A regulator that inhibits transcription by targeting an intersubunit interaction of the RNA polymerase holoenzyme

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The structures of the bacterial RNA polymerase holoenzyme have provided detailed information about the intersubunit interactions within the holoenzyme. Functional analysis indicates that one of these is critical in enabling the holoenzyme to recognize the major class of bacterial promoters. It has been suggested that this interaction, involving the flap domain of the β subunit and conserved region 4 of the σ subunit, is a potential target for regulation. Here we provide genetic and biochemical evidence that the σ region 4/β-flap interaction is targeted by the transcription factor AsiA. Specifically, we show that AsiA competes directly with the β-flap for binding to σ region 4, thereby inhibiting transcription initiation by disrupting the σ region 4/β-flap interaction.

The bacterial RNA polymerase (RNAP) holoenzyme consists of a catalytically proficient core enzyme (subunit structure $\alpha_2\beta\beta'\omega$) and a σ subunit that confers on the holoenzyme the ability to recognize specific promoter sequences (1). The primary σ factor in *Escherichia coli* is σ^{70}, and the σ^{70}-containing holoenzyme (Eσ^{70}) typically recognizes promoters defined by two conserved sequence elements (the -10 and -35 hexamers) positioned roughly 10 and 35 bp upstream of the transcription start point (1). Most σ factors share four conserved regions (2), and of these, regions 2 and 4 interact with the -10 and -35 elements, respectively (1). Nevertheless, intact σ^{70} recognizes specific promoter sequences only in the context of the holoenzyme because of critical conformational changes in σ^{70} that take place when it associates with the core enzyme (3). These include an increase in the interdomain distance between regions 2 and 4 that is caused by an interaction between σ region 4 and the β-flap domain (4). The σ region 4/β-flap interaction is essential for the recognition of $-10/-35$ promoters because it positions regions 2 and 4 of σ^{70} for simultaneous interaction with the promoter -10 and -35 elements (4). This role of the σ region 4/β-flap interaction suggested that there may exist regulatory factors that inhibit transcription from $-10/-35$ promoters by disrupting the σ region 4/β-flap interaction (4, 5).

Here we examine the mechanism of action of the bacteriophage T4-encoded anti-σ factor AsiA. AsiA is known to bind tightly to region 4 of σ^{70} and inhibit transcription from $-10/-35$ promoters when complexed with the holoenzyme (6–10). The finding that AsiA binds to region 4 of σ^{70} and inhibits transcription specifically from $-10/-35$ promoters raises the possibility that AsiA works by disrupting the σ^{70} region 4/β-flap interaction, a model that is consistent with recent NMR analysis (11). We provide genetic evidence that AsiA and the β-flap interact with overlapping determinants on σ^{70} region 4. We then present complementary biochemical and biophysical evidence that AsiA works by a competitive binding mechanism, inhibiting transcription from $-10/-35$ promoters by disrupting the σ region 4/β-flap interaction in the context of the holoenzyme.

Materials and Methods

Mutant Screen. Mutagenic PCR was used to introduce random mutations into the appropriate fragment of plasmid pBRα-σ^{70} D581G as described (12). A pool of mutant α-σ^{70} D581G chimeras was transformed into strain F$'$93+62 (13) bearing the test promoter (p*lac*2λop) and a linked *lacZ* reporter gene on an F$'$ episome and containing pAC$\Delta$$-35\lambda$cI–AsiA K20A (a derivative of pAC$\Delta$$-35\lambda$cI–AsiA; ref. 13). The resulting transformants were plated on indicator medium containing 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) (40 μg/ml) and the β-galactosidase inhibitor tPEG (250 μM), permitting the identification of colonies containing α-σ^{70} D581G mutants that caused a reduction in λcI–AsiA-stimulated transcription from the test promoter. Plasmid DNA from these colonies was isolated, and the mutant α-σ^{70} D581G chimeras were assayed for their abilities to stimulate transcription from p*lac*Cons-35C.1 (identical in sequence to p*lac*Cons-35C from the transcription start point to position -62; see ref. 12) in strain BG18. Mutants were sought that stimulated transcription from p*lac*Cons-35C.1 as well or nearly as well as the parent chimera.

β-Galactosidase Assays. Cells were grown in LB supplemented with the appropriate antibiotics at the following concentrations: carbenicillin (100 μg/ml), chloramphenicol (25 μg/ml), and kanamycin (50 μg/ml). Isopropyl-β-D-thiogalactoside (IPTG) was provided at the indicated concentrations. SDS·CHCl$_3$-permeabilized cells were assayed as described (13). Assays were performed at least three times in duplicate on separate occasions, with similar results. Values are the averages from one experiment; duplicate measurements differed by <5%.

Proteins. N-terminally his-tagged versions of wild-type σ^{70} and the σ^{70} mutants were purified as described after overproduction from the corresponding derivative of vector pLHN12-His (14). *E. coli* RNAP core enzyme was purchased from Epicentre, and holoenzymes were assembled by incubation of RNAP core (62.5 nM) with a 5-fold molar excess of the appropriate σ^{70} at 37°C for 10 min. C-terminally his-tagged AsiA was purified as described (15) and AsiA-containing RNAP holoenzymes were assembled by preincubating the appropriate σ^{70} (in 5-fold molar excess over RNAP core) with different concentrations of AsiA in σ storage buffer (14) at 4°C for 10 min. RNAP core (62.5 nM) was then added, and the mixtures were incubated at 37°C for 10 min.

***In Vitro* Transcription Assays.** Assays were performed by preincubating template DNA (10 nM) in transcription buffer (10 mM
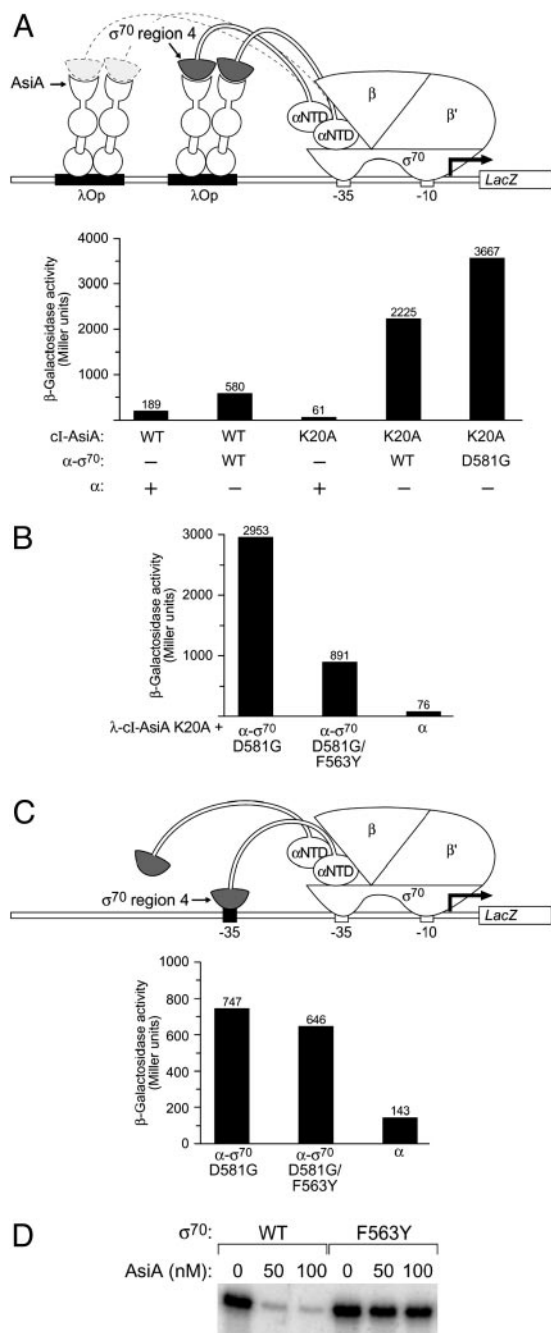

Fig. 1. Bacterial two-hybrid assay used to screen for mutations in region 4 of σ^{70} that disrupt the interaction with AsiA. (*A*) The diagram depicts test promoter p*lac*2λop (previously called p*lac*COP-93+OL2–62, ref. 13), which bears a consensus λ operator sequence centered 93 bp upstream and the λ operator O$_L$2 centered 62 bp upstream from the initiation point of the *lac* core promoter. In strain F′93+62 this test promoter is linked to *lacZ* on an F′ episome. Replacement of the RNAP α-CTD by a fragment of σ that harbors region 4 permits interaction with the AsiA moiety of either of two DNA-bound λcI–AsiA fusion proteins. The graph shows the effect of the λcI–AsiA fusion protein (with or without the K20A substitution) on transcription *in vivo* from p*lac*2λop in the presence of the α-σ^{70} chimera (λcI–AsiA and λcI–AsiA K20A) or the α-σ^{70} D581G chimera (λcI–AsiA K20A only). F′93+62 cells harboring compatible plasmids directing the synthesis of the indicated proteins were grown in the presence of 50 μM IPTG and assayed for β-galactosidase activity. Plasmids pACΔ–35λcI–AsiA (13) and derivative pACΔ–35λcI–AsiA K20A directed the synthesis of the λcI–AsiA and λcI–AsiA K20A fusion proteins, and plasmids pBRα-σ^{70} (28), pBRα (28), and pBRα-σ^{70} D581G (12) directed the synthesis of the α-σ^{70} chimera, full-length α, and the α-σ^{70} D581G chimera. (*B*) Effect of F563Y substitution in σ moiety of α-σ^{70} D581G chimera on

MgCl$_2$/90 mM KCl/40 mM Tris·HCl, pH 8.0/125 μg/ml BSA/5 mM DTT/10% vol/vol glycerol). RNAP holoenzyme (with or without AsiA) was then added to a final concentration of 10 nM and the reactions incubated for 10 min at 37°C to allow open complex formation. Transcription was initiated by addition of 200 μM GTP (initiating nucleotide), 5 μM ATP, 5 μM CTP, 3 μM [α-^{32}P]UTP at 2 mCi/ml, and 100 μg/ml heparin (1 Ci = 37 GBq). The total volume of each reaction was 25 μl. Reactions were incubated for 15 min at 37°C and quenched by the addition of 25 μl of stop solution (95% vol/vol formamide/20 mM EDTA/0.05% wt/vol bromophenol blue/0.05% wt/vol xylene cyanol). Samples were electrophoresed on 6% (wt/vol) polyacrylamide sequencing gels. Bands were visualized by PhosphorImager, and the data were analyzed by IMAGEQUANT.

Fluorescence Resonance Energy Transfer (FRET) Analysis. *AsiA/RNAP holoenzyme complexes.* σ^{70} labeled at residue 581 by cpm (Molecular Probes) was prepared by using mutant σ^{70} containing a single reactive cysteine at position 581 as described (3). AsiA labeled at position 86 with fluorescein (Molecular Probes) was prepared by using mutant AsiA containing a single reactive cysteine at position 86 as described (15). To assess the binding of AsiA to RNAP holoenzyme containing wild-type or mutant σ^{70}, equal amounts of labeled AsiA/holoenzyme complex and unlabeled holoenzyme were mixed together as follows. Core (12.5 pM) was combined with 11 pM σ^{70}-cpm and 10 pM AsiA–FAM (fluorescein malemide) in 200 μl of buffer F containing 50 mM Tris·HCl (pH 8), 100 mM NaCl, and 0.5 mg/ml BSA in a quartz cuvette. The mixture was incubated for 20′ at 37°C to ensure that labeled holoenzyme–AsiA complex reached equilibrium. Under these conditions, at least 90% of labeled σ^{70} is bound to the core (data not shown; see also ref. 4). Then, 5 μl containing 12.5 pM core and 11 pM unlabeled wild-type or mutant σ^{70} were added to the labeled holoenzyme-AsiA complex. The mixture was incubated at 37°C for 5 min before measurements were taken. Fluorescence emission spectra with the excitation at 380 nm (cpm excitation) were recorded from 400 to 600 nm. Fluorescence spectra were recorded by using an Aminco Bowman Series 2 spectrofluorometer (Spectronic Instruments, Rochester, NY). Spectra were acquired with the scan rate of 2 nm/sec and with excitation and emission slits of 2 and 4 nm, correspondingly. The ratio of fluorescence intensity

transcription *in vivo* from p*lac*2λop in presence of the λcI–AsiA K20A fusion protein. F′93+62 cells harboring compatible plasmids directing the synthesis of the indicated proteins were grown in the presence of 50 μM IPTG and assayed for β-galactosidase activity. Plasmid pACΔ–35λcI–AsiA K20A directed the synthesis of the λcI–AsiA K20A fusion protein and plasmids pBRα-σ^{70} D581G, pBRα-σ^{70} D581G/F563Y, and pBRα directed the synthesis of the α-σ^{70} D581G chimera, the α-σ^{70} D581G/F563Y chimera, and full-length α. (*C*) Substitution F563Y in σ moiety of α-σ^{70} chimera does not affect the ability of the tethered σ^{70} region 4 moiety to bind DNA. The diagram depicts test promoter p*lac*Cons–35C.1 (see *Materials and Methods*), which contains a consensus −35 element centered 55 bp upstream from the transcription start site. In strain BG18, this test promoter is linked to *lacZ* on an F′ episome. Replacement of the RNAP α-CTD by a fragment of σ that harbors region 4 containing the D581G substitution permits interaction with the −35 element centered at position −55. The graph shows the effect of the F563Y substitution in the σ moiety of the α-σ^{70} D581G chimera on transcription *in vivo* from p*lac*Cons–35C.1. BG18 cells harboring compatible plasmids directing the synthesis of the indicated proteins were grown in the presence of 50 μM IPTG and assayed for β-galactosidase activity. Plasmids pBRα-σ^{70} D581G, pBRα-σ^{70} D581G/F563Y, and pBRα directed the synthesis of the α-σ^{70} D581G chimera, the α-σ^{70} D581G/F563Y chimera, and full-length α. (*D*) Eσ^{70} F563Y is resistant to inhibitory effect of AsiA on transcription from a −10/−35 promoter. Shown are results of single round *in vitro* transcription assays performed on −10/−35 promoter T7A2 in the absence or presence of increasing concentrations of AsiA (50 or 100 nM) by using wild-type Eσ^{70} or mutant Eσ^{70} bearing the F563Y substitution in σ^{70}.

integrated between 500 and 600 nm (acceptor emission) to the intensity integrated between 400 and 500 nm (donor emission) was used as an apparent parameter describing the extent of FRET between the donor and acceptor. The value of this parameter is directly proportional to the amount of $E\sigma^{70}$-cpm–AsiA–FAM complex in solution. For these experiments, wild-type RNAP core enzyme was prepared by *in vitro* reconstitution as described (16), and unlabeled wild-type and mutant σ^{70} subunits were purified as described (14).

AsiA/σ^{70} complexes. Complexes between labeled σ^{70} and labeled AsiA were formed by mixing the proteins in 200 μl of buffer F in a quartz cuvette at a ratio of 1:1.5 (50 nM σ^{70}-cpm and 75 nM AsiA–FAM). Relative binding affinities of σ^{70} mutants were assessed by adding various amounts of unlabeled protein to the mixture of labeled σ^{70} and AsiA. Addition of unlabeled σ^{70} (wild type or mutant) led to a substitution of labeled σ^{70} in the σ^{70}-cpm–AsiA–FAM complex by the unlabeled competitor protein, resulting in diminished intensity of the FRET signal.

Results

Design of Genetic Screen. To probe the mechanism of transcription inhibition by AsiA, we took a genetic approach. Previous work suggested that the interaction of AsiA with σ^{70} region 4 is required for AsiA-mediated transcription inhibition (8–10). Therefore, we sought to identify amino acid residues in region 4 of σ^{70} that participate directly in the interaction with AsiA through the use of a bacterial two-hybrid assay (17, 18). In this assay, contact between a protein domain fused to a component of RNAP and an interacting protein (or protein domain) fused to a DNA-bound protein (here, the cI protein of bacteriophage λ) activates transcription of a *lacZ* reporter gene (see Fig. 1*A*). In particular, we made use of a λcI–AsiA fusion protein and an α-σ^{70} chimera in which the C-terminal domain of α (the α-CTD) has been replaced by a fragment of σ^{70} (residues 528–613) encompassing region 4 (Fig. 1*A*). The λcI–AsiA fusion protein activated transcription from test promoter p*lac*2λop $\approx$3-fold specifically in cells containing the α-σ^{70} chimera (Fig. 1*A*; see also ref. 13).

To increase the sensitivity of our genetic screen, we sought to identify a λcI–AsiA variant that would activate transcription more strongly in our two-hybrid assay. Because AsiA forms stable homodimers that must first dissociate for AsiA to bind to σ^{70} region 4 (11, 19, 20), we hypothesized that the ability of the fused AsiA moiety to activate transcription might be limited by its tendency to dimerize in the context of the dimeric λcI–AsiA fusion protein (13). To test this hypothesis, we introduced into the AsiA moiety an amino acid substitution (K20A) that specifically disrupts the formation of AsiA homodimers without inhibiting the ability of AsiA to interact with σ^{70} region 4 (R.J.B.U. and J.U., unpublished data). The resulting λcI–AsiA K20A variant stimulated transcription from promoter p*lac*2λop $\approx$36-fold in the presence of the α-σ^{70} chimera (Fig. 1*A*), providing strong support for the hypothesis that the interaction of the fused AsiA moieties of the λcI–AsiA dimer competes with the interaction of the AsiA and σ^{70} region 4 moieties in the two-hybrid assay.

To identify amino acid substitutions in σ^{70} region 4 that specifically affected its interaction with AsiA and eliminate from consideration those that disrupted the structural integrity of the σ^{70} moiety, we took advantage of a related genetic assay that reports on the ability of the tethered σ^{70} region 4 moiety to bind to a -35 element (12). In this assay, the interaction of the region 4 moiety with an ectopic -35 element positioned upstream of the core promoter elements activates transcription from the test promoter depicted in Fig. 1*C* (promoter p*lac*Cons-35C.1). We showed previously that although the wild-type σ^{70} moiety does not bind detectably to the ectopic -35 element of p*lac*Cons-35C, a variant bearing amino acid substitution
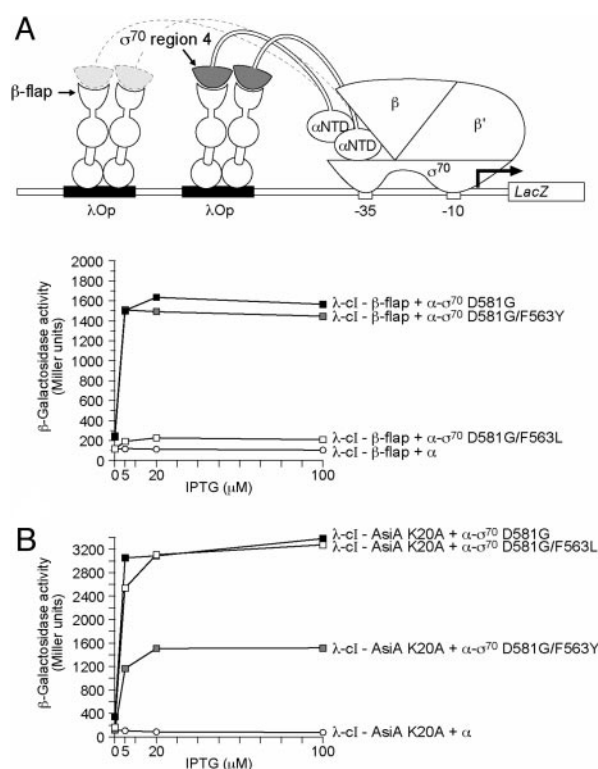

Fig. 2. Effects of substitutions at position 563 of σ^{70} on the interactions with AsiA K20A and the β-flap moiety of RNAP. (*A*) The effects of two different substitutions at position 563 of σ^{70} on the σ^{70} region 4/β-flap interaction. The diagram depicts test promoter p*lac*2λop. The graph shows the effects of the λcI-β-flap fusion protein on transcription *in vivo* from p*lac*2λop in the presence of α-σ^{70} chimeras bearing different amino acids at position 563 of the tethered σ^{70} region 4 moiety. F'93+62 cells harboring compatible plasmids directing the synthesis of the indicated proteins were grown in the presence of different concentrations of IPTG and assayed for β-galactosidase activity. Plasmid pACλcI-β-flap (4) directed the synthesis of the of λcI-β-flap fusion protein, and plasmids pBRα-σ^{70} D581G (12), pBRα-σ^{70} D581G/F563L, pBRα-σ^{70} D581G/F563Y, and pBRα directed the synthesis of the α-σ^{70} D581G chimera, the α-σ^{70} D581G/F563L chimera, the α-σ^{70} D581G/F563Y chimera, and full-length α. (*B*) The effect of two different substitutions at position 563 of σ^{70} on the σ^{70} region 4/AsiA K20A interaction. The graph shows the effects of the λcI–AsiA K20A fusion protein on transcription *in vivo* from p*lac*2λop in the presence of α-σ^{70} chimeras bearing different amino acids at position 563 of the tethered σ^{70} region 4 moiety. F'93+62 cells harboring compatible plasmids directing the synthesis of the indicated proteins were grown in the presence of different concentrations of IPTG and assayed for β-galactosidase activity. Plasmid pAC$\Delta-35\lambda$cI–AsiA K20A directed the synthesis of the λcI–AsiA K20A fusion protein, and plasmids pBRα-σ^{70} D581G, pBRα-σ^{70} D581G/F563L, pBRα-σ^{70} D581G/F563Y, and pBRα directed the synthesis of the α-σ^{70} D581G chimera, the α-σ^{70} D581G/F563L chimera, the α-σ^{70} D581G/F563Y chimera, and full-length α.

D581G does (12). Thus, we used the D581G variant of the α-σ^{70} chimera in our genetic screen, having first verified that the D581G substitution did not compromise the ability of the AsiA moiety of the λcI–AsiA fusion protein to interact with σ^{70} region 4 in our two-hybrid assay (Fig. 1*A*).

Identification and Characterization of a σ^{70} Mutant That Is Specifically Defective in the Interaction with AsiA. We introduced random mutations into the gene fragment encoding the σ^{70} moiety of an α-σ^{70} D581G chimera. After screening the mutagenized library of α-σ^{70} D581G chimeras, we identified an amino acid substitution, F563Y, that reduced λcI–AsiA K20A-stimulated transcription from test promoter p*lac*2λop, but had only a slight effect on the ability of the tethered σ^{70} moiety to activate
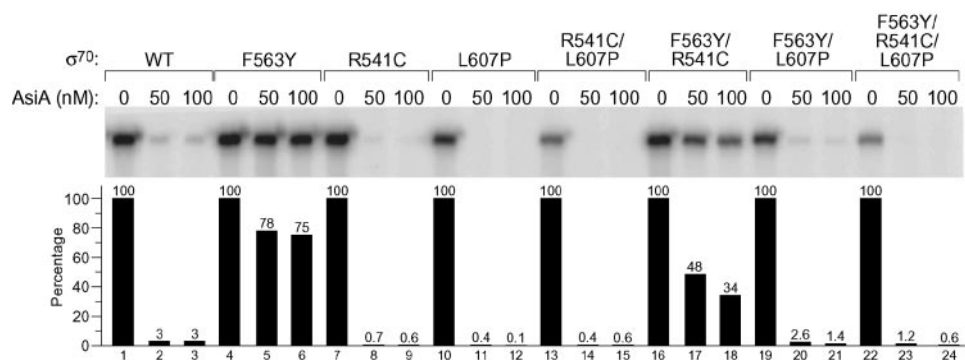
Fig. 3. Mutations that weaken the σ^{70} region 4/β-flap interaction restore sensitivity of Eσ^{70} F563Y to AsiA. Shown are the results of single round *in vitro* transcription assays performed on T7A2 promoter in the absence or presence of increasing concentrations of AsiA (50 or 100 nM) by using wild-type or mutant RNAP holoenzymes. For each RNAP, the transcripts were quantified (by IMAGEQUANT) to determine the magnitude of the inhibitory effect of AsiA. Thus, the amount of transcript produced in the absence of AsiA was set at 100% for each holoenzyme. Note that all of the mutant holoenzymes except those containing σ^{70} with both the R541C and L607P substitutions initiated transcription from T7A2 approximately as well as Eσ^{70} WT (compare lanes 4, 7, 10, 16, and 19 with lane 1). Eσ^{70} R541C/L607P and Eσ^{70} F563Y/R541C/L607P initiated transcription from T7A2 only ≈50% as efficiently as Eσ^{70} WT. Disruption of the interaction of σ^{70} with the β-flap is expected to cause defects in the recognition of −10/−35 promoters, and analysis of the effects of the R541C and L607P substitutions indicated that it was necessary to introduce both of these substitutions into σ^{70} to detect a significant defect in the recognition of strong −10/−35 promoters *in vitro* (B.E.N., unpublished data). No defects in the recognition of a control extended −10 promoter were detected with σ^{70} mutants bearing substitutions R541C and L607P either singly or in combination (B.E.N., unpublished data).

transcription from test promoter p*lac*Cons-35C.1 (Fig. 1 *B* and *C*). We conclude that substitution F563Y specifically weakens (but does not abolish) the interaction of σ^{70} region 4 with AsiA.

We then wished to determine whether or not introduction of the F563Y substitution into full-length σ^{70} would affect the ability of AsiA to inhibit transcription from a −10/−35 promoter (T7A2) *in vitro*. Reconstituted holoenzymes Eσ^{70} and Eσ^{70} F563Y were equally proficient in initiating transcription from this promoter in the absence of AsiA (Fig. 1*D*). As expected, AsiA inhibited transcription by the wild-type holoenzyme (Fig. 1*D*, lanes 1–3). In contrast, the mutant holoenzyme containing σ^{70} F563Y was almost completely refractory to the inhibitory effect of AsiA (Fig. 1*D*, compare lanes 4–6 with lanes 1–3). Thus, amino acid substitution F563Y in σ^{70} both weakened the σ^{70} region 4/AsiA interaction in the two-hybrid assay and disrupted AsiA-mediated transcription inhibition *in vitro*, indicating that the σ^{70} region 4/AsiA interaction detected in the two-hybrid assay is required for AsiA to function as an inhibitor of transcription.

Substitutions of σ^{70} Residue 563 Affect both the σ^{70}/AsiA and the σ^{70}/β-Flap Interactions. Two independent lines of evidence implicate residue F563 of σ^{70} in the interaction of σ^{70} region 4 with the β-flap. First, the RNAP holoenzyme structures indicate that the residue corresponding to F563 of σ^{70} lies at the region 4/β-flap interface (21, 22). Second, we found that a substitution (F278L) affecting the corresponding residue of σ^{38} (the stationary phase-specific σ factor in *E. coli*) specifically disrupted the interaction of σ^{38} region 4 with the β-flap (B.E.N., unpublished data). We therefore wished to compare the effects of the F563Y and F563L substitutions in σ^{70} on both the σ^{70} region 4/β-flap and the σ^{70} region 4/AsiA interactions. Accordingly, we introduced the F563L substitution into the σ^{70} moiety of the α-σ^{70} D581G chimera. Whereas the F563L substitution in σ^{70} region 4 nearly abolished the stimulatory effect of the λcI–β-flap fusion protein from test promoter p*lac*2λop (Fig. 2*A*), it had no effect on the ability of the λcI–AsiA K20A fusion protein to stimulate transcription from the same promoter (Fig. 2*B*). Conversely, we found that amino acid substitution F563Y in σ^{70} region 4, which reduced the stimulatory effect of the λcI–AsiA K20A fusion protein, had no effect on the ability of the λcI–β-flap fusion protein to stimulate transcription (Fig. 2 *A* and *B*). We conclude,

therefore, that the F563L substitution specifically affects the interaction of σ^{70} region 4 with the β-flap, whereas the F563Y substitution specifically affects its interaction with AsiA, suggesting that the molecular details of the two interactions differ.

Evidence That AsiA Competes with the β-Flap for Binding to σ^{70} Region 4. The identification of an amino acid residue (F563) that apparently participates in both the σ^{70} region 4/β-flap and the σ^{70} region 4/AsiA interactions suggested that AsiA and the β-flap interact with overlapping determinants of σ^{70} region 4. Therefore, AsiA and the β-flap would be expected to compete for binding to σ^{70} region 4 in the context of the RNAP holoenzyme. This competitive binding model for AsiA-mediated transcription inhibition implies that the relative affinities of AsiA and the β-flap for σ^{70} region 4 should dictate whether AsiA can effectively inhibit transcription from −10/−35 promoters. To test this competitive binding model, we asked whether weakening the interaction of σ^{70} F563Y with the β-flap could restore the susceptibility of Eσ^{70} F563Y to AsiA. To do this, we took advantage of two amino acid substitutions in σ^{70} (R541C and L607P) that specifically disrupt the interaction between σ^{70} region 4 and the β-flap (B.E.N., unpublished data). We first used the two-hybrid assay to test whether these substitutions had any effect on the interaction of σ^{70} region 4 and AsiA. When introduced into σ^{70} region 4 either singly or in combination, neither the R541C substitution nor the L607P substitution significantly affected the σ^{70} region 4/AsiA K20A interaction (data not shown).

We then introduced substitutions R541C and L607P, singly and in combination, into full-length σ^{70} already containing the F563Y substitution. We used these mutant σ^{70} proteins as well as σ^{70} R541C, σ^{70} L607P, σ^{70} R541C/L607P, σ^{70} F563Y, and σ^{70} WT to reconstitute a set of holoenzymes and examined the abilities of these holoenzymes to initiate transcription from the T7A2 promoter in the absence or presence of AsiA (Fig. 3; see also Table 1). As in the experiment of Fig. 1*D*, we found that AsiA efficiently inhibited transcription by Eσ^{70} WT (Fig. 3, lanes 1–3) but was unable to inhibit transcription by Eσ^{70} F563Y (Fig. 3, lanes 4–6). Furthermore, AsiA inhibited transcription by Eσ^{70} R541C, Eσ^{70} L607P, and Eσ^{70} R541C/L607P even more efficiently than it inhibited transcription by Eσ^{70} WT (Fig. 3, compare lanes 7–9, 10–12, and 13–15, with lanes 1–3). Finally, we

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Table 1. Sensitivity of mutant RNAP holoenzymes to AsiA

σ^{70} mutant	Sensitivity of σ^{70}-containing holoenzyme to AsiA *in vitro*
Wild type	+ + + +
F563Y*	+
R541C†	≥ + + + +
L607P	≥ + + + +
R541C/L607P	≥ + + + +
F563Y/*R541C*	+ +
F563Y/*L607P*	≥ + + + +
F563Y/*R541C/L607P*	≥ + + + +

*Boldface indicates that the F563Y substitution disrupts the interaction of σ^{70} region 4 with AsiA (but not with the β-flap).

†Italics indicate that the designated substitutions disrupt the interaction of σ^{70} region 4 with the β-flap (but not with AsiA).

found that introduction of substitutions R541C and/or L607P into σ^{70} F563Y enabled AsiA to inhibit transcription by the corresponding doubly and triply substituted holoenzymes (Fig. 3, lanes 16–18, 19–21, and 22–24). Moreover, the efficiency with which AsiA inhibited transcription by each of these holoenzymes correlated with the severity of the β-flap-binding defect of the corresponding mutant σ^{70}. That is, σ^{70} substitutions R541C, L607P, and R541C/L607P resulted in increasingly severe β-flap-binding defects (B.E.N., unpublished data), and correspondingly, Eσ^{70} F563Y/R541C, Eσ^{70} F563Y/L607P, and Eσ^{70} F563Y/R541C/L607P were increasingly susceptible to inhibition by AsiA (Fig. 3, lanes 16–24). These findings demonstrate that the effect of weakening the σ^{70} region 4/AsiA interaction can be suppressed by weakening a competing interaction between σ^{70} region 4 and the β-flap.

To assay directly the binding of AsiA to the wild-type and mutant holoenzymes in solution, we used a FRET-based binding assay. FRET donor and acceptor probes were incorporated into AsiA and σ^{70} by using single-cysteine mutants of the proteins. The labeled polypeptides were mixed with RNAP core to form the AsiA-containing holoenzyme. Association of acceptor-labeled AsiA with donor-labeled holoenzyme produced a FRET signal. The labeled holoenzyme–AsiA complex was then mixed with an equal amount of unlabeled holoenzyme (containing either wild-type or mutant σ^{70}), and the decrease in FRET was

quantified. The decrease in FRET reflects the ability of the unlabeled holoenzyme species to compete with the labeled wild-type holoenzyme for the binding of labeled AsiA. Compared to the wild-type holoenzyme, the holoenzyme containing the σ^{70} F563Y mutant competed poorly for the binding of AsiA, as expected (Fig. 4A). Furthermore, this defect in the ability of the σ^{70} F563Y-containing holoenzyme to bind AsiA was suppressed by the introduction into σ^{70} F563Y of substitutions R541C and/or L607P, which weaken the σ^{70} region 4/β-flap interaction (Fig. 4A). The results of the FRET analysis thus paralleled the results of the *in vitro* transcription assays (Fig. 3), indicating that the ability of AsiA to inhibit transcription correlates with its ability to bind the holoenzyme.

We performed a control FRET experiment to confirm that the introduction of substitutions R541C and L607P into the σ^{70} F563Y mutant did not affect the affinity of AsiA for free σ^{70}. In this experiment, donor/acceptor complexes were formed with labeled AsiA and labeled σ^{70}. Increasing concentrations of unlabeled wild-type or mutant σ^{70} were then added, and the decrease in FRET was quantified. The decrease in FRET signal reflects the ability of each added σ^{70} to compete with the labeled σ^{70} for the binding of labeled AsiA (Fig. 4B). The results confirmed that the binding of AsiA to free σ^{70} was weakened significantly by the F563Y substitution and that this binding defect was not reversed by either the R541C or the L607P substitution (or the combination of the two) (Fig. 4B).

Discussion

Our findings provide strong support for the idea that AsiA targets the σ^{70}/β-flap interaction, inhibiting transcription from $-10/-35$ promoters by displacing σ^{70} region 4 from the β-flap in the context of the holoenzyme. We note that this competitive binding model for AsiA-mediated transcription inhibition may provide an explanation for the previous demonstration that AsiA binds more readily to free σ^{70} than to the σ^{70}-containing holoenzyme (23). Our data are consistent with the results of a recent analysis of the σ^{70} region 4/AsiA interaction by NMR (11). This analysis identified amino acid side chains in σ^{70} region 4 that are contacted by AsiA in the complex that forms when AsiA is combined with a pair of σ^{70} peptides comprising subregions 4.1 and 4.2. The identified side chains (which include F563) are distinct from those that participate in contacts with the
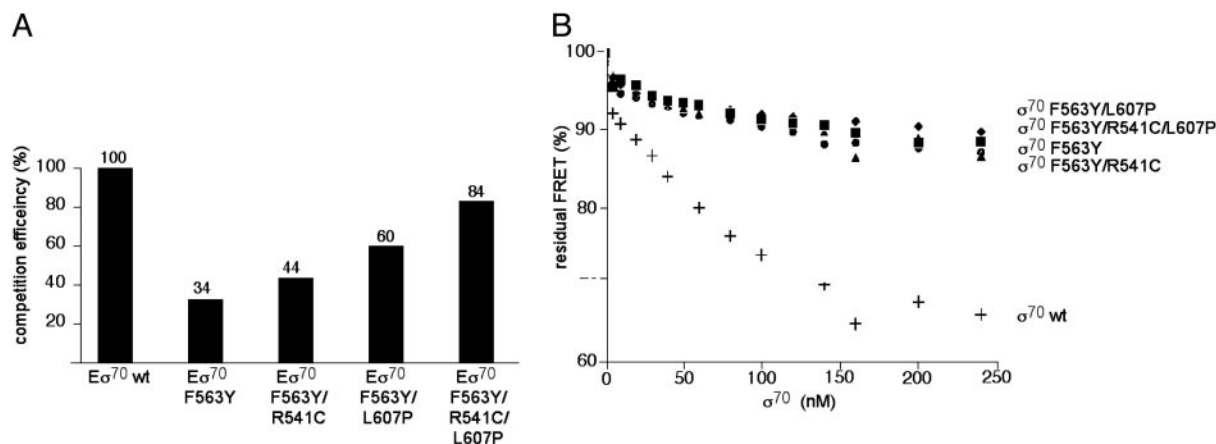

Fig. 4. Effects of substitutions in σ^{70} on AsiA binding in the context of RNAP holoenzyme (A) and free σ^{70} (B). (A) The indicated RNAP holoenzymes were added to a ternary complex containing RNAP core and fluorescently labeled σ^{70} and AsiA. The decrease in FRET signal was determined and converted to competition efficiency (assumed to be 100% for wild-type RNAP holoenzyme). (B) A complex between fluorescently labeled σ^{70} and AsiA was formed and the effect of the addition of increasing concentrations of unlabeled wild-type or mutant σ^{70} subunits on the FRET signal was determined (plus signs, wild-type σ^{70}; circles, σ^{70} F563Y; triangles, σ^{70} F563Y/R541C; diamonds, σ^{70} F563Y/L607P; squares, σ^{70} F563Y/R541C/L607P). The decrease in FRET signal reflects the ability of each added σ^{70} to compete for labeled AsiA.

−35 element as seen in the crystal structure of a σ region 4–DNAcomplex (24), but map instead to a hydrophobic pocket that binds the β-flap in the context of the holoenzyme (21, 22). Additional evidence indicated that the binding of AsiA to the σ^{70}-containing holoenzyme resulted in a decrease in the interdomain distance between regions 2 and 4 of σ^{70}, consistent with the expected disruption of the σ^{70} region 4/β-flap interaction (11).

We found that the ability of AsiA to inhibit transcription by the σ^{70}-containing holoenzyme depends on the relative affinities of AsiA and the β-flap for σ^{70} region 4, supporting a competitive binding mechanism for AsiA-mediated transcription inhibition. Specifically, we showed that, when the interaction between σ^{70} region 4 and AsiA is weakened by substitution F563Y in σ^{70}, AsiA fails to inhibit transcription unless the interaction between σ^{70} region 4 and the β-flap is also weakened by the introduction of one or more additional substitutions into σ^{70} (see Table 1). A prediction of the competitive binding model for AsiA action is that AsiA's ability to inhibit transcription would be compromised not only by amino acid substitutions that weaken the interaction of AsiA with σ^{70} region 4, but also by those that strengthen the interaction of σ^{70} region 4 with the β-flap. We have recently identified amino acid substitutions in σ^{70} region 4 that strengthen its interaction with the β-flap in the two-hybrid assay (ref. 4 and S.J.G., unpublished data). As predicted by the competitive binding model, we found that these substitutions, which did notweaken the interaction of σ^{70} region 4 with AsiA, blocked the ability of AsiA to inhibit transcription (B.D.G., unpublished data).

In addition to its role as an inhibitor of σ^{70}-dependent transcription from −10/−35 promoters, AsiA functions as a coactivator of T4 middle gene transcription (25, 26). T4 middle promoters (which are recognized by the σ^{70}-containing holoenzyme) bear a binding site for the phage-encoded regulator MotA that is located at approximately the position of the −35 element of a standard −10/−35 promoter (25). Activation of these promoters depends on a protein–protein interaction between DNA-bound MotA and residues near the C terminus of σ^{70} (27). Because this portion of σ^{70} has also been implicated in the β-flap interaction (21, 22), we speculate that, by disrupting the σ^{70} region 4/β-flap interaction, AsiA may facilitate the MotA/σ^{70} region 4 interaction.

The demonstration that AsiA works by disrupting the σ region 4/β-flap interaction suggests that it will be worthwhile to search for other regulators that target this interaction in the context of the σ^{70}-containing holoenzyme as well as other holoenzyme species. Moreover, because evidence indicates that the strength of the σ region 4/β-flap interaction varies with different σ factors (4), different holoenzyme species are likely to be differentially susceptible to such regulators.

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