MotA. We report that the interaction between AsiA and $\sigma^{70}$ occurs with a 1:1 stoichiometry. When preincubated with RNA polymerase, AsiA is a potent inhibitor of open complex formation at the lac UV5 promoter, whereas it does not perturb preformed open or intermediate promoter complexes. DNase I footprinting and electrophoretic mobility shift analyses of RNA polymerase-DNA complexes formed at the T4 early promoter P15.0 show that AsiA blocks the initial RNA polymerase binding step that leads to the formation of specific closed promoter complexes. A contrasting result is obtained on the T4 middle promoter PrIIB2, where AsiA stimulates the formation of both closed complexes and open complexes. Therefore, we propose that AsiA modulates initial DNA binding by the RNA polymerase, switching promoter usage at the level of closed complex formation.

Bacteriophage T4 development in Escherichia coli is regulated mainly at the transcriptional level. A number of phase-encoded transcription factors modify both the structure and the function of the host RNA polymerase (RNAP), which is responsible for the transcription of all T4 genes. These RNAP modifications result in the sequential utilization of the three classes of T4 promoters: early, middle, and late (1). Early promoters are transcribed immediately upon infection by unmodified host RNAP, which recognizes their bacterial-like promoter elements situated at $-10$ and $-35$ relative to the transcription start site (2). Shorty thereafter, the $a$ subunits of the RNAP are ADP-ribosylated by two phase-encoded proteins, Alt and Mod, and the RNAP is tightly bound by the products of T4 genes rpbA and asIA (3). Middle promoters contain an E. coli-like $-10$ consensus sequence, but lack an identifiable $-35$ region. This is replaced by a "Mot box" ((t/a)(t/a/TGCTT(t/c))A), a 9-bp sequence centered around $-30$, which is the binding site for the T4 transcriptional activator MotA (3, 4). Although it is possible that ADP-ribosylation and association with the RpbA protein modulate transcription in vivo, transcription initiation from a middle promoter in vitro requires only the E. coli RNAP holoenzyme, and the purified T4 proteins MotA and AsiA (5–7).

AsiA is a 10.6-kDa protein that was first identified through its tight association with the $\sigma^{70}$ subunit of RNAP, and the resulting inhibition of transcription at E. coli and T4 early promoters (6, 8–10). The interaction between AsiA and $\sigma^{70}$ is thought to decrease the affinity of $\sigma^{70}$ for the core polymerase (8, 10) and diminish the binding of RNA polymerase to phages T4 and T7 DNA (9). Nonetheless, AsiA is a coactivator of middle transcription, which also requires the $\sigma^{70}$ subunit (6). Therefore, the activity of AsiA cannot be merely a result of $\sigma^{70}$-core dissociation. In light of its dual role, and the relative simplicity of the prokaryotic system in which it functions, the study of AsiA should provide some insight into how the activity of a major sigma factor is regulated through specific protein-protein interactions.

Numerous kinetic and structural studies of the complexes formed between the E. coli RNAP holoenzyme (R) and several promoter sequences (P) have resulted in the following basic model for productive transcription initiation (11–13).

$$
\begin{array}{c}
R + P \rightarrow RP_C \rightarrow RP_2 \rightarrow RP_0 \rightarrow RP_{\text{AP}} \rightarrow RP_k \\
\text{NTP} \quad \text{NTP} \\
\end{array}
$$

Scheme 1

In this multistep process (reviewed in Ref. 11), RP$_C$ represents the initial closed complex, which is characterized by its sensitivity to competitors such as heparin, and its rapid equilibrium with free DNA. A slow isomerization step leads to the formation of a strained intermediate, RP$_I$. This transition, which renders the complex insensitive to challenge by polynucleotides, is proposed to involve substantial untwisting or bending of the DNA in the spacer region (14–16). However, it is not until the appearance of the open complex, RP$_O$, that strand separation is detected near the transcription start site. The addition of ribonucleotides (NTP) allows for the formation of the initiated complex (RP$_{\text{init}}$), in which abortive products (AP), are reiteratively synthesized (see, e.g., Ref. 17). This natural cycling ceases upon promoter clearance and the entry into the stable ternary elongation complex RP$_E$.

Transcription regulators that act at nearly every point in this model have been described. The series of experiments presented below is an attempt to identify the step (or steps) in...
this pathway that are affected by AsiA. To accomplish this, we studied the inhibitory effects of AsiA on both the well characterized E. coli promoter lac UV5, and the T4 early promoter P15.0. A comparison is drawn between these results and the stimulatory effects of AsiA observed with the T4 middle promoter PrIIB2.

EXPERIMENTAL PROCEDURES

Materials—QIAquick spin columns were from QIAGEN. Sephacryl-100 was purchased from Pharmacia. Affi-Gel Blue Gel was from Bio-Rad. Enzymes were obtained from the following suppliers: T4 polynucleotide kinase from New England Biolabs, Taq Plus from Stratagene, and DNase I from Boehringer Mannheim. Radioactively labeled nucleotide triphosphates were obtained from ICN.

Plasmids and DNA Fragments—Plasmid pOMlacUV5 is a derivative of pOM70 (18). At its unique EcoRI site, it contains a 203-bp lac insert giving rise to a 125-nucleotide transcript originating from the lac UV5 promoter (19). This plasmid also harbors a strong promoter responsible for the synthesis of RNA I, a 108-nucleotide transcript involved in the regulation of plasmid replication (18). The 203-bp DNA fragment lac L8UV5 used for abortive transcription was obtained as described (19).

In the absence of CRP (the cyclic AMP receptor protein), the L8 mutation at position 68 of the lac UV5 promoter has no effect on the transcriptional activities monitored in the core enzyme, and was referred to as lac UV5 in the text. The DNA fragments containing the T4 early promoter P15.0 (132 bp), or the T4 middle promoter PrIIB2 (158 bp) were obtained by performing polymerase chain reaction on T4 DNA using Taq Plus polymerase. The primers used to amplify the P15.0 fragment were uniquely end-labeled using T4 polynucleotide kinase and [γ-32P]ATP. The PrIIB2 fragment used previously (6) was uniformly radiolabeled by performing polymerase chain reaction in the presence of [α-32P]dCTP. The fragments were purified using spin columns.

Enzymes, Proteins, and Reaction Buffers—E. coli RNA polymerase was purified according to the method of Burgess and Jendrisack (20). A Coomassie Blue-stained SDS-polyacrylamide gel of the purified enzyme was scanned with a densitometer and the ratio of 15-AsiA to the core enzyme was estimated to be 0.65–0.70. Phage T4 MotA and AsiA proteins were purified as described (6) with an additional purification step for AsiA on Affi-Gel Blue Gel, which eliminated a contaminating DNase activity (21). The resulting AsiA preparation was more than 95% homogeneous.

GST and DNase I Footprinting and Electrophoretic Mobility Shift Assays—Reactions for GSTase digestion contained 0.05 lU of GSTase (labeled P15.0 DNA labeled on the non-template strand) at 10 nM in a 10-μl volume, in the presence of 50 nm RNAP holoenzyme where indicated. The DNA (or protein) was preincubated at either 4 °C or 37 °C for 10 min, then challenged for 1 min with non-specific competitor (0.015 μg/μl P15.0 in 0.15 M EDTA), before loading onto 10% polyacrylamide native gels running at 200 V, and being loaded onto 5% polyacrylamide native gels running at 200 V, and then electrophoresed through a 5% native polyacrylamide gel (as described below). The retarded complex was visualized by autoradiography, cut from the gel, and eluted overnight in elution buffer (20 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, 0.2% SDS, and 1 M LiCl).

RESULTS

AsiA Binds σ70 with a 1:1 Stoichiometry—To study the stoichiometry of the physical interaction between σ70 and the AsiA protein, we used [GST]-σ70 fusion protein, GSTσ506, which contains the C-terminal 108 amino acids of σ70 fused to the GST moiety (described in Ref. 22). To ensure that GSTσ506 harbors the full site for AsiA binding, we analyzed by electrophoretic mobility shift the interaction of 32P-labeled AsiA with both full-length σ70 and GSTσ506 (data not shown). Measuring the fraction of AsiA bound to an excess of either σ70 or 32P chromotography paper prespotted with 100 mM EDTA. The abortive transcription product ApApUpU was separated from TUP by ascending chromatography in water-ammonium sulfate-isopropanol (WASP) solvent (23). The incorporation of pu in the product was quantitated with the help of a PhosphorImager.

Single Round Transcription—The transcription protocol for pOM-lacUV5 is described in the legend to Fig. 3. Transcription buffer contained 30 mM Tris-HCl, pH 8.0, 100 mM KC1, 3 mM MgCl2, 0.1 mM EDTA, 0.2 mM DTT, and 100 μg/ml acetylated bovine serum albumin. Elongation reactions, in a final volume of 12 μl, contained 100 μM ATP, CTP, and GTP, 10 μM UTP, 0.6 μCi of [α-32P]UTP, and 200 μg/ml heparin. Transcription on the linear template P15.0 and PrIIB2 was initiated by the addition of [α-32P]UTP and ApAp at the concentrations described for native gel shift experiments, with the addition of rNTPs at the concentrations given above, and heparin (as indicated) to a final concentration of 670 μM. All RNA was analyzed by electrophoresis of the reaction products on 7% polyacrylamide gels containing 7 M urea. Quantification of the transcripts was performed on a PhosphorImager (Molecular Dynamics) using the ImageQuant software.

DNase I Footprinting and Electrophoretic Mobility Shift Assays—Reactions for DNase I digestion contained P15.0 DNA (labeled on the non-template strand) at 10 nM in a 10-μl volume, in the presence of 50 mM RNAP holoenzyme where indicated. The DNA (or protein) was preincubated at either 4 °C or 37 °C for 10 min, then challenged for 1 min with non-specific competitor (0.015 μg/μl P15.0 in 0.15 M EDTA), before loading onto 10% polyacrylamide native gels running at 200 V, and then electrophoresed through a 5% native polyacrylamide gel (as described below). The retarded complex was visualized by autoradiography, cut from the gel, and eluted overnight in elution buffer (20 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, 0.2% SDS, and 1 M LiCl). All reactions were phenol-extracted and ethanol-purified, and the DNA dissolved in 4 μl of formamide loading dye. The samples were then electrophoresed through an 8% polyacrylamide, 7 M urea sequencing gel. Base positions were determined using G + A sequencing ladders generated as described below. The retarded complex was visualized by autoradiography, cut from the gel, and eluted overnight in elution buffer (20 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, 0.2% SDS, and 1 M LiCl).

Complexes for gel shift experiments on the 5'-labeled P15.0 fragment were formed at 4 °C in standard reaction buffer, plus acetylated bovine serum albumin (100 μg/ml) under the conditions given in the figure legend. After 10 min, these complexes were challenged with 2 μl of loading solution (30% glycerol), which contained heparin (final concentration 670 μg/ml), AsiA (final concentration 100 μM), or both, as indicated. The reactions were incubated for 1 min (or as indicated) before being loaded onto 5% polyacrylamide native gels running at 200 V, and electrophoresed for 2 h at 6 °C. The radioactivity was measured using a PhosphorImager (Molecular Dynamics) and the ImageQuant software.

Complexed DNA was defined as the radioactivity present in the discrete RNAP-DNA complexes (Fig. 5a, designated by arrows). In Fig. 5b, this quantity is plotted as the fraction of the total radioactivity present in each lane.

Mobility shift experiments were performed on the PrIIB2 DNA under conditions described previously for middle-mode transcription from this promoter (6). Concentrations given below represent the final concentrations present before loading. Reactions containing the 32P-labeled PrIIB2 fragment (2.5 μM) and MoA (100 μM) were preincubated on ice for 5 min. Core polymerase or RNAAP holoenzyme (5 μM) was added, followed by AsiA (0, 10, or 25 nm, as indicated). Nonspecific competitor (0.015 μg/μl poly(dl-dc)) was also supplied to the samples at 37 °C. All reactions (10 μl) were incubated at either 4 °C or 37 °C for 15 min before adding 2 μl of loading solution, which contained heparin (670 μg/ml), as shown in the figure legend. The resulting complexes were resolved as described above.

RESULTS

AsiA Binds σ70 with a 1:1 Stoichiometry—To study the stoichiometry of the physical interaction between σ70 and the AsiA protein, we used [GST]-σ70 fusion protein, GSTσ506, which contains the C-terminal 108 amino acids of σ70 fused to the GST moiety (described in Ref. 22). To ensure that GSTσ506 harbors the full site for AsiA binding, we analyzed by electrophoretic mobility shift the interaction of 32P-labeled AsiA with both full-length σ70 and GSTσ506 (data not shown). Measuring the fraction of AsiA bound to an excess of either σ70 or
**σ^70-AsiA Interaction**

Fig. 1. The co-elution of GST(506) and the AsiA protein. The proteins were passed through Sephacyrl 100HR sizing columns, either separately (A and B), or together (C). The column fractions were precipitated in 10% trichloroacetic acid, washed with acetone, and subjected to 16.5% SDS-PAGE. The gels were stained with Coomassie Blue.

The intermediate bands observed between GST(506) monomer, eluting at fraction 28; 46-kDa dimer, eluting at fraction 21). Lysozyme (14 kDa, eluting at fraction 39) and 434 repressor (23-kDa arrow and fractionated together (the position of each protein is indicated by an arrow). The column was calibrated (data not shown) by size filtration of lysozyme (14 kDa, eluting at fraction 29) and 434 repressor (23-kDa monomer, eluting at fraction 28; 46-kDa dimer, eluting at fraction 21). The intermediate bands observed between GST(506) and AsiA are due to impurities in the GST(506) preparation and do not affect this interaction.

GST(506), yielded similar binding curves (data not shown), demonstrating that the region of σ^70 present in GST(506) is sufficient for AsiA binding. This evidence confirms and quantifies the data of Severinova et al. (26), who recently localized the AsiA binding region to within the C-terminal 63 amino acids of σ^70.

We then used gel filtration to determine the stoichiometry of the interaction between GST(506) and AsiA. The GST(506) fusion (41 kDa) and the AsiA protein (10 kDa) were initially passed over sizing columns separately, and their elution profiles determined by electrophoresis of the column fractions on SDS-polyacrylamide gels (Fig. 1, A and B). In Fig. 1C, AsiA was preincubated with an equimolar amount of GST(506) before gel filtration. Identical analysis of these column fractions shows that the AsiA protein clearly coelutes with GST(506) in fractions 21–28 (Fig. 1C); this is significantly earlier than the position at which AsiA elutes alone (Fig. 1B, fractions 28–31). The interaction of GST(506) with the AsiA protein was specific to the σ^70–61 portion of this fusion protein, since AsiA does not coelute with an unfused GST moiety (data not shown).

We estimated the molar ratio of the proteins in AsiA–GST(506) complexes by scanning densitometry of SDS-polyacrylamide gels similar to that shown in Fig. 1C (described under “Experimental Procedures”). Table I, part A, presents the quantitative analysis of the column fractions from co-chromatography of 3 nmol of each protein, yielding a definitive stoichiometry of 1:1. To demonstrate the uniqueness of this complex, the experiment was repeated with 6 nmol of AsiA and 2 nmol of GST(506). These results are shown in Table I, part B. For all fractions that could be quantitated accurately, the ratio of GST(506) to AsiA is still remarkably close to 1.0.

The Effects of AsiA on Abortive Initiation and Open Complex Formation—Abortive transcription assays were used to analyze the effect of AsiA on open promoter complex formation and initiation by the E. coli RNAP at the lac UV5 promoter. We measured the steady-state levels of the tetranucleotide ApApUpU released during the abortive cycling of RNAP at this promoter in the absence or presence of increasing concentrations of AsiA (see “Experimental Procedures”). Fig. 2 shows that AsiA is a potent inhibitor of one or more of the steps leading to the formation of the transcriptionally competent open complexes. Since this method assays only the events leading to RPinit, AsiA activity must precede promoter clearance. Furthermore, for the low AsiA concentrations, five experimental points can be fitted to a straight line which, when extrapolated, intersects the x axis at −100 nM AsiA. This reflects the effective concentration of σ^70 present in these reactions (see “Experimental Procedures”), indicating that the 1:1 stoichiometry established for the free AsiA–σ^70 complex (Table I), is maintained when the complex is part of the functional RNAP holoenzyme.

To refine our understanding of AsiA function, single round in vitro transcription experiments were designed to analyze the effects of AsiA on the formation of open complexes at the lac UV5 promoter, as well as its effects on such preformed complexes (legend to Fig. 3). RNAP was first preincubated with increasing concentrations of AsiA prior to the addition of the DNA template (supercoiled pOMlacUV5). RNAP-promoter complex formation was then allowed for 10 min at 37 °C, before a single round of transcription was initiated. Fig. 3A shows that AsiA inhibits the synthesis of the 125-nucleotide lac UV5 transcript, and the 108-nucleotide RNA I transcript, to the same extent. The quantitation of this effect is shown in Fig. 3C. A very steep inhibition profile is reproducibly obtained with a residual activity of approximately 10% at the highest AsiA concentration tested. In contrast, when open complexes were allowed to form prior to the addition of AsiA, the levels of transcript generated are not affected, even in the presence of a...
30-fold molar excess of AsiA over the RNAP (Fig. 3, B and C). These results imply that although AsiA inhibits a step leading to the formation of the open complex RP_O, its presence affects neither the stability of such a preformed complex nor the subsequent steps in the transcription pathway.

AsiA and Closed Complex Formation—We next investigated the effects of AsiA on the formation and stability of RNAP-promoter complexes formed earlier in the series of events which lead to promoter opening. Previous studies have demonstrated that different transient intermediates can be accumulated by varying the incubation temperature. The closed complex, RC, is favored at temperatures of 8 °C or lower, intermediate complexes RP_I are seen between 8 °C and 21 °C, while the open complex RP_O is predominant above 22 °C. The lac UV5 promoter has been extremely well characterized in this regard, and much is known about its specific kinetic properties. Challenge experiments demonstrate that, like RP_O, the RP_I complexes are not perturbed by nonspecific competitors such as poly(dA-dT), and that RP_C disappears rapidly in their presence (12, 27). Thus, the addition of poly(dA-dT) to preformed complexes allowed for the determination of the dissociation rate (k_d) and the half-life (t_1/2) of the complexes which form in the range of 14 °C to 37 °C (12).

We chose 16 °C, a temperature that favors the accumulation of RP_I, to perform similar challenge experiments on lac UV5. However, rather than challenging the complex with a nonspecific competitor, we added AsiA in a 5-fold molar excess over the RNAP holoenzyme, and then monitored the decay of the open complex formation. The quantitation of transcriptionally active species was assayed by quantitating the amount of transcript expressed relative to that of the uninhibited control.

![Graph](image-url)  
**Fig. 2.** AsiA inhibits open complex formation at the lac UV5 promoter, as measured by the abortive initiation assay. Prior to open complex formation, RNA polymerase (125 nM) was incubated with AsiA at the concentrations indicated on the abscissa. The lac UV5 fragment (2 nM) was added to these reactions, followed by the substrates ApA and [a-32P]UTP (see “Experimental Procedures”). The presence of transcriptionally active species was assayed by quantitating the abortive transcription product. The amount of transcript is expressed relative to that of the uninhibited control.

![Graph](image-url)  
**Fig. 3.** AsiA inhibits single round transcription when present before complex formation. A, RNA polymerase (20 nM at this step) and AsiA at the indicated concentrations were incubated (6 μl) for 10 min at 37 °C in transcription buffer. pOMlac UV5 DNA (3 μl) was then added to a final concentration of 5 nM, and the mixture was further incubated for 10 min. B RNAP was incubated with pOMlac UV5 DNA at the concentrations given above in 6 μl at 37 °C for 15 min. AsiA was then added (3 μl) as indicated, and the incubation continued for 5 min. In both cases, elongation was initiated by adding rNTPs in a volume of 3 μl. After 5 min, the reaction was terminated by mixing equal volumes of reaction mixture and formamide loading buffer prior to electrophoresis on a 7% polyacrylamide sequencing gel. C, quantitation of the single round transcripts. AsiA was added to RNA polymerase either before (●) or after (○, □) open complex formation. The quantitation of transcription formation is expressed as in Fig. 2.
Promoter DNA at 4 °C, leading to the appearance of a discrete RNAP holoenzyme is able to form specific complexes with the molecules bound nonspecifically to the same DNA fragment. The complexes that remain at the origin are dissociated as well. This biphasic behavior is consistent with previous data, implying a partition between at least two distinct species at 4 °C, which differ in their sensitivity to heparin. However, none of these discrete species are initially sensitive to challenge by AsiA; instead, a slower continuous rate of dissociation is observed.

DNase I footprinting was used to further characterize the specific polymerase-DNA complexes formed at the P15.0 promoter. RNAP-DNA mixtures were subjected to digestion by DNase I prior to electrophoresis through nondenaturing polyacrylamide gels. The DNA engaged in protein complexes was isolated as described under “Experimental Procedures” and analyzed on sequencing gels. Fig. 6 presents the DNase I cleavage of the free P15.0 DNA fragment, as well as the specific RNAP-DNA complexes seen in the absence of AsiA at 4 °C and 37 °C. The species formed in the presence of AsiA failed to produce any detectable footprint, reflecting their nonspecific character (data not shown). The footprint exhibited by the complexes formed at 37 °C (Fig. 6, lane 4) spans the region between −56 and +15, which is characteristic of either RP1 or RP2. Although our analysis does not allow us to differentiate between these two species, it is likely that RP2, which predominates at 37 °C. Moreover, the DNase I cleavage pattern displays the typical sites of hypersensitivity at positions −47, −39, −37, −27, and −26 on the nontemplate strand, that have been previously documented for the open complex (11, 31). This contrasts with the footprint displayed at 4 °C (Fig. 6, compare lanes 3 and 4), which shows weaker protection between +1 and +15. The cleavage pattern is also distinct; it lacks the intense reactivity at positions −47, −37, −27, and −26, and exhibits a unique cleavage at −14. The footprint observed at 4 °C suggests the presence of RP2 (11), wherein the promoter DNA is not fully protected downstream of the transcription start site and has not yet adopted the bent conformation thought to be characteristic of open or intermediate complexes (11, 14–16). There are two possibilities for the observed partial protection in the region +1 to +15 (refer to Fig. 6): (i) the RNAP bound in the RP2 complexes at P15.0 sterically hinders cleavage in this area by DNAse I, or (ii) the complexes footprinted at 4 °C reflect an equilibrium between RP2 and either the intermediate or open complexes. This latter explanation is supported by the prior data on the heparin sensitivity of these complexes (Fig. 5), and the discovery that, on certain “extended −10” promoters, the closed complex is capable of isomerization at low temperatures (32).

The fact that closed complexes are readily formed in the absence of AsiA, but not in its presence, indicates that AsiA inhibits the formation of RP2. Additionally, the initial resistance of the preformed 4 °C complexes to AsiA challenge sug-

**FIG. 4.** The intermediate complex RP is insensitive to AsiA. All reactions were performed in the abortive transcription buffer (see “Experimental Procedures”), except that 25 mM Hepes was substituted for 40 mM Tris-HCl, RNAP (1 μm) and lac UV5 fragment (20 ng) were incubated at 16 °C for 1 h. A 5-fold molar excess of AsiA (5 μm) was then added to this solution at time zero. Aliquots of this mixture (7.5 μl) were removed at time points between 0.5 and 32 min and quickly mixed with 42.5 μl of standard abortive assay solution prewarmed at 37 °C. The amount of RNAP engaged in transcriptionally competent complexes was then measured using the abortive transcription assay as described above. A logarithmic plot of [α-32P]UTP incorporation is presented as a function of time. Values presented are derived from two separate experiments.
suggests that the RP C complexes are not actively dissociated by AsiA. Therefore, we propose that AsiA acts on the free RNAP holoenzyme, to modify the initial interactions between the polymerase and the promoter DNA.

AsiA Stimulates Complex Formation on a T4 Middle Promoter—As mentioned previously, a physiological role of the AsiA protein is, when bound to s70, that of a coactivator of T4 middle transcription. It has been demonstrated that open complex formation at middle promoters not only requires RNAP holoenzyme, AsiA and MotA, but that these components are sufficient (6). Moreover, Hinton et al. (7) have shown that heparin-resistant complexes form between T4 modified RNAP holoenzyme (containing AsiA), MotA protein, and middle promoter DNA.

We have undertaken a step in defining the complexes at the strong middle promoter PrIIB2, by analyzing through electrophoretic mobility shift their formation and stability at both 4 °C and 37 °C. These experiments, like those performed on the P15.0 promoter, allowed us to compare the effects of AsiA on both the presumed closed and open promoter complexes. Preformed MotA-PrIIB2 DNA complexes were incubated at a given temperature with RNAP holoenzyme or core RNA polymerase (in the presence or absence of AsiA), challenged with heparin (as indicated) and run on native gels at 6 °C (see “Experimental Procedures” and legend to Fig. 7). Fig. 7A shows that the core polymerase-DNA species (arrows; nonspecific complexes), are unchanged by the addition of AsiA, confirming that the s70 subunit is required for AsiA function. This provides strong evidence for the idea that the AsiA-σ70 interaction is not simply dissociating σ70 from core, and is consistent with previous reports that the intact holoenzyme, bound to AsiA, is required for efficient middle-mode recognition (6).

In addition, Fig. 7A shows that the formation of discrete complexes between the RNAP holoenzyme and the promoter DNA is strongly enhanced by the addition of AsiA, at either 4 °C (lanes 5–7) or 37 °C (lanes 11–16). The presence of AsiA not only increases the quantity of RNAP-promoter complex observed, but leads to the formation of a complex that has a slightly lower mobility than that detected in its absence (compare arrows; +AsiA, −AsiA). This slower moving species requires not only the RNAP and AsiA, but also MotA, since, in its absence, this complex is not observed (Ref. 7 and data not shown).

The RNAP-promoter complexes that form at 4 °C are sensitive to heparin challenge, regardless of the presence of AsiA (lanes 8–10), suggesting that they represent closed complexes, whereas the heparin-resistant species observed at 37 °C are likely to correspond to open complexes (lanes 14–16). Since AsiA is a positive effector of complex formation at both temperatures, it must function at the step common to the formation of both closed complexes and the heparin-resistant species. This step is the initial binding equilibrium, which suggests that both the inhibitory and stimulatory roles of AsiA could proceed through the same mechanism (i.e. regulation of initial binding to the promoter DNA).
To investigate in greater detail the complexes between RNAP and PrIIB2 promoter DNA, we assayed their transcriptional competence under the conditions described for electrophoretic mobility shift assays. Complexes formed at 4 °C or 37 °C were supplied with all four rNTPs, in the presence or absence of heparin, and incubated at 37 °C to allow elongation. No transcription activity was detected from any of the 4 °C complexes after heparin challenge (Fig. 7A, lanes 4–6), supporting their identification as RPC complexes. However, in the presence of AsiA and the absence of heparin, the addition of rNTPs yielded transcripts during a 3-min incubation at 37 °C. This indicates that, at 4 °C, only the complexes containing AsiA are capable of isomerizing quickly into transcriptionally active species. In fact, the amount of transcript observed from these complexes was indistinguishable from that of the corresponding complexes formed at 37 °C (lane 2) or 37 °C (lane 4). The RNAP-promoter complexes were separated from free DNA before analysis on the sequencing gel.

This confirms that although AsiA is needed for efficient middle-mode recognition, under certain conditions, a small amount of transcription may occur in its absence (33). A comparison of the level of transcript formed with or without a challenge by the competitor heparin shows that the 37 °C complexes are largely heparin-resistant. This supports the conclusions drawn from the above gel shift experiments that these are stable, open complexes (RPO). Therefore, while the mobility shift assay indicates that AsiA stimulates the initial binding of RNAP to the PrIIB2 promoter, the analysis of the transcription data suggests that the slower moving complexes that form in the presence of AsiA (Fig. 7A, arrow designating +AsiA) also possess greater transcription potential than their −AsiA (Fig. 7A, arrow) counterparts. Thus, it is possible that, in addition to increasing the amount of RP C complexes formed, AsiA confers greater stability upon this closed complex, or aids in its transition toward a transcriptionally active species.

FIG. 6. DNase I footprints of the complexes between RNAP and the P15.0 promoter. The P15.0 fragment, labeled on the nontemplate strand, was subjected to treatment by DNase I as described under “Experimental Procedures.” Lanes 1 and 2 show the cleavage of the free P15.0 DNA by DNase I at either 4 °C (lane 1) or 37 °C (lane 2). Lanes 3 and 4 display the footprints of the RNAP-DNA complexes formed at 4 °C (lane 3) or 37 °C (lane 4). The RNAP-promoter complexes were separated from free DNA before analysis on the sequencing gel.

FIG. 7. AsiA stimulates recognition of the MotA-bound middle promoter PrIIB2 at both 4 °C and 37 °C. A, preformed complexes between MotA and PrIIB2 DNA were incubated with core polymerase (lanes 2–4) or RNAP holoenzyme (lanes 5–16) in the presence of AsiA (at the concentrations indicated). The resulting complexes, formed at either 4 °C or 37 °C, were loaded on a nondenaturing gel, after a challenge by heparin (as indicated). The relative mobilities of the free PrIIB2 DNA and nonspecific (core) complexes are noted at left (arrows), while the arrows at right indicate the slightly different positions of the RNAP-PrIIB2 complexes with or without the AsiA protein. B, in vitro transcription from the RNAP-DNA complexes at either temperature. Holoenzyme-promoter complexes were formed as described above, supplied with rNTPs in the presence or absence of heparin (as indicated), and shifted to 37 °C to allow elongation. PrIIB2 run-off transcripts are shown by arrows.

DISCUSSION

Biochemical analyses of RNA polymerase-associated proteins in bacteriophage T4-infected E. coli cells led many years ago to the identification of the 10-kDa anti-α70 factor (8), recently shown to be encoded by the T4 asiA gene (10). Although several roles have been proposed for the AsiA protein (reviewed in Ref. 34), its precise regulatory function has remained elusive for more than 2 decades. It is known that, in the presence of AsiA, in vitro transcription from E. coli and T4 early promoters is inhibited, while recognition of MotA-dependent T4 middle
AsiA interacts with the $\sigma^{70}$ subunit to alter the RNAP specificity has remained unclear. The results presented here are a step toward understanding the role of AsiA in T4 gene expression. We have characterized both the structural interactions between AsiA and $\sigma^{70}$, and the functional consequences of this binding. Although the AsiA protein was initially characterized through its tight association with $\sigma^{70}$, the region of $\sigma^{70}$ responsible for this interaction was just recently determined (26). In this work, we confirm that the region encompassing amino acids 506–613 of $\sigma^{70}$ associates tightly with AsiA, and demonstrate a 1:1 stoichiometry for this interaction, both in solution (Fig. 1, Table 1), and as a part of the RNAP holoenzyme (Fig. 2). Then, using a simplified model for transcription initiation (Scheme 1), we proceed stepwise to isolate the point at which AsiA functions in transcription inhibition, as well as investigating the validity of this result as a general mechanism for the role of AsiA in stimulation of T4 middle-mode synthesis.

Since the above model was largely devised from work on the E. coli lac UV5 promoter, this promoter was chosen for use in our initial experiments. The abortive initiation assay (Fig. 2), demonstrates clearly that AsiA acts at a step preceding the formation of RP$_{70}$ (Scheme 1), and in particular, promoter clearance. In this assay, AsiA was capable of completely abolishing transcription when present at only a 2-fold molar excess over RNAP holoenzyme. This differs from the approximately 10% residual activity consistently obtained in single round run-off transcription assays, even with a large excess of AsiA. Since the abortive initiation assay provides for direct study of the events leading to transcription initiation, there exists no possibility of kinetic escape of the polymerase into an AsiA-insensitive elongation complex, as exists in the single round transcription assay. This distinction between the two assays is most likely the source of the difference in residual polymerase activity observed at high AsiA concentrations (Figs. 2 and 3).

Our results show that, when bound to AsiA, the RNAP holoenzyme cannot form open or intermediate complexes with the lac UV5 promoter (Figs. 3, A and C, and 4). However, once these complexes have formed, AsiA is unable to affect their stability or transcription potential (Figs. 3, B and C, and 4), suggesting that AsiA acts at a step preceding the formation of either of these specific complexes (i.e. at the initial binding step or the isomerization of RP$_C$ to RP$_T$). We then pursued our studies using the strong T4 early promoter, P15.0, where the closed complex RP$_C$ is sufficiently stable to support analysis. The DNase I footprints displayed at P15.0 indicate that the RNAP contacts this promoter through a molecular mechanism similar to that previously described for other promoters (Ref. 31 and Fig. 6). The presumed open complex (formed at 37 °C) shows the expected region of protection (between −55 and +17), and the typical positions of hypersensitivity. In contrast, the 4 °C complexes (Fig. 6), exhibit a footprint that reveals the presence of a considerably different species, presumably the initial closed complex. The unusual strength of T4 early promoters, and specifically P15.0, is reflected in both the ability to isolate and analyze the RP$_C$ complexes, and the existence of a more stable, heparin-resistant species at 4 °C. This could be due to the presence of an "extended −10" region on the P15.0 promoter, which is thought to facilitate the recognition of promoter sequences by the $\sigma^{70}$ subunit (29, 30) as well as permitting low temperature isomerization (32). We have not determined whether these heparin-resistant species correspond to intermediate or open complexes, since the previously presented data from analysis at the lac UV5 promoter show conclusively that AsiA activity precedes both complexes.

Our failure to detect specific RNAP-DNA complexes at 4 °C in the presence of AsiA suggests that the AsiA-RNAP interaction inhibits the formation of the initial closed complexes. In contrast, AsiA does not immediately alter the quantity, or change the equilibrium, of preformed species. Hence, it is unlikely that AsiA functions directly at the reverse binding step, to dissociate the specific promoter-bound complexes. Therefore, these data suggest that the target for AsiA activity is the free RNAP.

An attempt to extend this conclusion to the role of AsiA in the stimulation of middle transcription is supported by the in vitro transcription and electrophoretic mobility shift data obtained on PrIIB2. The 4 °C complexes formed at this promoter show the heparin sensitivity typical of the closed complex. Complex formation at 4 °C is significantly stimulated by the presence of AsiA, to an extent similar to that observed at 37 °C, suggesting that AsiA enhances the initial binding of RNAP to T4 middle promoters. Therefore, AsiA may function in the stimulation of middle promoter recognition at the same basic step that is implicated in the inhibition of transcription from E. coli and T4 early promoters. The opposite effects of the AsiA protein could thus be achieved through a common mechanism, involving a modification of DNA binding by $\sigma^{70}$. However, this would not preclude the AsiA-$\sigma^{70}$ interaction from affecting additional steps in the stimulation of T4 middle mode transcription, as there are a number of transcription activators that are thought to enhance initial promoter binding as well as subsequent step(s) in the transcription pathway (35). Moreover, the results from both the electrophoretic mobility shift and transcription assays at 37 °C (Fig. 7, A and B), suggest that the presence of AsiA may also facilitate isomerization and/or open complex formation on PrIIB2.

The question remains: how does AsiA, bound to $\sigma^{70}$, inhibit transcription from one class of promoters, while stimulating the recognition of another? Perhaps, some insight is provided by an analysis of the structure and function of the domains of $\sigma^{70}$ involved in this interaction. Sequence comparison and genetic analysis of numerous $\sigma$ factors have identified four highly conserved regions, which can be further divided into subregions (36, 37). The study of these subregions has implicated specific domains in the binding of $\sigma$ to core (38), and in promoter recognition (22, 39–43). The conserved domain 2.4 of $\sigma^{70}$ interacts with the −10 consensus promoter sequence (39–42), while the putative helix-turn-helix motif of domain 4.2 recognizes the −35 promoter element (22, 39, 43). Recent analysis of $\sigma^{70}$-AsiA binding has demonstrated that a small fragment containing only conserved domain 4 of $\sigma^{70}$ is capable of binding to AsiA (26). Thus, AsiA may interact with this domain in a manner that interferes with, or substitutes for, these promoter contacts. This would prevent the initial RNAP binding to promoters requiring recognition of the −35 region (such as T4 early and host promoters), while redirecting the AsiA-containing holoenzyme to the recognition of MotA-dependent T4 middle promoters. Alternatively, AsiA could bind $\sigma^{70}$ within the putative “contact site II” (30), which extends from conserved region 3.2 to the upstream end of 4.2. Deletion analyses have shown that this region encompasses the sites of interaction for several E. coli class II activators (30), which, like the T4 MotA protein, bind to promoter DNA at positions overlapping the −35 region. Conceivably, AsiA binding within contact site II could cause $\sigma^{70}$ to adopt a conformation unable to form an initial closed complex on host or T4 early promoters, yet with stimulated ability to interact with MotA-dependent T4 middle promoters.

Although the precise details of AsiA function are not yet known, the general mechanism for AsiA activity is likely to include the modification of initial interactions between $\sigma^{70}$ and
the promoter DNA, reducing initial binding affinity for the canonical E. coli or T4 early promoters, and stimulating recognition of T4 middle promoters bearing the transcription activator MotA. In this way, the presence or absence of the MotA protein on the promoter DNA seems to be the major determinant of AsiA activity. MotA, bound in the −30 region of the promoter, may serve to stimulate AsiA-holoenzyme binding, by interacting directly with the AsiA protein, the σ70 subunit, or other regions of the RNA polymerase. One possibility is that the two T4-encoded proteins interact directly, with AsiA serving as a co-activator of MotA-dependent transcription in the conventional eukaryotic sense, while hindering recognition of promoters lacking MotA. Alternatively, binding to AsiA may induce changes in σ70 that block the recognition of the consensus sequence in the −35 region, but stimulate σ70 interaction with MotA and/or the middle promoter DNA.

The AsiA protein, acting in concert with the RNAP and MotA, could therefore function as a molecular switch, that modulates transcription at its initial step, to efficiently regulate gene expression during T4 development.

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