The *Escherichia coli* RNA Polymerase-Anti-σ70 AsiA Complex Utilizes α-Carboxyl-terminal Domain Upstream Promoter Contacts to Transcribe from a −10/−35 Promoter*

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During infection of *Escherichia coli*, the phage T4 early protein AsiA inhibits open complex formation by the RNA polymerase holoenzyme Eσ70 at −10/−35 bacterial promoters through binding to region 4.2 of the σ70 subunit. We used the −10/−35 lacUV5 promoter to study the properties of the Eσ70-AsiA complex in the presence of the glutamate anion. Under these experimental conditions, inhibition by AsiA was significantly decreased. KMnO₄ probing showed that the observed residual transcriptional activity was due to the slow transformation of the ternary complex Eσ70-AsiA-lacUV5 into an open complex. In agreement with this observation, affinity of the enzyme for the promoter was 10-fold lower in the ternary complex than in the binary complex Eσ70-lacUV5. A tau plot analysis of abortive transcription reactions showed that AsiA binding to Eσ70 resulted in a 120-fold decrease in the second-order on-rate constant of the reaction of Eσ70 with lacUV5 and a 55-fold decrease in the rate constant of the isomerization step leading to the open complex. This ternary complex still responded to activation by the cAMP-catabolite activator protein complex. We show that compensatory Eσ70/promoter upstream contacts involving the C-terminal domains of σ subunits in Eσ70 become essential for the binding of Eσ70-AsiA to the lacUV5 promoter.

In *Escherichia coli* RNA polymerase, the core enzyme E (subunit composition αββ′ω) associates with the σ subunit to form the holoenzyme Eσ, the species able to initiate transcription at specific promoter sites on bacterial or phage genomes. The nature and properties of the σ subunit present in a holoenzyme at a given promoter, together with the ability of the α-carboxyl-terminal domain (α-CTD)³ to recognize an upstream element of the promoter, determine whether and how often transcription will start at this site (1). These principles are nicely illustrated by the regulation of transcription during the development of phage T4 in *E. coli*. Here, all the phage genes are transcribed by the host RNA polymerase, the structure and molecular properties of which are modified by phage-coded proteins, resulting in the sequential utilization of early, middle, and late promoters (2, 3). Immediately after infection, T4 early promoters, with their bacterium-like −10 and −35 recognition sequences, are transcribed by Eσ70, the host holoenzyme harboring σ70, the major host σ factor. T4 middle promoters contain a −10 element closely matching the −10 consensus sequence for σ70, but the −35 element is replaced by a so-called MotA box, a −30 binding site for the phage-coded middle transcriptional activator MotA (4, 5). In addition to Eσ70 and MotA, middle mode transcription also requires the presence of another phage early protein, the anti-σ factor AsiA (6). Upon binding the MotA box, MotA protein activates recognition of middle promoters by a holoenzyme Eσ70-AsiA complex (7, 8). Here, AsiA appears to act as a molecular device that switches σ70 from the early to the middle class of T4 promoters. Transcription at late promoters is closely coupled with T4 DNA replication and requires a novel T4-encoded σ subunit, gp55 (9).

Because it is a coactivator of transcription from T4 middle promoters and, simultaneously, a transcription inhibitor of bacterial or T4 early promoters (3), AsiA might also be able to cause the rapid arrest of transcription from early promoters, concomitant with the start of MotA-dependent middle transcription. Assigning this function is a long-standing and unresolved question in phage T4 biology (10). It has been recently shown, however, that this transcription shutoff also occurs in the absence of AsiA (11), although the same study confirmed that transcription of *E. coli* genes is rapidly and strongly inhibited in *vivo* when AsiA is overproduced.

*In vitro* transcription studies have helped to outline the mechanism of inhibition by AsiA. This 10-kDa protein binds to region 4.2 of σ70. In the Eσ70-AsiA complex, this binding blocks the normal interaction between σ70 and the −35 upstream promoter element (12–14). Although AsiA strongly inhibits open promoter complex formation and transcription by Eσ70 from a −10/−35 promoter like lacUV5 or the T4 early promoter P15.0, the holoenzyme is resistant to AsiA inhibition at promoters that, like *galP1*, lack a −35 consensus motif and contain an “extended −10” motif (12, 13, 15, 16). These observations led to a simple model in which the interactions of domain 4.2 in σ70 with the −35 promoter element or with AsiA are mutually exclusive. This model explains the effect of AsiA on Eσ70 at a −10/−35 promoter under classical assay conditions (13). However, at lacUV5, these experimental conditions preclude a detailed analysis of the repression mechanism. In this study, we have looked for conditions allowing the maintenance of residual transcriptional activity. For this purpose, we selected a salt that enhances the interactions between the RNA polymerase partners. Replacing the chloride anion by glutamate increases the affinity of RNA polymerase for its promoters (17, 18). Such a change is also likely to improve the interaction between protein partners (19). We expected, and indeed observed, that in the presence of the Glu anion, Eσ70 could still form a

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† The abbreviations used are: α-CTD, α-carboxyl-terminal domain; bp, base pair; CAP, catabolite activator protein; KGlu, potassium glutamate.
kinetically complex in the presence of AsiA. This allowed us to quantify free energy changes occurring in the overall reaction and to identify specific compensatory interactions between the holoenzyme and the promoter that permit RNA polymerase to overcome the otherwise strong inhibition brought about by AsiA.

**EXPERIMENTAL PROCEDURES**

**Materials—**High Pure spin columns and the *Pseu* polymerase were from Roche Molecular Biochemicals (Mannheim, Germany). *T. pyrojulicioide kinase* was purchased from New England Biolabs. Anti-AsiA antibodies were from Worthington. Magnetic DynaBeads were from Dynal, Inc., and the 8–25% polyacrylamide PhastGels were from Amersham Pharmacia Biotech.

**Plasmids and DNA Fragments—**A 207-bp lacUV5 DNA fragment (20) was inserted at the EcoRI site of plasmid pCD10 (21) upstream of the *rnnB* T1 and T2 terminators to yield plasmid pGO1. A 665-bp lacUV5 fragment comprising the two terminators was isolated by polymerase chain reaction with template pGO1 and the following primers: 5′-CGCGGACTTCCCGACCGGTTTCCCAGTCACGAC and 5′-GGATTTGTCCTACTCACGGAG. A 220-bp lacUV5 fragment was isolated by polymerase chain reaction using primer pBR-lacUV5 as a template (20) together with primers A1 (5′-GGCTGATACGCCGCTTTCG) and B1 (5′-GCTGAGCCGCTTTCG). Primer A1 was end-labeled with T4 polynucleotide kinase and α-32P-ATP (3000 Ci/mmol) or used with unlabeled primer B1 in a polymerase chain reaction to prepare the 220-bp lacUV5 fragment labeled at the 5′-end on the non-template strand. This fragment was used in the gel shift assay and the DNase I footprinting experiments. Primer A1 biotinylated at the 5′-end was used in a polymerase chain reaction to obtain the biotinylated 220-bp lacUV5 fragment.

**Purified Proteins and Standard Reaction Conditions—** *E. coli* RNA polymerase holoenzyme was purified according to Burgess and Jendrisak (22) as modified by Marschall et al. (21). AsiA protein from phage T4 was purified as previously described (16). Core RNA polymerase was prepared according to Lederer et al. (23), and α-32P-UTP was obtained using the overproducing strain M5219/pMRG8 and the published purification procedure (24). Catabolite activator protein (CAP) was prepared as described by Ghoshuni et al. (25), and the Δσ25 RNA polymerase was a gift from Dr. Evelyne Richet. The experiments described below were performed in buffer A containing 40 mM Hepes (pH 8.0), 10 mM MgCl2, 500 μg/ml bovine serum albumin, 1 mM dithiothreitol, and either 100 mM KCl or the indicated KGlu concentration.

**Formation of the Ternary RNA Polymerase—**The biotinylated lacUV5 DNA fragment was prepared as described above. After purification, the biotinylated fragment was immobilized on Dynal streptavidin magnetic beads (12). RNA polymerase holoenzyme (40 pmol) and an 8-fold molar excess of AsiA were incubated in a 30-μl reaction for 30 min at 37 °C in buffer A containing 100 μM KGlu and 0.125% (v/v) Tween instead of bovine serum albumin. The binding reaction was then added to the immobilized lacUV5 fragment, and the mixture was incubated for 60 min at room temperature. The beads were collected by centrifugation, and the supernatant containing the unbound proteins was withdrawn (see Fig. 2, lane 3). The beads were then washed twice with KGlu buffer, and the bound proteins were eluted after a 1-h incubation at room temperature in 1% SDS (see Fig. 2, lane 3). The samples and controls were analyzed by electrophoresis under denaturing conditions on an 8–25% (w/v) polyacrylamide gradient PhastGel.

**Single Round Transcription Assays—**Prior to assembly for transcription reactions, RNA polymerase, AsiA, and the lacUV5 template were separately diluted in ice in buffer A containing either 100 mM KCl or the indicated KGlu concentration (100–400 mM). Runoff transcription reactions were carried out at 37 °C under the following conditions: 30 nM RNA polymerase holoenzyme, 2 nM lacUV5 template, the indicated molar excess of AsiA over the polymerase concentration, 100 μM ATP, 100 μM CTP, 100 μM GTP, 10 μM UTP, 0.5 μCi of α-32P-UTP, and 250 μg/ml heparin. RNA polymerase and AsiA were first mixed at 37 °C for 15 min. Template was added to allow open complex formation for 5 min, followed by addition of the nucleotides and heparin. Elongation reactions lasted 10 min and were stopped by mixing equal volumes of samples and 20 μM EDTA in xylene cyanol-containing 0.9% formamide. Following heating at 65 °C, the samples were electrophoresed on a 7% polyacrylamide sequencing gel, and the transcripts were quantified with a PhosphorImage.

**Abortive Transcription—**Abortive transcription reactions (26, 27) were carried out at 37 °C in buffer A containing the indicated KGlu concentrations and the final concentrations of the following components: 500 μM ApA, 50 μM UTP, 2 nM lacUV5 DNA fragment, and 0.5 μCi of α-32P-UTP. The lag assay in Fig. 5A was performed with 30 nM RNA polymerase (final concentration), previously incubated for 15 min either with buffer or with an 8-fold molar excess of AsiA. The experiments with reconstituted RNA polymerases were performed using a 30-min lag assay at a 100 μM final concentration of the different enzymes. Tau plot analysis (26, 28) was carried out to measure the average time τ, required for open complex formation at lacUV5 with and without AsiA previously bound to the holoenzyme. For each reaction, the amount of UTP incorporated was plotted versus time. The two parameters τ, (minutes) and final steady-state velocity V, (mole of UTP incorporated) were determined using a Kaleidograpic program that performed a least-squares fit of the data to the following equation: Y = V t − τ,exp(−t τ,exp(−t)), where Y is the amount of product, and t is time in minutes (28).

**Gel Retardation Assays—**Stock solutions of AsiA and RNA polymerase were prepared on ice in buffer A with 100 mM KCl or KGlu. When present, AsiA was in 6-fold molar excess relative to the highest polymerase concentration used. Incubations were at 37 °C. RNA polymerase-AsiA complexes were first formed during 15 min, and the enzymes were then incubated for 80–90 min with the radioactively labeled lacUV5 fragment at 0.02–0.065 mM. After addition of heparin (55 μg/ml), the samples were loaded onto 5% native polyacrylamide gels prepared in Tris borate/EDTA buffer and electrophoresed at 120 V at room temperature.

**DNase I Footprinting—**Complexes with the labeled lacUV5 promoter (at a 3 nM final concentration) were formed during 50 min at 37 °C in buffer A containing 200 mM KGlu or 100 mM KGlu, using a 6-fold molar excess of AsiA or the CAP-CAP complex as indicated in Fig. 7, with purified RNA polymerase or reconstituted Δσ25 RNA polymerase (each at a 100 nM final concentration). Complexes were then treated with DNase I (at a 80 μg/ml final concentration) for 30 s or 15 s in the absence of RNA polymerase. Protected bands were identified on the gels using the migration of the same fragment treated for the G + A sequencing reaction (29).

**RESULTS**

**Single Round Transcription Analysis in KGlu Buffers—**Transcription inhibition by AsiA of *E. coli* RNA polymerase holoenzyme, has been conveniently analyzed with well defined DNA templates (13, 15, 16). Here, we performed single round transcriptions with a DNA linear fragment containing the lacUV5 promoter upstream of a strong transcriptional terminator and generating a 178-nucleotide transcript. This construct was used to assess the effect of increasing AsiA concentrations (relative to holoenzyme) on runoff transcription reactions when the assays were performed in KGlu buffers of increasing ionic strength as compared with KCl, after a 30-min incubation time with the promoter in each case. In the absence of AsiA, the effect of the KGlu buffers (100–400 mM KGlu) was a 25–30% decrease in transcriptional activity relative to that measured in 100 mM KCl, an effect that was ionic strength-independent. Fig. 1 shows that the nature and concentration of the buffer both have a marked effect on the extent of transcription inhibition by AsiA. Strong inhibition was observed in 100 mM KCl and more so in 400 mM KGlu. In contrast, at all other KGlu concentrations (100–300 mM), transcription was significantly less inhibited, and the corresponding residual activities reached a 75–80% plateau as the AsiA concentration was raised. We therefore utilized the 100–300 mM KGlu concentration range to investigate in greater detail the behavior of the enzyme-inhibitor complex, the *Eo*70/AsiA entity, relative to the lacUV5 promoter.

**Existence of a Ternary Complex Formed at the lacUV5 Promoter by *Eo*70/AsiA—**The results described above prompted us to check whether AsiA was present in a stable ternary complex formed with *Eo*70 and the promoter in the presence of 100–300 mM KGlu. For this experiment, we used a biotinylated lacUV5 DNA fragment immobilized on streptavidin-agarose magnetic beads. The proteins found to be bound to this DNA fragment were recovered and analyzed on an SDS-polyacrylamide gel (12). AsiA (in an 8-fold molar excess over enzyme) was first
added to the holoenzyme in 100 mM KGlu (Fig. 2, lane 1), and the mixture was incubated with the lacUV5 fragment to allow open complex formation. Following this treatment, the holoenzyme was eluted from the beads (Fig. 2, lane 3). Based on the relative Coomassie Blue staining intensities, this eluted fraction was found to contain AsiA in a stoichiometric ratio relative to the ω subunit present in this holoenzyme preparation (Fig. 2, lane 5). As a control, the same experiment was performed in the presence of 100 mM KCl. In this case, in contrast to the result observed in KGlu, previous incubation of E70 with an 8-fold molar excess of AsiA led to the elution from the immobilized promoter of an extremely low quantity of E70, containing no detectable amount of AsiA (data not shown). Furthermore, in DNase footprinting experiments performed in the presence of 100 mM KCl with E70-AsiA on the lacUV5 fragment, the only species that could be detected was a residual binary complex, E70-lacUV5 (see Fig. 7B below). Therefore, in the presence of KGl, AsiA appeared to take part in a stable ternary complex at the lacUV5 promoter.

**KGl Increases the Affinity of the Holoenzyme:**

**AsiA Complex for the lacUV5 Promoter**—Given the possibility of forming in KGl buffers a ternary complex between RNA polymerase, AsiA, and lacUV5, we used gel shift assays to determine the effect of AsiA on holoenzyme affinity for the lacUV5 promoter. We measured the formation of heparin-resistant complexes in KGl and KCl buffers in the absence and presence of AsiA. Fig. 3 shows the gel retardation assay of complexes formed in 100 mM KGl, and Fig. 3B shows an analogous experiment performed in the presence of 100 mM KCl. In either buffer, a clear retarded complex was observed at all holoenzyme concentrations. After quantification by phosphorimaging, the gel shift data were fitted to the equation of a rectangular hyperbola using a nonlinear regression program. The results can be summarized as follows. In KGl buffer, even in the presence of AsiA, a fractional saturation value close to 1 was observed at high holoenzyme concentrations. In 100 mM KGl, an apparent dissociation constant (Kd) of 0.35 ± 0.07 nM was found for E70/promoter binding. This value was >10-fold higher when AsiA was previously bound to the holoenzyme (Kd = 4.7 ± 1.6 nM).

**KMnO4 Reactivity of the E70-AsiA/lacUV5 Complex in Potassium Glutamate**—Potassium permanganate has been used to probe exposed pyrimidines in single-stranded DNA (30). KMnO4 footprinting experiments were performed to confirm that the complex visualized in the gel retardation experiments in the presence of AsiA was an open complex. We first compared the KMnO4 reactivities of the complexes formed in KGl with and without AsiA. KMnO4 sensitivity was observed in the binary complex E70-lacUV5 at positions +2, +1, −2, −9, and −11 on the template strand of lacUV5 (data not shown), characteristic of the open complex formed by E70 at this promoter (30). When AsiA was previously bound to E70, the same positions were found to react with KMnO4, although more slowly. The time course of establishment of this process was monitored. At zero time, E70-AsiA was incubated in 100 mM KGl with the labeled promoter fragment (50 nM E70, 300 nM AsiA, and 2 nM DNA), and the change in KMnO4 reactivity was measured as a function of time. The results were quantified by comparison with the footprinting signal obtained with E70 after allowing 60 min for maximal open complex formation. The kinetics observed with the ternary complex fit a single exponential (time constant η close to 26 min), and the value measured at 60 min was 85% of the control (Fig. 4). This kinetic profile clearly differs from the fast opening of the promoter in the binary complex (Fig. 4, see the control at 10 min). In the presence of KGl, the species E70-AsiA is therefore able to form an open complex at the lacUV5 promoter, with a time

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**Fig. 1.** Effect of reaction conditions on AsiA inhibition of single round transcription by holoenzyme E70 from lacUV5. Runoff transcription reactions were carried out as described under “Experimental Procedures.” The graph shows the percent activity relative to that of controls in the absence of AsiA. The reactions were run with 30 nM E70 and 2 nM lacUV5 DNA fragment in buffer A containing the following salts: 100 mM KCl (○) and 100 mM (●), 200 mM (▲), 300 mM (□), and 400 mM (+) KGlu.

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**Fig. 2.** SDS-polyacrylamide gel analysis of the ternary E70-AsiA/lacUV5 complex. E70 and AsiA (8-fold molar excess) were incubated in buffer A containing 100 mM KGl with the biotinylated lacUV5 fragment on magnetic beads as described under “Experimental Procedures.” The graph shows the percent activity relative to E70/promoter binding. This value was >10-fold higher when AsiA was previously bound to the holoenzyme (Kd = 4.7 ± 1.6 nM). In 100 mM KCl and in the absence of AsiA, the binary complex displayed a Kd of 1 ± 0.1 nM, indicative of weaker binding of the enzyme to the promoter. In the same buffer and in the presence of 600 nM AsiA, the formation of a retarded species was greatly affected as the enzyme concentration increased. An apparent Kd of 100 ± 10 nM was calculated for the enzyme from the gel shift assay. Taken together, these results indicate a qualitative but clear-cut trend: shifting from glutamate to chloride destabilizes the binary complex by a factor of 2.8 in the absence of AsiA. In its presence, the RNA polymerase affinity for the promoter is even more affected because the apparent affinity drops by a factor of 22–25 under the conditions tested. These figures illustrate the destabilizing character of the Cl− buffers often used in vitro to study systems involving DNA/protein interactions.
course that is nevertheless significantly slower than for the uninhibited holoenzyme.

Kinetic Analysis of Open Complex Formation by the Holoenzyme AsIA Species—The process studied above by KMnO4 reactivity could also be monitored using an abortive initiation assay that probes only the kinetically competent species. By this method, we analyzed the kinetics of binding of the holoenzyme to the promoter without and with AsIA previously bound to the enzyme. When the reaction was initiated by addition of Eσ70 AsIA instead of Eσ70 alone (40 nM in each case), we systematically observed an important increase in the latency time required to reach the steady-state rate of oligonucleotide synthesis. This increase was monitored at several KGlu concentrations (Fig. 5A). No lag could be easily measured with the holoenzyme alone. When AsIA was previously bound to the holoenzyme, a marked and roughly constant latency time was observed in the presence of 100–300 mM KGlu (Fig. 5A). We chose then 200 mM as a convenient KGlu concentration and performed a tau plot analysis to determine the [Eσ70] dependence of the observed lag time $\tau_{\text{obs}}$ without and with an 8-fold excess of AsIA. At excess RNA polymerase over promoter concentration, the tau plot analysis is based upon a simple two-step model (Equation 1) for open complex formation (31, 32),

$$R + P \xrightarrow{K_B} R_P \xrightarrow{k_f} R_{P0}$$

where $R$ is Eσ70, $K_B$ is the equilibrium binding constant of $R$ to the promoter $P$, and $k_f$ is the isomerization first-order rate constant (31). Utilizing Equation 1 in the present case is thus an attempt to analyze the effect of binding AsIA to $R$ in terms of this two-step model. Fig. 5B shows the considerable effect induced by AsIA upon the holoenzyme concentration dependence of the lag time $\tau$, and Table I reports the kinetic parameters derived from these measurements. A strong kinetic penalty is brought about by the addition of AsIA. The overall second-order association constant $K_{Bf}$ is decreased by $\approx 120$-fold. In terms of the two-step model of Equation 1, this effect is mainly expressed at the isomerization step of the pathway. Relative to the situation encountered with the free enzyme $R$,
there is a 55-fold decrease in $k_f$ in the presence of excess AsiA. Despite a very large uncertainty in the values of $K_R$ (a ratio of 2 is observed between the average $K_R$ constants), we can safely conclude that, under these experimental conditions, AsiA is not a competitive inhibitor of RNA polymerase binding to the lacUV5 promoter.

Transcription by $E_\sigma^{70}$ from the galP1 promoter or from a promoter sequence lacking a $\zeta_{\text{35}}$ hexamer has been shown to be essentially insensitive to inhibition by AsiA (12, 13, 15). In agreement with this, we did not observe the marked lags described above with lacUV5 when we used a consensus galP1 promoter that lacks a $\zeta_{\text{35}}$ consensus sequence (data not shown). Thus, the increased lags at lacUV5 specifically reflect an imperfect interaction between $E_\sigma^{70}$-AsiA and this promoter with its functional $\zeta_{\text{35}}$ hexamer. We therefore looked for indications of modified interactions between the polymerase and the upstream regions of the promoter that could account for the observed transcriptional activity of $E_\sigma^{70}$-AsiA at this $\zeta_{\text{10}}$/35 promoter.

**The Ternary Complex $E_\sigma^{70}$-AsiA-lacUV5 Is Susceptible to Activation by CAP—At the lacUV5 promoter, the cAMP-CAP complex is known to bind a site centered at $\zeta_{\text{161.5}}$ and to activate open complex formation (33). We have analyzed the effect of the cAMP-CAP complex on the kinetics of open complex formation by $E_\sigma^{70}$-AsiA. Fig. 6 shows that when cAMP-CAP was incubated with the promoter before addition of 30 nM $E_\sigma^{70}$ and with an 8-fold molar excess of AsiA, the observed lag time was reduced from 52 to 18 min; and interestingly, the two kinetics reached the same final steady-state rate after the lag period. The cAMP-CAP complex is therefore able to activate the holoenzyme entity $E_\sigma^{70}$-AsiA. We suspected that this effect could be mediated through the formation of a quaternary $E_\sigma^{70}$-AsiA-lacUV5-CAP complex (see below).

**Role of Upstream Contacts in the Properties of the Ternary Complex—**We used reconstituted holoenzymes to compare the behavior of normal $E_\sigma^{70}$ and of $E_\sigma^{70}(\Delta \alpha)$, a holoenzyme containing $\alpha$ subunits with deletions in their CTDs (34, 35). Using the abortive initiation assay, we first measured, in the presence of 180 mM KGlu, the extent of inhibition caused by AsiA (6-fold molar excess) for three different holoenzymes, each at a 100 nM final concentration: native $E_\sigma^{70}$, reconstituted $E_\sigma^{70}$, and reconstituted $E_\sigma^{70}(\Delta \alpha)$. Each holoenzyme was incubated for 30 min with lacUV5 prior to the assay. The native holoenzyme was 30% inhibited. Reconstituted $E_\sigma^{70}$ was 60% inhibited, whereas reconstituted $E_\sigma^{70}(\Delta \alpha)$ was almost totally inhibited (97%). An $\alpha$-CTD deletion was therefore able to almost totally suppress the partial insensitivity to AsiA conferred to $E_\sigma^{70}$ by the interactions enhanced by the presence of glutamate. Thus, promoter upstream contacts involving the $\alpha$-CTD appear to be essential to counteract the inhibitory effect of AsiA bound to $\sigma^{70}$. Upstream contacts between the promoter and the C-terminal domain of an $\alpha$ subunit of $E_\sigma^{70}$ are known to be further stabilized by the cAMP-CAP complex bound upstream (36).

To test this possibility, we used the reconstituted holoen-
enzymes $E_{sigma}^{70}$ and $E_{sigma}^{70}(Deltaalpha)$ in DNase I footprinting experiments to probe the structure of these complexes with and without AsiA. The ternary complex formed by $E_{sigma}^{70}$-AsiA showed an extended protection pattern (Fig. 7A, lane 3) with, however, notable differences compared with that produced by $E_{sigma}^{70}$ (lane 4). The most visible difference was the strong hypersensitive bands around position −35 in the $E_{sigma}^{70}$-AsiA pattern. This footprint also showed an increased protection around positions −57 to −62 relative to that of $E_{sigma}^{70}$ (Fig. 7A, compare lanes 3 and 4). Under similar conditions and in the presence of 100 mM KCl (Fig. 7B), the RNA polymerase footprint on the DNA fragment was barely detectable, and we were unable to observe the presence of the hyperreactive band that we considered as the signature of the ternary open complex (Fig. 7B, lane 5).

In KGlu, we looked further for the presence of AsiA in the footprint when the cAMP-CAP complex was used to activate the system. As in the pattern afforded by the ternary complex, bands at position −35 were strongly visible in presence of $E_{sigma}^{70}$-AsiA lacUV5 and CAP, strengthening the hypothesis that a quaternary complex forms in the presence of cAMP-CAP (Fig. 7A, lane 11). Also, addition of AsiA to a preformed $E_{sigma}^{70}$-lacUV5 complex did not yield the pattern characteristically perturbed by AsiA (Fig. 7A, lanes 5 and 13), confirming that the inhibitor cannot bind to $sigma^{70}$ once the holoenzyme has already formed an open complex (13). Finally, when it was preincubated with AsiA, $E_{sigma}^{70}(Deltaalpha)$ failed to form any stable complex, and the lacUV5 fragment (5′-labeled on the non-template strand) yielded a pattern similar to that obtained with naked DNA (Fig. 7A, compare lanes 2 and 6). These results confirmed the conclusion that, in the presence of the glutamate anion, AsiA is part of a stable and functional open complex formed by $E_{sigma}^{70}$ at the lacUV5 promoter. Moreover, this particular open complex is susceptible to activation by cAMP-CAP, as demonstrated by the DNase I protection pattern showing evidence for the presence of the quaternary complex $E_{sigma}^{70}$-AsiA lacUV5-CAP.

### DISCUSSION

In this study, we describe the behavior of the $E_{sigma}^{70}$-AsiA complex in the presence of KCl and KGlu, two salts widely used in transcription studies. We have studied the mechanism of inhibition exerted by AsiA when it is bound to $E_{sigma}^{70}$ acting upon a −10/−35 promoter in greater detail (13, 15, 16). By run-off transcription analysis on the lacUV5 promoter, we show that the species $E_{sigma}^{70}$-AsiA is much less inhibited in KGl than in KCl (Fig. 1). These observations suggested the existence of a transcriptionally active ternary complex at lacUV5, a typical bacterial promoter with −10 and −35 recognition sequences. Active ternary complexes containing AsiA have been previously observed at several promoters that do not require a −35 motif for open complex formation (12, 13, 15). A number of convergent observations support the hypothesis of a ternary complex at a −10/−35 promoter. First, we showed that AsiA was present in a stoichiometric amount when the $E_{sigma}^{70}$-AsiA entity was first incubated with lacUV5 in KGlu (Fig. 2), but not in KCl. Thus, we ruled out the possibility that, in the presence of KGl, a spurious loss of AsiA could explain the restoration of $E_{sigma}^{70}$ activity. Furthermore, in Glu− buffer, only the extended DNase I footprint formed by $E_{sigma}^{70}$-AsiA at lacUV5 possessed a distinctive AsiA “signature” in the −35 region (Fig. 7A, lanes 3 and 11). In contrast, with AsiA bound to the holoenzyme in KCl, we observed only a weak protection against DNase I, which we interpreted as due to residual amounts of the binary $E_{sigma}^{70}$/lacUV5 complex (Fig. 7B, lane 5). A third piece of evidence comes from an investigation of the structure of the ternary complex by laser UV photoreactivity.2 Laser UV photo-footprinting of a RNA polymerase-promoter complex allows precise probing of changes in the local structure of DNA (37). When we formed an open complex at lacUV5 with $E_{sigma}^{70}$ in 200 mM KGlu, thymine dimer formation at positions −34 and −33 was suppressed, probably due to contacts between $sigma^{70}$ and the −35 region (37). In contrast, no such effect was found when AsiA was previously bound to $E_{sigma}^{70}$. However, in this case, we observed signals in the −10 region, indicative of contacts that are normally present in open complexes (38). This again strongly suggests that even when access to open complex formation via the −35 region is prevented by AsiA, RNA polymerase can nevertheless form a transcriptionally competent species possessing the downstream structural characteristics of a normal open complex. The presence of a significant fraction of binary complex would have resulted in a corresponding decrease in the −34 dimer signal. This was not observed. We conclude that the effects we observed in the presence of glutamate belong to a real and probably unique ternary complex referred to as $E_{sigma}^{70}$-AsiA lacUV5.

AsiA inhibits open complex formation by binding directly to the conserved region 4.2 of the $sigma^{70}$ subunit. At a −10/−35 promoter, this binding interferes with the interactions between $sigma^{70}$ region 4.2 and the −35 motif (12–14). Hindrance of these

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2 G. Orsini and M. Buckle, unpublished results.
crucial interactions with concurrent inhibition of open complex formation has been recently described in the case of the gene 2 protein of bacteriophage T7 (39). Here, however, the inhibitor gp2 binds to the β9 subunit of Es70 and thus indirectly disrupts the 235/Es70 region 4.2 interaction (39). Both inhibitors T4 AsiA and T7 gp2 are believed to operate in a mutually exclusive mode: either the inhibitor is bound to the enzyme with ensuing inhibition, or the polymerase is bound to the promoter without inhibition. Moreover, in the latter case, the inhibitor can no longer be bound to the enzyme (13, 39).

By analyzing the mode of action of AsiA in the presence of the glutamate anion, we show that this mutual exclusion can be by-passed. As for many other DNA/protein systems in which Cl− has been replaced by Glu−, our results illustrate the general observation that, in the presence of Glu−, there is a substantial increase in the affinity of the proteins for their binding sites on DNA (17, 40). The apparent Kd values determined by gel shift assay (see above and Fig. 3) are totally in line with previous reports on the “glutamate effect” (40). Using the abortive initiation assay and a simple two-state model (Equation 1) for open complex formation, we showed by tau plot analysis that the rate constant k1 of the isomerization step was decreased 55-fold when AsiA was present in the complex (Table I). Inhibition by AsiA was therefore strongly maintained, as shown by the global 120-fold effect on the second-order association constant KdKf. In a parallel study, we used the abortive initiation assay to assess the stability of the ternary complex by heparin challenge (data not shown). The binary complex (without AsiA) formed in 200 mM KGlu was absolutely stable for >1 day. The ternary complex was much less stable and showed a first-order decay with a half-life of ~18 h. This value is considerably larger than the half-life associated with the conversion of the closed to the open complex. The final ternary open complex formed in KGlu is therefore clearly more stable than the closed intermediate.

The transcriptional activity of the ternary complex strongly implied the existence of a compensatory mechanism. It is well documented that the α subunit of RNA polymerase can also participate in promoter recognition through specific interactions between α-CTD and upstream regions of the −35 hexamer called “UP elements” (41–43). In glutamate, we show here that the contribution to the binding energy brought about by α-CTD binding to lacUV5 is sufficient to partially overcome inhibition by AsiA and to allow the formation of a ternary Es70-AsiA:lacUV5 active complex. Using the reconstituted holoenzyme Es70(Δα), we show that the deletion of the α-CTD totally prevents the formation of the ternary complex. This complex displays RNA polymerase/promoter upstream contacts
as revealed by a characteristic DNase I footprinting pattern that depends on α-CTD binding upstream of position −40 (Fig. 7, lanes 3 and J). A similar differential pattern was previously observed in the absence of AsiA at promoters where the UP elements are essential (34, 43). Also, α-CTD binding can be facilitated by the binding of the CAMP-CAP complex at a site centered at −61.5. Interactions between α-CTD and CAP allow one of the two α-CTDs to anchor more tightly in the minor groove around position −43, upstream of the −35 hexamer (44). Interestingly, this interaction occurs regardless of whether AsiA is bound to the adjacent ρ0 subunit, and it allows CAP to activate the ternary complex Eρ0AsiAlacUV5, thereby forming a quaternary transcriptionally competent complex (Fig. 6).

The compensatory mechanism documented here at lacUV5 has also been observed, almost unmodified, on lacPS, a parent and weaker promoter in which the −10 region is altered, but not the sequences participating in upstream contacts. Conversely, when, at lacUV5, the UP element is reinforced by insertion of a proper canonical sequence, the inhibition due to AsiA was found to be already attenuated in the KCl-containing buffer. It is therefore likely that a better anchoring of the RNA polymerase:AsiA complex can conceivably take place on the UP element at any promoter, but that a clear balance favoring this repositioning might or might not require the presence of potassium glutamate depending on the relative strengths of the interactions at the −35 hexamer and at the UP element.

At this point, we will briefly consider the transcription properties of the Eρ0AsiA complex relative to phage T4 early and MotA-dependent middle promoters. AsiA inhibits complex formation at the T4 early promoter P15.0, possessing both a −35 recognition element and an extended −10 motif (16). When assayed in KCl buffer, transcription from the T4 middle promoters PuvX, PriB1, PriB2, and P1 is strictly dependent on assayed in KCl buffer, transcription from the T4 middle recognition element and an extended 2 region.

2 G. Orsini and A. Kolb, unpublished results.

3 It is therefore likely that a better anchoring of the RNA polymerase:AsiA complex can conceivably take place on the UP element at any promoter, but that a clear balance favoring this repositioning might or might not require the presence of potassium glutamate depending on the relative strengths of the interactions at the −35 hexamer and at the UP element.

To conclude this section, we will briefly consider the transcription properties of the Eρ0AsiA complex relative to phage T4 early and MotA-dependent middle promoters. AsiA inhibits complex formation at the T4 early promoter P15.0, possessing both a −35 recognition element and an extended −10 motif (16). When assayed in KCl buffer, transcription from the T4 middle promoters PuvX, PriB1, PriB2, and P1 is strictly dependent on the presence of both activators: MotA bound to the −30 middle promoter sequence, and AsiA bound to σ70 (8). Remarkably, when assayed in KGl, Eρ0AsiA is able to transcribe correctly from PuvX (5, 7). This basal transcription by Eρ0AsiA is not activated by MotA (45). Furthermore, in KGl, addition of AsiA to Eρ0AsiA inhibits both open complex formation at a PuvX DNA fragment and transcription from this middle promoter (7). Taken together, these observations emphasize the point that AsiA acting alone behaves as a repressor. It inhibits transcription from a MotA-dependent promoter in a manner reminiscent of its action upon a −10/−35 promoter. It is therefore likely that most of the RNA polymerase/promoter contacts at −35 (and at −30 as well) are perturbed by a local conformational change affecting σ70 when it binds AsiA. This perturbation is, in turn, relieved by the new contacts and therefore the new specificity conferred by the addition of MotA.

In this view, this study demonstrates that promoter upstream contacts mediated by α-CTD of Eρ0AsiA are able to reduce the strong structural and kinetic block brought about by AsiA. As a repressor, AsiA changes radically the potential use of the enzyme modules involved in the formation of contacts with the upstream region of the promoter. We propose that in vivo, this drastic change also occurs. Here, however, the α-CTDs are first ADP-ribosylated at the onset of T4 infection (46). This modification plays a major role in regulating promoter utilization since it irreversibly blocks the use of the rescued pathway documented here. The efficiency of the Eρ0AsiA complex will now crucially depend on the binding of MotA to those promoters containing the −30 middle promoter motif (47, 48) and on the establishment of the proper contacts between the σ70 subunit and MotA (49).

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