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The Bacteriophage T4 Inhibitor and Coactivator AsiA Inhibits *Escherichia coli* RNA Polymerase More Rapidly in the Absence of σ^{70} Region 1.1: Evidence that Region 1.1 Stabilizes the Interaction between σ^{70} and Core

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The N-terminal region (region 1.1) of σ^{70}, the primary σ subunit of *Escherichia coli* RNA polymerase, is a negatively charged domain that affects the DNA binding properties of σ^{70} regions 2 and 4. Region 1.1 prevents the interaction of free σ^{70} with DNA and modulates the formation of stable (open) polymerase/promoter complexes at certain promoters. The bacteriophage T4 AsiA protein is an inhibitor of σ^{70}-dependent transcription from promoters that require an interaction between σ^{70} region 4 and the −35 DNA element and is the coactivator of transcription at T4 MotA-dependent promoters. Like AsiA, the T4 activator MotA also interacts with σ^{70} region 4. We have investigated the effect of region 1.1 on AsiA inhibition and MotA/AsiA activation. We show that σ^{70} region 1.1 is not required for MotA/AsiA activation at the T4 middle promoter P_{uvsX}. However, the rate of AsiA inhibition and of MotA/AsiA activation of polymerase is significantly increased when region 1.1 is missing. We also find that RNA polymerase reconstituted with σ^{70} that lacks region 1.1 is less stable than polymerase with full-length σ^{70}. Our previous work has demonstrated that the AsiA-inhibited polymerase is formed when AsiA binds to region 4 of free σ^{70} and then the AsiA/σ^{70} complex binds to core. Our results suggest that in the absence of region 1.1, there is a shift in the dynamic equilibrium between polymerase holoenzyme and free σ^{70} plus core, yielding more free σ^{70} at any given time. Thus, the rate of AsiA inhibition and AsiA/MotA activation increases when RNA polymerase lacks region 1.1 because of the increased availability of free σ^{70}. Previous work has argued both for and against a direct interaction between regions 1.1 and 4. Using an *E. coli* two-hybrid assay, we do not detect an interaction between these regions. This result supports the idea that the ability of region 1.1 to prevent DNA binding by free σ^{70} arises through an indirect effect.

RNA synthesis in *Escherichia coli* is accomplished by an RNA polymerase holoenzyme consisting of a core of five subunits (two α's, β, β′, and ω) and a σ specificity factor (reviewed in references 15 and 30). Core polymerase contains RNA synthesizing activity, but the specific start site for transcription initiation is determined by the DNA binding specificity of the particular σ factor. In *E. coli*, σ^{70} is the primary σ factor that is responsible for initiation of transcription during exponential growth. Sequence and structural analyses of various σ proteins from many prokaryotes indicate that primary σ factors share four main regions of conservation (regions 1 to 4) (15, 23). Sequence recognition capability in σ^{70} is provided by alpha helices within regions 2 and 4, which interact directly with a promoter's −10 and −35 DNA elements, respectively.

Although most σ family members share conservation in regions 2 through 4, the sequence of region 1.1, the N-terminal, highly negatively charged portion, is conserved only among primary σ proteins (15, 23, 30). Within σ^{70}, region 1.1 is composed of the first 100 residues. There is no evidence to suggest that region 1.1 interacts directly with promoter DNA. Nonetheless, its presence has a major impact on σ^{70}-DNA interactions. In free σ^{70}, the presence of region 1.1 prevents both specific and nonspecific binding to DNA (9). Early work suggested region 1.1 prevents this binding by directly interacting with region 4. However, subsequent work using nuclear magnetic resonance (NMR) spectroscopy of segmental, isotopically labeled σ^{A} of *Thermotoga maritima*, a σ^{70} analog, has failed to detect an interaction between regions 1.1 and 4.2 (5).

Besides its function in free σ^{70}, the presence of region 1.1 also modulates the ability of RNA polymerase holoenzyme to form stable, open complexes with certain promoters. Given the particular promoter, the presence of region 1.1 can either inhibit or promote the formation of the stable pretranscription complex (39, 40). Thus, region 1.1 provides important functions both when σ^{70} is free and when it is present within RNA polymerase. However, how region 1.1 works is not yet clear.

The bacteriophage T4 AsiA protein has two functions. It inhibits σ^{70}-dependent transcription from promoters that require an interaction between σ^{70} region 4 and the −35 DNA element and it coactivates transcription from T4 middle promoters that use σ^{70} containing RNA polymerase and the T4 transcription activator MotA (reviewed in reference 18). The MotA protein binds to a DNA motif (a MotA box) centered at

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position −30 of middle promoter DNA, and, in addition, both AsiA and MotA interact with σ^{70} region 4. A recent NMR structure of the AsiA/σ^{70} region 4 complex shows that AsiA contacts multiple σ^{70} residues, including residues that make direct contact with base moieties in the −35 element when RNA polymerase binds to a typical σ^{70}-dependent promoter (22).

Because the presence of region 1.1 modulates the interaction of σ^{70} with host promoter DNA and AsiA interacts with σ^{70} residues that contact the −35 element, we wondered whether σ^{70} region 1.1 was important for MotA/AsiA activation and AsiA inhibition. We show here that region 1.1 is not required for MotA/AsiA activation at a T4 middle promoter. However, RNA polymerase lacking region 1.1 is more rapidly inhibited by AsiA and more rapidly activated by MotA/AsiA. Our previous work indicated that AsiA binds efficiently to free σ^{70} but poorly, if at all, to holoenzyme (19). Our evidence supports the idea that AsiA inhibits more rapidly when region 1.1 is absent, because the absence of region 1.1 destabilizes the interaction of σ^{70} with core, resulting in more available free σ^{70} at any given time.

MATERIALS AND METHODS

Proteins and buffers. MotA and AsiA were purified as described previously (16). The N-terminal His_6-tagged σ^{fl}, which contains the sequence MRGSHHH HHHGSSGLVPRGSELGTRL fused to the start of the σ^{70} protein, and the N-terminal His-tagged $\sigma^{\Delta 1.1}$, which contains the sequence MRGSHHHHHHT DPHASSVP fused to residue 100 of the σ^{70} protein, were purified from the σ^{fl} and $\sigma^{\Delta 100}$ plasmids (40), respectively, as described previously (39). Transcription assays indicated that either σ saturated core at a ratio of ~2:1 (I. Hook-Barnard and D. M. Hinton, unpublished data). Our sequencing of the σ genes within these plasmids has revealed that σ^{fl} also contains the substitution V36L and that both σ^{fl} and $\sigma^{\Delta 1.1}$ contain the substitution N149D. (V36 is missing in the N-terminal deletion in $\sigma^{\Delta 1.1}$.) We have found no detectable difference in activity between σ^{fl} and purified wild-type σ^{70} (13; data not shown). N-terminal His_6-tagged σ mutants with N-terminal deletions of 50 ($\sigma^{\Delta 50}$), 75 ($\sigma^{\Delta 75}$), or 133 ($\sigma^{\Delta 133}$) residues were the generous gifts of A. Dombroski and C. W. Bowers. *E. coli* RNA polymerase core was purchased from Epicenter Technologies.

Incubation buffer I contained 23 mM Tris-Cl, pH 7.9; 37 mM Tris-acetate, pH 7.9; 37 mM NaCl; 22% glycerol; 0.56 mM EDTA; 0.18 mM dithiothreitol (DTT); 0.004% Triton X-100; 139 mM potassium glutamate; 3.7 mM magnesium acetate; 93 μg/ml bovine serum albumin (BSA); and 0.09 mM 2-mercaptoethanol. Incubation buffer II contained 34 mM Tris-Cl, pH 7.9; 26 mM Tris-acetate, pH 7.9; 42 mM NaCl; 34% glycerol; 0.17 mM DTT; 0.71 mM EDTA; 0.006% Triton X-100; 97 mM potassium glutamate; 2.6 mM magnesium acetate; and 65 μg/ml BSA. Incubation buffer III contained 29 mM Tris-Cl, pH 8; 26 mM Tris-acetate, pH 7.9; 38 mM NaCl; 28% glycerol; 0.65 mM EDTA; 0.15 mM DTT; 0.005% Triton X-100; 97 mM potassium glutamate; 2.6 mM magnesium acetate; 65 μg/ml BSA; and 0.06 mM 2-mercaptoethanol. DNA buffer I contained 7.4 mM Tris-Cl, pH 7.9; 51 mM Tris-acetate, pH 7.9; 57 mM NaCl; 0.66 mM EDTA; 0.13 mM DTT; 2.1% glycerol; 190 mM potassium glutamate; 5.1 mM magnesium acetate; 130 μg/ml BSA; 425 μM each of ATP, GTP, and CTP; and 110 μM [α-^{32}P]UTP (~1×10^4 dpm/pmol). DNA buffer II was identical to DNA buffer I except that it lacked ribonucleoside triphosphates (rNTPs). DNA buffer III contained 4.2 mM Tris-Cl, pH 7.9; 63 mM Tris-acetate, pH 7.9; 0.58 mM EDTA; 0.16 mM DTT; 240 mM potassium glutamate; 6.3 mM magnesium acetate; 160 μg/ml BSA; 525 μM each of ATP, GTP, and CTP; and 13 μM [α-^{32}P]UTP (~1×10^5 dpm/pmol). DNA buffer IV contained 5.4 mM Tris-Cl, pH 7.9; 52 mM Tris-acetate, pH 7.9; 16 mM NaCl; 0.6% glycerol; 0.61 mM EDTA; 0.13 mM DTT; 190 mM potassium glutamate; 5.2 mM magnesium acetate; 130 μg/ml BSA; 0.06 mM 2-mercaptoethanol; 525 μM each of ATP, GTP, and CTP; and 13 μM [α-^{32}P]UTP (~1×10^5 dpm/pmol). NTP solution I contained 2 mM each of ATP, CTP, and GTP and 0.5 mM [α-^{32}P]UTP (1×10^4 dpm/pmol). Tris-borate-EDTA (TBE) (1×) contained 2.5 mM EDTA and 89 mM Tris-borate, pH 8.3.

DNA. pDKT90 (25) contains the promoters P_{uvsX} and P_{minor} in the vector pTZ19U (United States Biochemical Co.). $pP_{uvsX/sigma}$ (19), which was derived from pDKT90, contains the canonical σ^{70} −35 region TTGACA located 16 bp upstream of the P_{uvsX} −10 region (TATAAT). pP_{wt}, which was derived from $pP_{uvsX/sigma}$, is identical to $pP_{uvsX/sigma}$ except for an extra T immediately downstream of the −35 element that results in a 17-bp spacer between the −10 and −35 regions. Plasmid DNA was isolated as described previously (17) and then purified by centrifuging to equilibrium in an ethidium bromide-cesium chloride gradient. Linear templates for in vitro transcription reactions, obtained after digestion with restriction enzymes, were purified by phenol extraction followed by ethanol precipitation.

The pBRα-σ^{70} chimera plasmid (11) and its derivative pBRα-σ^{70} (D581G) (21), used for the two-hybrid assays, have been described previously. The α-σ^{70} chimera proteins from these plasmids are composed of the N-terminal domain of α (amino acids 1 to 248) fused in frame to the C-terminal region (amino acids 528 to 613) of wild-type σ^{70} or σ^{70} containing the substitution D581G. The pACλcI32 plasmid, used to construct pcI-$\sigma^{1.1}$, has been described previously (20). The σ^{70} region 1.1 fragment (corresponding to amino acids 1 to 100) cloned into pACλcI32 was obtained as a PCR product of pLNH12 (29), which contains the wild-type σ^{70} gene, by using *Pfu*Turbo DNA polymerase (Stratagene) and primers that contained the necessary NotI or BglII sites to allow ligation with pACλcI32 that had been previously cleaved with NotI and BglII. The PCR product was cloned into pACλcI32 by use of standard techniques. DNA sequence analysis of pcI-$\sigma^{1.1}$, performed by MGW Biotech, confirmed the expected sequence.

In vitro transcription assays. Transcription reactions were assembled as indicated in the figure legends. Upon addition of rNTPs, reactions were incubated at 37°C. Multiple-round transcription reactions were incubated for 7.5 min. Single-round transcription reactions were treated with 150 ng of rifampin 20 s after the addition of rNTPs and then incubated for an additional 7 min. Reactions were collected on dry ice. Gel load solution (1× TBE, 7 M urea, 0.1% bromophenol blue, 0.1% xylene cyanol FF) was added at a volume of five times that of the reaction aliquots, and the solution was heated at 95°C for 2 min before electrophoresis on 4% polyacrylamide-7 M urea denaturing gels run in 0.5× TBE. After autoradiography, films were scanned using a Desktop Plus scanner and quantified using Diversity One software from Protein Databases, Inc., or using a Powerlook 2100XL densitometer and Quantity One software from Bio-Rad, Inc.

Two-hybrid assay. β-Galactosidase assays were performed as described previously (11), using the strain KS1 (11) and the desired pBRα-σ and pcI plasmids, except that in some cases cultures were grown in M9 medium plus Casamino Acids (plus appropriate antibiotics) rather than LB media.

RESULTS

Region 1.1 of σ^{70} is not required for MotA/AsiA-dependent activation of transcription from P_{uvsX}. Previously, portions of region 4 of σ^{70} (amino acids 551 to 563 and 580 to 598) as well as the far C terminus of the protein (amino acids 604 to 613) have been shown to be important for the interaction of σ^{70} with AsiA and MotA, respectively (reviewed in reference 18). To investigate the effect of region 1.1 of σ^{70} on MotA/AsiA activation, we examined transcription from the T4 middle promoter P_{uvsX} by using polymerases containing either the full-length σ^{70} (σ^{fl}) or σ^{70} with various N-terminal deletions. Like other T4 middle promoters, P_{uvsX} has an excellent match to a σ^{70} −10 element, but it has a poor match to the σ^{70} −35 element. Instead, it has a MotA box at position −30. When using polymerase with σ^{fl} ($E\sigma^{fl}$) in multiple-round transcription reactions, we observed a pattern of P_{uvsX} transcription that was similar to that previously documented for wild-type holoenzyme (16). Transcription, which was seen with polymerase alone (Fig. 1, lane 1), was not significantly affected by MotA (Fig. 1, lane 2) but was greatly inhibited by AsiA (Fig. 1, lane 3). The presence of both MotA and AsiA resulted in a higher level of P_{uvsX} transcription (Fig. 1, lane 4).

The amount of P_{uvsX} RNA obtained with polymerase containing a σ^{70} with an N-terminal deletion of 50 ($\sigma^{\Delta 50}$) (Fig. 1, lanes 5 to 8), 75 ($\sigma^{\Delta 75}$) (Fig. 1, lanes 9 to 12), or 99 ($\sigma^{\Delta 1.1}$) (Fig. 1, lanes 13 to 16) residues was less than that observed with $E\sigma^{fl}$. However, all of these polymerases were activated by the

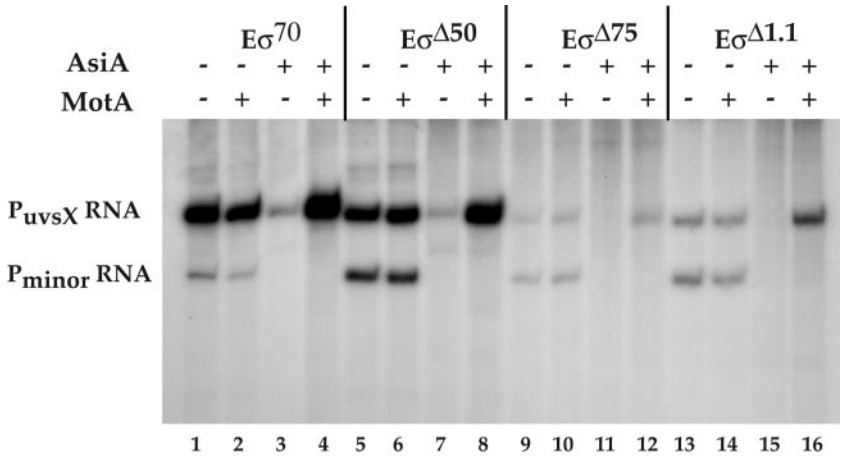

FIG. 1. MotA/AsiA activation of P_{uvsX} does not require region 1.1 of σ^{70}. Polymerase, reconstituted with 0.4 pmol core and 1 pmol of the indicated σ, was preincubated with or without 9 pmol AsiA in 2.15 μl of incubation buffer I for 30°C for 15 min before the addition of 2.35 μl of DNA buffer II containing 0.04 pmol of P_{uvsX} DNA and, as indicated, 1.9 pmol MotA. After an incubation at 37°C for 10 min, transcription was initiated by the addition of 0.5 μl of NTP solution I. The positions of the P_{uvsX} and P_{minor} RNAs on the denaturing polyacrylamide gel are indicated. Quantitation indicated that the relative level of P_{uvsX} RNA [(level of P_{uvsX} RNA with MotA/AsiA)/(level of P_{uvsX} RNA by polymerase alone)] was similar for each σ: 1.6 ($E\sigma^{fl}$), 1.7 ($E\sigma^{\Delta 50}$), 1.9 ($E\sigma^{\Delta 75}$), and 2.0 ($E\sigma^{\Delta 1.1}$).

addition of MotA and AsiA (Fig. 1, lanes 8, 12, and 16). The level of activated P_{uvsX} transcription (observed in the presence of polymerase, MotA, and AsiA) relative to the level observed with polymerase alone was quite similar, ranging from 1.6 to 2.0, and the level of activated P_{uvsX} transcription relative to that observed with polymerase plus AsiA was 10-fold or greater in each case. Thus, the presence of region 1.1 is not required for MotA/AsiA activation of P_{uvsX}. Polymerase containing a deletion of 133 amino acids from the N terminus ($E\sigma^{\Delta 133}$), which removes region 1.2 as well as region 1.1, did not produce P_{uvsX} RNA either in the absence or in the presence of MotA and AsiA (data not shown). This result is consistent with the very poor activity of $E\sigma^{\Delta 133}$ observed with other promoters (40).

Although removal of residues within region 1.1 did not affect the increase in the level of P_{uvsX} RNA with MotA/AsiA relative to that seen with polymerase alone, it did significantly affect transcription from another promoter, P_{minor} (Fig. 1, lanes 1, 5, 9, and 13). Our previous investigations have indicated that the relative level of P_{minor} RNA is greater with $E\sigma^{\Delta 1.1}$ than with $E\sigma^{fl}$ because, in the absence of region 1.1, polymerase forms stable pretranscription complexes at this promoter more rapidly than it does when region 1.1 is present (39).

AsiA inhibits and MotA/AsiA activates more rapidly in the absence of σ^{70} region 1.1. Transcription from σ^{70}-dependent promoters that require an interaction with the −35 region of promoter DNA is significantly inhibited when RNA polymerase is associated with AsiA (7, 31, 33, 36). Previous work has indicated that this association of AsiA with RNA polymerase occurs efficiently by a two-step process: first AsiA binds to σ^{70} and then the AsiA/σ^{70} complex binds to core (19). Thus, AsiA inhibition of holoenzyme is a process that depends on the dynamic equilibrium between holoenzyme and free σ^{70} plus core.

As seen in Fig. 2A, this association of AsiA with polymerase can be monitored by assaying the level of transcription when AsiA is incubated with free σ^{fl} before the addition of core versus the level when AsiA is incubated directly with holoenzyme. In this assay, we used the $P_{uvsX/sigma}$ promoter, which is derived from the T4 middle promoter P_{uvsX} but contains a canonical σ^{70} −35 sequence rather than a MotA box (39). Thus, $P_{uvsX/sigma}$ models an excellent σ^{70}-dependent promoter. Incubation of AsiA with free σ^{fl} at either 4°C or 30°C before the addition of core or incubation of AsiA with $E\sigma^{fl}$ for 15 min at 30°C completely inhibited the subsequent single round of $P_{uvsX/sigma}$ transcription, indicative of the formation of the AsiA-bound polymerase (Fig. 2A, lanes 3, 4, and 8). However, incubation of AsiA with $E\sigma^{fl}$ for 15 min at 4°C did not inhibit transcription (Fig. 2A, lane 7).

As seen with σ^{fl}, incubation of AsiA with $\sigma^{\Delta 1.1}$ before the addition of core also completely inhibited $P_{uvsX/sigma}$ transcription (Fig. 2B, lanes 3 and 4). However, in contrast to the results seen with $E\sigma^{fl}$, incubation of AsiA with $E\sigma^{\Delta 1.1}$ at 4°C reduced the levels of $P_{uvsX/sigma}$ and P_{minor} RNAs to 29% and 11%, respectively, of those seen without AsiA (Fig. 2B, lane 7 versus lane 5). Thus, in the absence of region 1.1, the AsiA-associated polymerase forms much more readily at 4°C.

To quantify the effect of region 1.1 on the formation of AsiA-associated polymerase, we performed time course experiments in which we incubated AsiA with holoenzyme for various times at either 4°C or 30°C (Fig. 3) before a single round of transcription using P_{uvsX} in the absence of MotA. Our results indicated that at 4°C about half of the $E\sigma^{\Delta 1.1}$ was inhibited after an incubation with AsiA for 10 min, whereas there was no detectable inhibition of $E\sigma^{fl}$ at this temperature. At 30°C, $E\sigma^{\Delta 1.1}$ was inhibited about two times more rapidly than was $E\sigma^{fl}$. Thus, in the absence of region 1.1, the rate of AsiA inhibition of polymerase is significantly increased at either 4°C or 30°C. To determine whether the rate of MotA/AsiA activation was also increased in the absence of region 1.1, we repeated the time course experiments using both the T4 middle promoter P_{uvsX} and the σ^{70}-dependent promoter P_{wt} and either $E\sigma^{fl}$ or $E\sigma^{\Delta 1.1}$ (Fig. 4). We found that the absence of

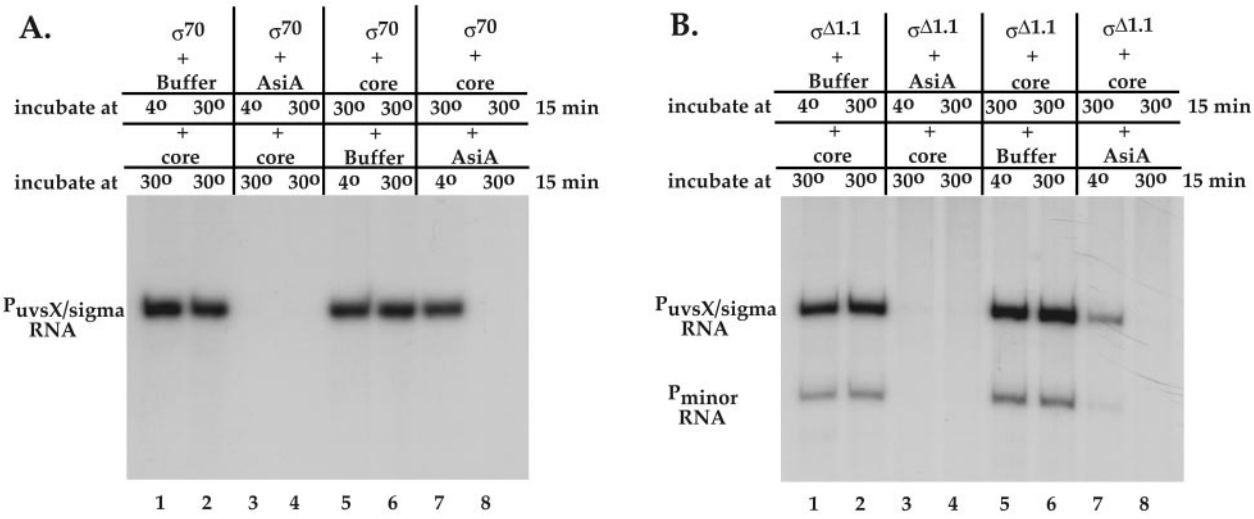

FIG. 2. Preincubation of AsiA with $E\sigma^{\Delta 1.1}$ at 4°C inhibits subsequent transcription. AsiA (9 pmol), core (0.4 pmol), and either 1 pmol σ^{fl} (A) or 1 pmol $\sigma^{\Delta 1.1}$ (B) were preincubated as indicated, resulting in a final volume of 2.15 μl of incubation buffer I. The protein mixtures were added to 0.02 pmol of P_{uvsX} DNA in 2.35 μl of DNA buffer I and incubated at 37°C for 20 s before the addition of 0.5 μl of rifampin (300 μg/ml). The positions of the P_{uvsX} and P_{minor} RNAs on the denaturing polyacrylamide gel are indicated.

region 1.1 increases the rates of inhibition and activation similarly.

Region 1.1 does not interact with σ^{70} region 4 in a two-hybrid assay. We investigated whether σ^{70} regions 1.1 and 4 interact directly by performing *E. coli* two-hybrid assays, which have previously been used to detect interactions between σ^{70} region 4 and other proteins or protein domains (10, 21, 32). We tested both wild-type σ^{70} region 4 and σ^{70} region 4 containing the substitution D581G. This substitution is needed to observe the interaction between σ^{70} region 4 and the β-flap region of core (21). We failed to detect an interaction between σ^{70} region 1.1 and either wild-type σ^{70} region 4 or σ^{70} region 4 containing D581G by using this assay (data not shown).

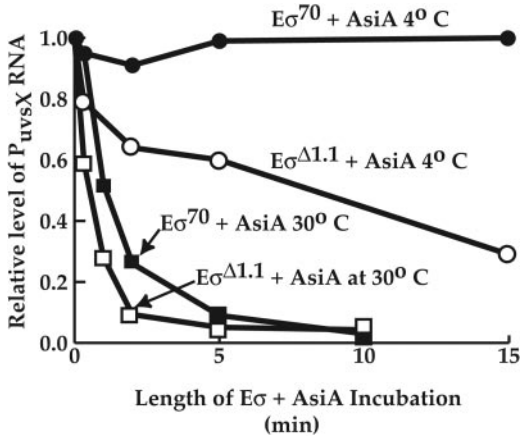

FIG. 3. AsiA inhibition of $E\sigma^{\Delta 1.1}$ is more rapid than its inhibition of $E\sigma^{fl}$ at either 4°C or 30°C. Transcriptions were performed as for Fig. 2A and B, lanes 7 and 8, except that reactions contained 0.5 pmol of the indicated σ and 0.2 pmol of core and AsiA was incubated with $E\sigma^{fl}$ or $E\sigma^{\Delta 1.1}$ at 4°C or 30°C for the indicated time before the start of transcription. The relative level of P_{uvsX} RNA [(amount of RNA observed after AsiA-plus-Eσ incubation for time *t*)/(amount of RNA observed without AsiA)] is shown versus the length of time (in min) of the AsiA-plus-Eσ incubation.

Holoenzyme exchanges $\sigma^{\Delta 1.1}$ for σ^{fl}. Although earlier work found no difference in the binding of σ^{fl} and $\sigma^{\Delta 1.1}$ to core (40), this result was based on an assay that monitored the loss of function by $E\sigma^{\Delta 1.1}$ at the P_{tac} promoter and used a transcription buffer with chloride as the predominant anion. We reinvestigated this question by taking advantage of the fact that the absence of region 1.1 results in different levels of holoenzyme activity at different promoters. Thus, in the following transcription assay, we could simultaneously observe P_{minor} transcription, which is favored by $E\sigma^{\Delta 1.1}$ (39), and P_{uvsX} transcription, which is favored by $E\sigma^{fl}$ (39). We also used a transcription buffer containing the physiologically relevant glutamate as the primary anion. We first reconstituted either $E\sigma^{fl}$ or $E\sigma^{\Delta 1.1}$ by incubation of core with the appropriate σ at 30°C. We then incubated $E\sigma^{fl}$ with a sixfold excess of $\sigma^{\Delta 1.1}$ or incubated $E\sigma^{\Delta 1.1}$ with a sixfold excess of σ^{fl} at 30°C or at 4°C before a single round of transcription using DNA that contained both P_{uvsX} and P_{minor}. As seen in Fig. 5, incubation of $E\sigma^{fl}$ with excess $\sigma^{\Delta 1.1}$ at either 30°C or 4°C did not affect the levels of P_{uvsX} and P_{minor} RNA. However, incubation of $E\sigma^{\Delta 1.1}$ with σ^{fl} at either temperature resulted in a rapid (at 30°C) or slower (at 4°C) change in the relative level of P_{minor} versus P_{uvsX} RNA, consistent with the conversion of the $E\sigma^{\Delta 1.1}$ to $E\sigma^{fl}$. These results suggest that holoenzyme which lacks region 1.1 is less stable than holoenzyme that contains region 1.1.

DISCUSSION

σ region 1.1 is the N-terminal, negatively charged domain that is common to all primary sigma factors. Previous work has indicated that for *E. coli* σ^{70}, region 1.1 affects the DNA binding properties of σ^{70} both when it is free and when it is bound to core in RNA polymerase holoenzyme. In free σ^{70}, the presence of region 1.1 prevents the interaction of free σ^{70} with

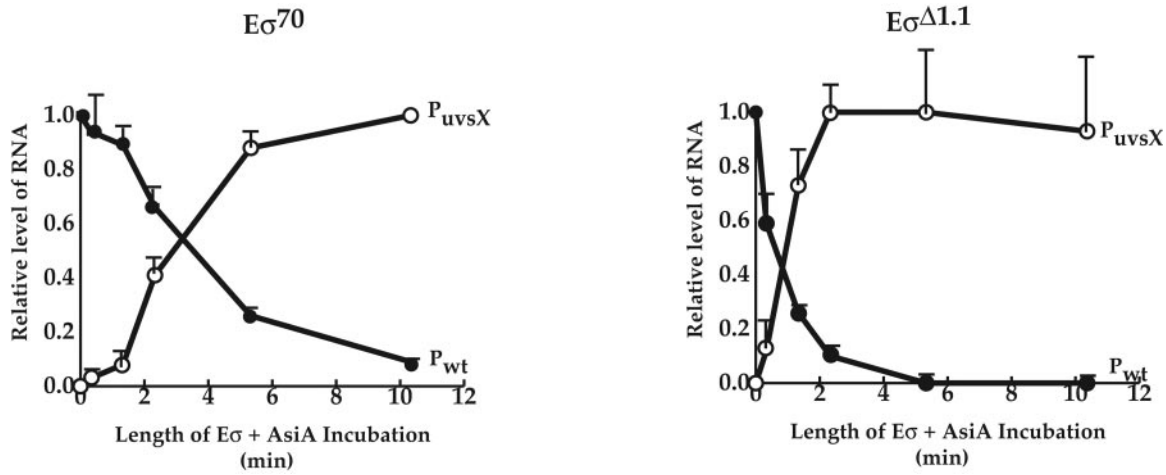

FIG. 4. AsiA inhibits and MotA/AsiA activates polymerase more rapidly in the absence of region 1.1. Reaction mixtures containing the indicated Eσ (reconstituted with 0.05 pmol core plus 0.2 pmol σ) were incubated at 30°C with 3 pmol AsiA in incubation buffer III for the indicated times. Transcription was initiated by mixing aliquots (3.1 μl) with 1.9 μl DNA buffer IV containing 1.9 pmol MotA, 0.01 pmol pDKT90 (P_{uvsX} plasmid) that had been restricted with BsaAI, and 0.01 pmol pP_{wt} that had been restricted with SspI. Reactions were incubated at 37°C for 20 s before the addition of 0.5 μl of rifampin at 300 μg/ml. The relative level of P_{uvsX} RNA is as follows: (amount of P_{uvsX} RNA observed after AsiA-plus-Eσ incubation for time t − amount of P_{uvsX} RNA observed without AsiA)/(amount of P_{uvsX} RNA observed after AsiA-plus-Eσ incubation for 10 min − amount of P_{uvsX} RNA observed without AsiA). The relative level of P_{wt} RNA is as follows: (amount of P_{wt} RNA observed after AsiA-plus-Eσ incubation for time t)/(amount of P_{wt} RNA observed without AsiA). These values are shown versus the length of time (in min) of the AsiA-plus-Eσ incubation.

DNA (9). In holoenzyme, its presence modulates the ability of holoenzyme to interact with particular promoters (39). Thus, region 1.1 plays an important role in controlling the interaction of DNA with the DNA binding regions of σ^{70}.

An understanding of exactly where region 1.1 is located within free σ^{70} and within holoenzyme would greatly help to elucidate how region 1.1 affects the interactions of σ^{70} with DNA. Unfortunately, efforts to determine the structure of this region have not yet been successful. However, information about the general location of region 1.1 has been inferred from other lines of evidence. Some work has argued that a direct interaction between regions 1.1 and 4.2 is responsible for the inability of free σ^{70} to bind to DNA. The addition of region 1.1 in *trans* prevents $\sigma^{\Delta 1.1}$ from recognizing the −35 element but not the −10 DNA element (8), and an I53A substitution in region 1.1, which renders holoenzyme unable to bind stably to λ P_R, is suppressed by deletion of residues 609 to 613 in region 4 (2). However, other results are not compatible with this idea. An engineered cysteine substitution at σ^{70} residue 59 within region 1.1 is more chemically reactive in free σ^{70} than when σ^{70} is bound to core (3). In addition, the NMR spectrum of segmentally labeled region 4.2 of free σ^{A}, the σ^{70} analog of *T. maritima*, is not affected by the presence or absence of region 1.1 (5). Thus, it has been suggested that region 1.1 prevents DNA binding by region 4.2 when σ^{70} is free because region 1.1 interacts directly with other portions of region 4, because the presence of region 1.1 places region 4.2 in a conformation that is unavailable to the DNA, or because the negatively charged region 1.1 provides a steric block to the DNA (5).

The position of σ^{70} region 1.1 in holoenzyme has been deduced from other analyses. Luminescence resonance energy transfer data indicated that when σ^{70} binds to core, regions 4.2 and 1 move relative to region 2 so that the spacing between regions 2 and 4.2 is correct for the distance between the −10 and −35 elements (4). Modeling of fluorescent resonance energy transfer data, protein-DNA photocrosslink analyses, and crystallographic structures indicate that in holoenzyme, region 1.1 acts as a DNA mimic, residing in the channel that will hold the downstream DNA when holoenzyme stably binds to a promoter and that upon formation of the stable polymerase/promoter complex, region 1.1 moves from this downstream DNA channel (26). Hydroxyl radical protein footprinting supports these conclusions. It indicates that the protection of region 1.1 in holoenzyme is lost upon the binding of polymerase to DNA (28). Thus, region 1.1 appears to be a highly flexible domain that can reside in different positions depending on whether σ^{70} is free, is in a complex with core, or is in the holoenzyme/promoter complex.

Both AsiA and MotA interact with portions of region 4 (7, 31, 32, 34), and structural analyses indicate that region 4 of σ^{70} is radically remodeled by AsiA (22). This remodeling is consistent with accumulated evidence suggesting that the σ^{70} region 4 DNA contacts must be reconfigured in order to allow MotA interaction with the MotA box sequence centered in the −30 region of T4 middle promoter DNA (reviewed in reference 18). Given that region 1.1 affects the DNA binding properties of σ^{70}, we supposed that the lack of region 1.1 might affect the ability of AsiA to inhibit transcription from σ^{70}-dependent promoters or of MotA/AsiA to activate transcription from T4 middle promoters. Our results indicate that region 1.1 is not required for transcription from P_{uvsX} RNA by polymerase/MotA/AsiA. However, we find that AsiA inhibits and MotA/AsiA activates RNA polymerase holoenzyme significantly more rapidly when region 1.1 is missing.

We have previously shown that AsiA-inhibited polymerase is formed efficiently by a two-step process (19). AsiA first binds to free σ^{70} and then this complex binds to core. AsiA inhibition of holoenzyme is a slow process that is consistent with the time

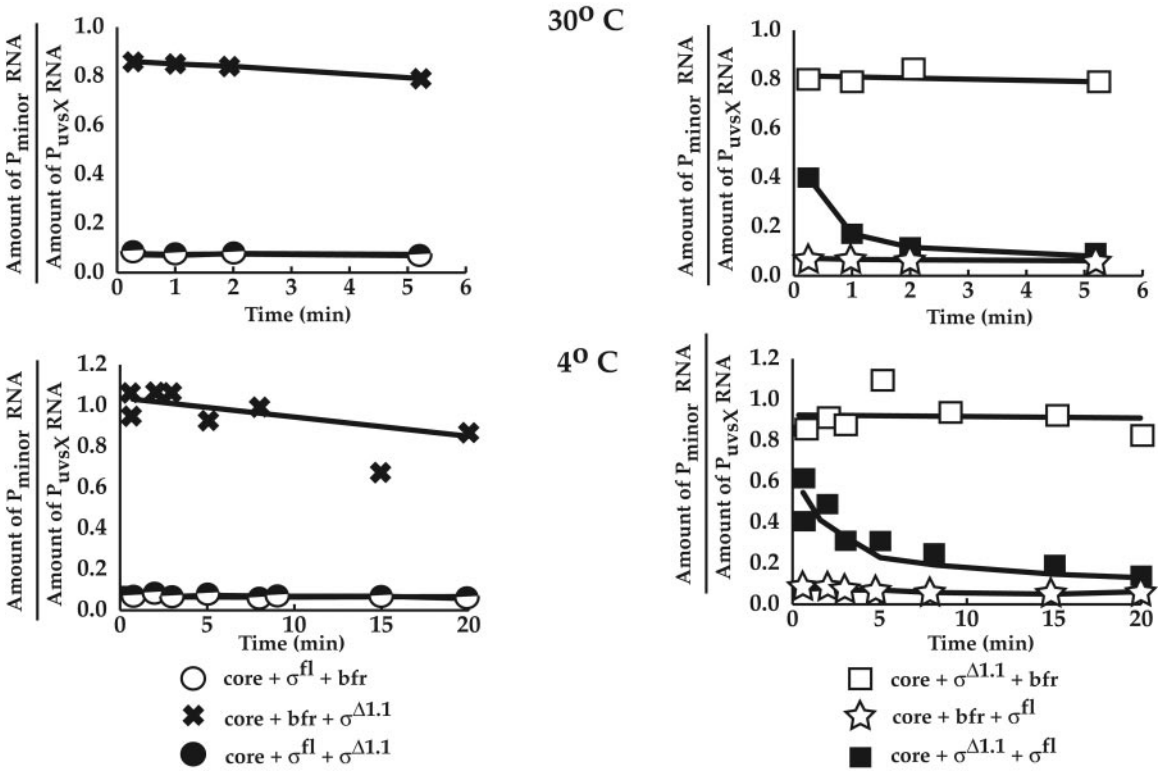

FIG. 5. Addition of free σ^{fl} converts $E\sigma^{\Delta 1.1}$ to $E\sigma^{fl}$. $E\sigma^{fl}$ or $E\sigma^{\Delta 1.1}$, reconstituted by incubating 0.05 pmol of core with 2.0 pmol of the appropriate σ for 15 min at 30°C, was then incubated either with 12 pmol of $\sigma^{\Delta 1.1}$ at 30°C (top left) or at 4°C (bottom left) or with 12 pmol of σ^{fl} at 30°C (top right) or at 4°C (bottom right) in 3.1 μl of incubation buffer (bfr) II for the indicated times. (The first time point was taken after an incubation of 15 s.) The protein mixtures were then added to 0.01 pmol of pDKT90 DNA in 1.9 μl of DNA buffer III and incubated at 37°C for 20 s before the addition of 0.5 μl of rifampin at 300 μg/ml.

needed for the dynamic equilibrium between holoenzyme and σ^{70} plus core to make free σ^{70} available for binding to AsiA (6, 12, 14, 24, 37, 41). Thus, two simple models could explain the ability of AsiA to inhibit $E\sigma^{\Delta 1.1}$ more readily than $E\sigma^{fl}$. In one model, the portions of σ^{70} region 4 that interact with AsiA might be available in free σ^{70} but not available in holoenzyme because of the position of region 1.1 in holoenzyme. Thus, the absence of region 1.1 would allow AsiA to bind directly to σ^{70} when it is present in holoenzyme. In an alternative model, the absence of region 1.1 would not allow AsiA to access holoenzyme directly, but rather it would affect the dynamic equilibrium between holoenzyme and σ^{70} plus core. Thus, in the absence of region 1.1, this equilibrium would be shifted toward more free σ^{70} and free core, increasing the concentration of free σ^{70} at any given time. AsiA would then be able to bind to the available free σ^{70} and the AsiA-associated polymerase would form more rapidly.

Our results are consistent with the second model. First, using a sensitive two-hybrid assay that has been used extensively to detect interactions of σ region 4 with other proteins and peptides (10, 21, 32), we do not detect an interaction of region 4 with region 1.1. Thus, our result supports other studies (3, 5) arguing that the effect of region 1.1 on the DNA binding capability of region 4.2 is indirect. Our evidence also argues that the increased rate of AsiA inhibition and MotA/AsiA activation when region 1.1 is missing is due to an indirect effect. We find that reconstituted $E\sigma^{fl}$ is immune to the presence of excess of $\sigma^{\Delta 1.1}$, while $E\sigma^{\Delta 1.1}$ is converted to $E\sigma^{fl}$ in the presence of excess σ^{fl} at either 4°C or 30°C. These results suggest that the presence of σ^{70} region 1.1 substantially increases the stabilization of the σ^{70}/core interaction. Previous work has determined that the interaction of σ^{70} with core is quite strong, with measured binding constants ranging from 2×10^{-7} to 5×10^{-10} M (12, 14, 24, 41). While the primary interaction of σ^{70} with core involves the interaction of σ^{70} regions 2.1 to 2.2 with the coiled-coil region of β' (1), the overall interface of σ^{70} with core is extensive, involving several regions of σ^{70} and β/β' (27, 28, 35, 38). The placement of the highly negatively charged region 1.1 into the downstream DNA channel of core, as predicted by modeling of fluorescent resonance energy transfer data (26), would provide multiple electrostatic interactions between σ^{70} and core. Our finding that the presence of region 1.1 helps to stabilize holoenzyme is compatible with this prediction and suggests that the loss of region 1.1/core interaction upon formation of the stable polymerase/promoter complex may contribute to the eventual release of σ^{70} from core when polymerase begins elongation.

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