The bacteriophage T4 AsiA protein contacts the ß-flap domain of RNA polymerase

The bacteriophage T4 AsiA protein contacts the β-flap domain of RNA polymerase

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To initiate transcription from specific promoters, the bacterial RNA polymerase (RNAP) core enzyme must associate with the initiation factor σ, which contains determinants that allow sequence-specific interactions with promoter DNA. Most bacteria contain several σ factors, each of which directs recognition of a distinct set of promoters. A large and diverse family of proteins known as "anti-σ factors" regulates promoter utilization by targeting specific σ factors. The founding member of this family is the AsiA protein of bacteriophage T4. AsiA specifically targets the primary σ factor in *Escherichia coli*, σ^{70}, and inhibits transcription from the major class of σ^{70}-dependent promoters. AsiA-dependent transcription inhibition has been attributed to a well-documented interaction between AsiA and conserved region 4 of σ^{70}. Here, we establish that efficient AsiA-dependent transcription inhibition also requires direct protein–protein contact between AsiA and the RNAP core. In particular, we demonstrate that AsiA contacts the flap domain of the RNAP β-subunit (the β-flap). Our findings support the emerging view that the β-flap is a target site for regulatory proteins that affect RNAP function during all stages of the transcription cycle.

anti-σ factor | transcription initiation | transcription regulation

The bacterial RNA polymerase (RNAP) holoenzyme consists of a catalytically-active multisubunit core enzyme ($\alpha_2\beta\beta'\omega$) in complex with a σ factor, which confers on the core enzyme the ability to initiate promoter-specific transcription (1). Bacteria typically contain a number of σ factors, each of which specifies recognition of a distinct class of promoters (2). The primary σ factor in *Escherichia coli* is σ^{70}, and the σ^{70}-containing holoenzyme is responsible for most transcription that occurs during the exponential phase of growth. In the context of the RNAP holoenzyme, σ^{70} makes direct contact with 2 conserved promoter elements that are separated by ≈17 bp, the −10 and −35 elements (consensus sequences TATAAT and TTGACA, respectively). RNAP holoenzyme can also initiate transcription from promoters that lack a recognizable −35 element, but carry an extended −10 element (consensus TGnTATAAT) (3). At extended −10 promoters, additional contacts between σ^{70} and the TG dinucleotide of the extended −10 element compensate for the lack of a −35 element (4). Primary σ factors share 4 regions of conserved sequence (regions 1–4), which have been further subdivided (1, 5). Structural work indicates that σ comprises 4 flexibly-linked domains: $\sigma_{1.1}$ (containing region 1.1), σ_2 (containing regions 1.2–2.4), σ_3 (containing regions 3.0 and 3.1), and σ_4 (containing regions 4.1 and 4.2) (1, 5–8). Regions 2, 3, and 4 contain DNA-binding domains responsible for recognition of the promoter −10 element, extended −10 element, and −35 element, respectively (1, 4, 5).

Holoenzyme formation critically depends on a high-affinity interaction between σ^{70} region 2 and a coiled-coil motif in the β'-subunit (the β' coiled coil, also referred to as the clamp helices) (9, 10). The interaction between σ^{70} region 2 and the β' coiled coil is also required for σ^{70} to make functional contact with the promoter −10 element (11). Interaction between σ^{70} region 4 and the flap domain of the β-subunit (the β-flap), although dispensable for holoenzyme formation, is required for sequence-specific interaction with the promoter −35 element (12). In particular, the σ^{70} region 4/β-flap interaction properly positions σ^{70} region 4 with respect to σ^{70} region 2 and thereby enables regions 4 and 2 to make simultaneous contact with promoter elements separated by ≈17 bp (12). Thus, the σ^{70} region 4/β-flap interaction is essential for recognition of the major class of *E. coli* promoters, those that depend on both a −10 and a −35 element (the −10/−35 class), but it is not strictly required for recognition of extended −10 promoters.

A large and diverse family of proteins known as "anti-σ factors" regulates utilization of particular classes of bacterial promoters by targeting specific σ factors (13, 14). Typically, anti-σ factors interact with core binding determinants in their cognate σ factors, thereby preventing their association with the RNAP core enzyme (15). The first anti-σ factor identified was the AsiA protein of bacteriophage T4, which targets σ^{70} (7, 16, 17); however, unlike most other well-characterized anti-σ factors, AsiA binds its cognate σ factor in the context of the RNAP holoenzyme (18). As a component of the σ^{70}-containing holoenzyme, AsiA inhibits transcription from the −10/−35 class of promoters, but does not inhibit transcription from extended −10 promoters (18).

Prior work has established that AsiA interacts directly with σ^{70} region 4 and that this interaction is required for AsiA-dependent transcription inhibition (7, 19–21). Two mechanistic consequences of the AsiA/σ^{70} region 4 interaction have been described. First, the interaction occludes determinants of σ^{70} region 4 that are required for the σ^{70} region 4/β-flap interaction (22, 23). Second, AsiA stabilizes an alternative conformation of region 4 in which its DNA-binding surface is deformed (24). Thus, AsiA inhibits transcription from −10/−35 promoters both by disrupting the σ^{70} region 4/β-flap interaction and by stabilizing a conformation of σ^{70} region 4 that is incompatible with sequence-specific binding to the −35 element.

Here, using a bacterial 2-hybrid assay, we demonstrate that AsiA interacts directly with the β-flap, and that AsiA can interact simultaneously with the β-flap and σ^{70} region 4. We further show that the AsiA/β-flap interaction is required for efficient AsiA-dependent transcription inhibition. Thus, in contrast to typical anti-σ factors, which require interactions only with σ to mediate their effects, AsiA requires contact with both its cognate σ factor and RNAP core.

Results

AsiA Interacts with the β-Flap. Based on the finding that AsiA prevents σ^{70} region 4 from interacting with the β-flap, we considered the possibility that, in the context of the AsiA-containing holoenzyme, interactions between AsiA and the

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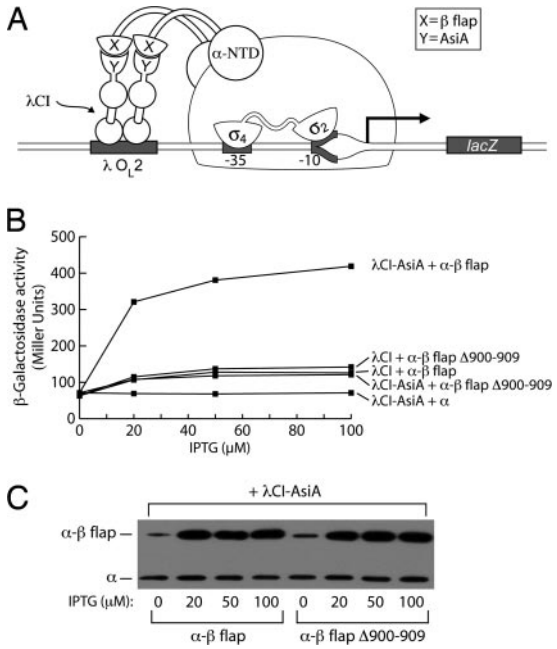

Fig. 1. AsiA interacts with the β-flap. (*A*) Bacterial 2-hybrid assay used to detect protein–protein interaction between AsiA and the β-flap. Diagram depicts how the interaction between AsiA, fused to the bacteriophage λ CI protein (λCI), and the β-flap, fused to the α-N-terminal domain (α-NTD), activates transcription from test promoter p*lac*O_L2–62, which bears the λ operator O_L2 centered 62 bp upstream of the *lac* core promoter start site. In reporter strain FW102 O_L2–62, test promoter p*lac*O_L2–62 is located on an F′ episome and drives the expression of a linked *lacZ* gene. (*B*) Results of β-galactosidase assays. The assays were performed with FW102 O_L2–62 cells containing 2 compatible plasmids, one encoding either λCI or a λCI–AsiA fusion protein, and the other encoding either α or the indicated α–β-flap fusion protein. The AsiA moiety of the λCI–AsiA fusion protein bore amino acid substitution K20A, which disrupts AsiA dimer formation, facilitating detection of protein–protein interactions that require prior dissociation of the AsiA dimer (23). The plasmids directed the synthesis of the fusion proteins (or λCI or α) under the control of IPTG-inducible promoters, and the cells were grown in the presence of increasing concentrations of IPTG. (*C*) Western blot analysis to assess intracellular levels of the α–β-flap fusion proteins. Samples from the cell lysates assayed for β-galactosidase (*B*) were processed for Western blot analysis as described (53). Bands corresponding to α–β-flap fusion proteins and chromosomally-encoded α are indicated. The results ruled out the possibility that the failure of the α–β-flap fusion protein lacking the flap-tip helix (Δ900–909) to interact with AsiA is attributable to protein instability.

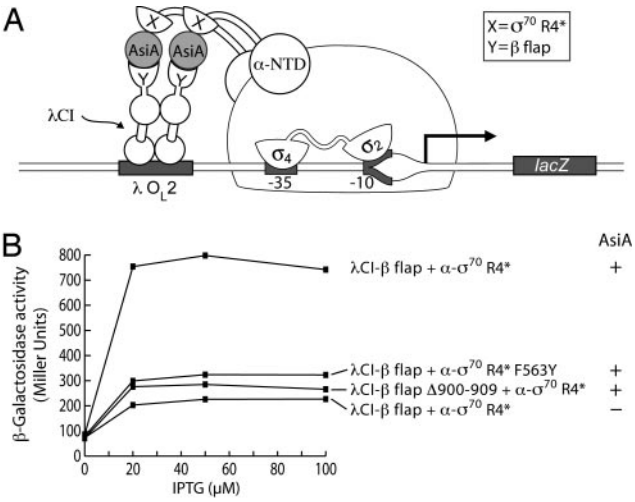

Fig. 2. AsiA interacts simultaneously with σ^{70} region 4 and the β-flap. (*A*) Bacterial 2-hybrid assay adapted to detect bridging interactions. Diagram depicts how simultaneous interactions between AsiA and the fused β-flap and σ^{70} region 4 moieties activate transcription from test promoter p*lac*O_L2–62. The asterisk indicates that the fused σ^{70} region 4 moiety contains the L607P substitution. (*B*) Results of β-galactosidase assays. The assays were performed with AY101 cells containing 3 compatible plasmids, one encoding the indicated λCI-β-flap fusion protein, a second encoding the indicated α-σ^{70} region 4 (L607P) fusion protein, and a third encoding either no protein or wild-type AsiA. The σ^{70} moiety of the α-σ^{70} region 4 fusion protein bore amino acid substitution D581G (in addition to substitution L607P); substitution D581G, which has been described (54), stabilizes the folded structure of the σ^{70} moiety of the fusion protein, facilitating the detection of its interactions in the 2-hybrid system. Strain AY101 contains a chromosomal mutation specifying σ^{70} substitution F563Y, which renders cellular σ^{70}-dependent transcription less susceptible to AsiA-mediated toxicity (55). The plasmids directed the synthesis of the fusion proteins (or AsiA) under the control of IPTG-inducible promoters, and the cells were grown in the presence of increasing concentrations of IPTG. Western blot analysis ruled out the possibility that the failure of AsiA to activate transcription in cells containing the λCI–β-flap (Δ900–909) is attributable to protein instability (Fig. S2*B*).

β-flap replace interactions between σ^{70} region 4 and the β-flap. As a first test of this model, we used a bacterial 2-hybrid assay (25–27) to determine whether AsiA can interact directly with the β-flap. In this assay, contact between a protein domain fused to a component of RNAP (here, the α-subunit) and a partner protein fused to a DNA-binding protein (here, the CI protein of bacteriophage λ) activates transcription of a *lacZ* reporter gene under the control of a test promoter bearing an upstream recognition site for the DNA-binding protein (here, a λ operator) (Fig. 1*A*). We previously used this 2-hybrid assay to study both the σ^{70} region 4/β-flap interaction (28) and the σ^{70} region 4/AsiA interaction (23, 29). To assay the ability of AsiA to interact with the β-flap, we used a λCI–AsiA fusion protein and an α-β-flap fusion protein (bearing the β-flap in place of the C-terminal domain of α). We found that *lacZ* transcription was increased significantly only in cells that contained both the λCI–AsiA and the α-β-flap fusion proteins (Fig. 1*B*), suggesting that AsiA can interact directly with the β-flap.

We next asked whether AsiA and σ^{70} region 4 interact with overlapping determinants of the β-flap. Prior work has established that the β-flap–tip helix (β residues 900–909) is the primary determinant of the σ^{70} region 4/β-flap interaction (30) and that removal of the β-flap–tip helix eliminates any detectable interaction between σ^{70} region 4 and the β-flap in the 2-hybrid assay (31). We found that removal of the β-flap–tip helix also eliminated the interaction between AsiA and the β-flap (Fig. 1 *B* and *C*), indicating that AsiA and σ^{70} region 4 interact with overlapping determinants of the β-flap.

AsiA Can Make Simultaneous Contact with the β-Flap and σ^{70} Region 4. The finding that AsiA and σ^{70} region 4 interact with overlapping determinants of the β-flap provides support for the idea that AsiA/β-flap interactions replace σ^{70} region 4/β-flap interactions when the AsiA-containing RNAP holoenzyme is formed. According to this model, AsiA would interact with both the β-flap and σ^{70} region 4 in the context of the AsiA-containing holoenzyme. This model thus specifies that AsiA should be able to make simultaneous contact with the β-flap and σ^{70} region 4. To test this prediction, we used a version of our 2-hybrid assay (32) that enabled us to ask whether AsiA could serve as a "bridge" between the β-flap (fused to λCI) and σ^{70} region 4 (fused to α).

Because σ^{70} region 4 and the β-flap interact directly, without the requirement for a bridging protein, our experimental strategy depended on our ability to genetically disrupt the σ^{70} region 4/β-flap interaction without affecting either the σ^{70} region 4/AsiA interaction or the AsiA/β-flap interaction. To accom-

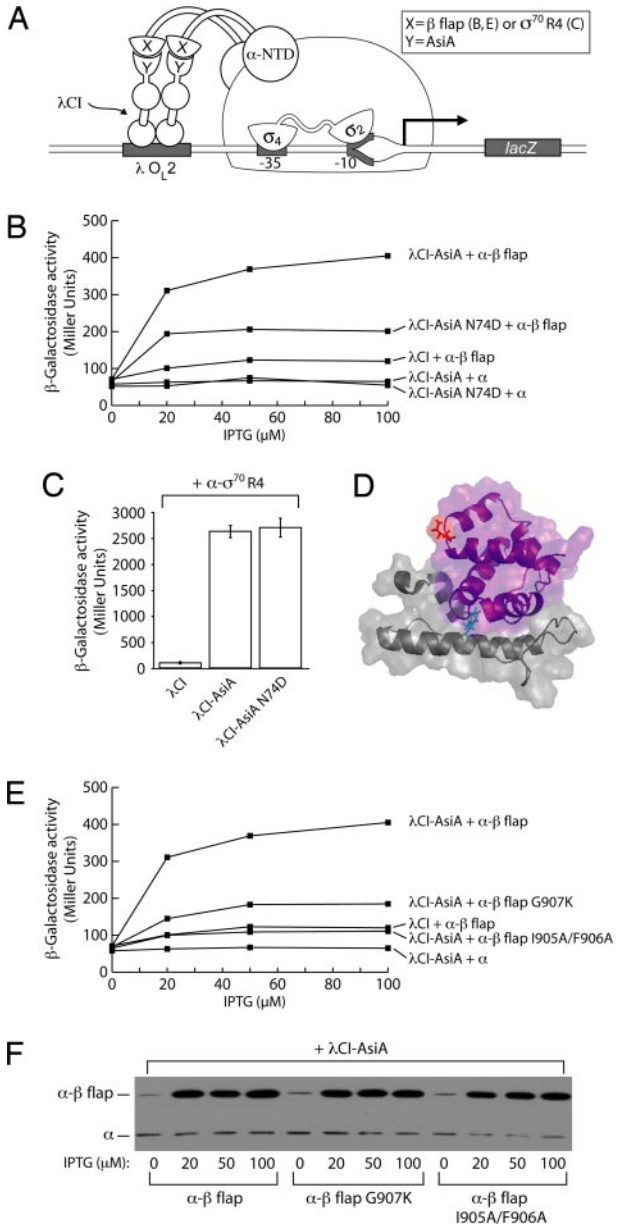

Fig. 3. Amino acid substitutions in AsiA and the β-flap that weaken the AsiA/β-flap interaction. (*A*) Bacterial 2-hybrid assay used to detect interactions of AsiA. Diagram depicts how the interaction between AsiA fused to λCI and either the β-flap (*B* and *E*) or σ^{70} region 4 (*C*) fused to the α-NTD activates transcription from test promoter p*lac*O_L2–62. The AsiA moiety of the λCI–AsiA fusion protein bore amino acid substitution K20A (see legend to Fig. 1*B*). (*B*) Substitution N74D in AsiA weakens the AsiA/β-flap interaction. Results of β-galactosidase assays performed with FW102 O_L2–62 cells containing 2 compatible plasmids, one encoding either λCI or the indicated a λCI–AsiA fusion protein, and the other encoding either α or the α–β-flap fusion protein. The plasmids directed the synthesis of the fusion proteins under the control of IPTG-inducible promoters and the cells were grown in the presence of increasing concentrations of IPTG. (We note that the specific effect of the AsiA N74D substitution on the AsiA/β-flap interaction excludes the formal possibility that the apparent interaction between AsiA and the β-flap is mediated by free σ^{70} forming bridging interactions between the fused AsiA and β-flap moieties.) (*C*) Substitution N74D in AsiA does not affect the σ^{70} region 4/AsiA interaction. Results of β-galactosidase assays performed as described in *B*, only with one plasmid encoding either λCI or the indicated λCI–AsiA fusion protein, and the other encoding an α-σ^{70} region 4 fusion protein. The σ^{70} moiety of the α-σ^{70} region 4 fusion protein bore amino acid substitution D581G (see legend to Fig. 2*B*). The cells were grown in the presence of 20 μM IPTG. The graph shows the averages of 3 independent measurements and SDs. (*D*) Structure of AsiA/σ^{70} region 4 complex (24) with AsiA and σ^{70} region 4 colored purple and gray, respectively. The complex is shown as a transparent space-filling model with protein backbones represented as ribbon diagrams. AsiA residue N74 (highlighted in red) is shown as a stick representation. σ^{70} residue F563, which lies at the AsiA/σ^{70} region 4 interface, is also highlighted (cyan). The figure was generated by using PyMOL (Protein Data Bank ID code 1TLH). (*E*) Substitutions G907K and I905A/F906A in the β-flap weaken the AsiA/β-flap interaction. Results of β-galactosidase assays performed with FW102 O_L2–62 cells containing 2 compatible plasmids, one encoding either λCI or the λCI–AsiA fusion protein, and the other encoding either α or the indicated α-β-flap fusion protein. The plasmids directed the synthesis of the fusion proteins under the control of IPTG-inducible promoters and the cells were grown in the presence of increasing concentrations of IPTG. (*F*) Western blot analysis to assess intracellular levels of the α-β-flap fusion proteins. Samples from the cell lysates assayed for β-galactosidase (*E*) were processed for Western blot analysis as described (53). Bands corresponding to α–β-flap fusion proteins and chromosomally-encoded α are indicated. The results ruled out the possibility that the disruptive effects of substitutions G907K and I905A/F906A are attributable to destabilization of the α–β-flap fusion protein.

plish this, we took advantage of a previously characterized amino acid substitution in σ^{70} region 4 (L607P) that disrupts the σ^{70} region 4/β-flap interaction (28), but does not significantly affect the σ^{70} region 4/AsiA interaction (Fig. S1). As expected, the λCI–β-flap fusion protein activated transcription only weakly in the presence of the α-σ^{70} region 4 (L607P) fusion protein (Fig. 2*B* and Fig. S1). Introduction of AsiA into cells containing these fusion proteins resulted in a significant (≈3.5-fold) increase in *lacZ* transcription (Fig. 2*B*). Control assays indicated that this high level of transcription was observed only in the presence of both fusion proteins (Fig. S2*A*), suggesting that AsiA was indeed serving as a bridge between the fused β-flap and σ^{70} region 4 moieties.

To establish that the observed AsiA-dependent increase in transcription required both the AsiA/β-flap interaction and the σ^{70} region 4/AsiA interaction, we genetically disrupted each of these interactions. To do this, we used either a λCI–β-flap fusion protein lacking the β-flap–tip helix or an α-σ^{70} fusion protein carrying amino acid substitution F563Y, which weakens the σ^{70} region 4/AsiA interaction (23). We found that disrupting either the AsiA/β-flap interaction or the σ^{70} region 4/AsiA interaction abolished the stimulatory effect of AsiA on *lacZ* transcription (Fig. 2*B*). We therefore conclude that AsiA can interact simultaneously with the β-flap and σ^{70} region 4.

Identification of Amino Acid Substitutions That Weaken the AsiA/β-Flap Interaction. To assess the mechanistic relevance of the AsiA/β-flap interaction as detected in the 2-hybrid assay, we sought to determine the effect of disrupting the AsiA/β-flap interaction on the ability of AsiA to inhibit σ^{70}-dependent transcription. For this purpose, we used the bacterial 2-hybrid assay (Fig. 3*A*) to identify amino acid substitutions in either AsiA or the β-flap that disrupted the AsiA/β-flap interaction. We found that a previously isolated AsiA substitution, N74D, specifically disrupted the AsiA/β-flap interaction, but did not affect the σ^{70} region 4/AsiA interaction (Fig. 3 *B* and *C*). Consistent with these genetic data, the structure of an AsiA/σ^{70} region 4 complex (24) indicates that N74 is exposed on a surface of AsiA that is distant from the AsiA/σ^{70} region 4 interface (Fig. 3*D*).

Although removal of the β-flap–tip helix abolishes the AsiA/β-flap interaction, the flap–tip helix is strictly required for the recognition of −10/−35 promoters. We therefore wanted to identify amino acid substitutions within the flap-tip helix that weakened the AsiA/β-flap interaction, but did not prevent recognition of −10/−35 promoters. We tested the effects of several previously characterized amino acid substitutions in the β-flap–tip helix (31) and found that substitution G907K moderately disrupted the AsiA/β-flap interaction, and amino acid substitutions I905A and F906A in combination strongly disrupted the AsiA/β-flap interaction (Fig. 3 *E* and *F*). Although

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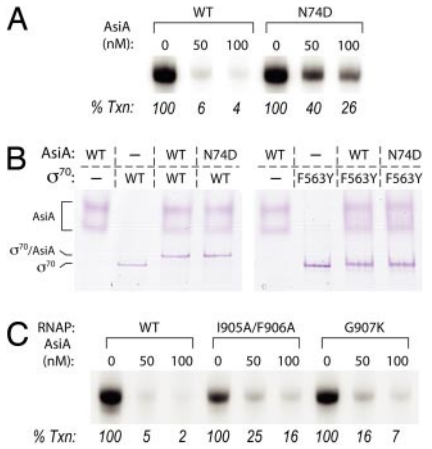

Fig. 4. Weakening the AsiA/β-flap interaction compromises AsiA-dependent transcription inhibition. (*A*) Substitution N74D in AsiA compromises AsiA-dependent transcription inhibition in vitro. Results of single-round in vitro transcription assays performed as described in *SI Text*, using wild-type RNAP holoenzyme in the absence or presence of increasing concentrations (50 or 100 nM) of the indicated AsiA protein. Radiolabeled transcripts and quantification from 1 representative experiment are shown (see Fig. S4*A* for averages and SDs of 3 independent experiments). Control assays indicated that purified AsiA proteins were free of contaminating σ^{70}. (*B*) Substitution N74D in AsiA does not affect protein stability in vitro. The indicated AsiA and σ^{70} proteins were incubated alone or in combination before electrophoresis through a native polyacrylamide gel. Proteins were visualized by Coomassie blue staining. Wild-type AsiA and AsiA bearing the N74D substitution formed electrophoretically-stable complexes with full-length wild-type σ^{70} (3rd and 4th lanes). Control assays indicated that σ^{70} substitution F563Y, which disrupts the σ^{70} region 4/AsiA interaction (23), prevented the formation of electrophoretically-stable complexes (compare last 2 lanes with 3rd and 4th lanes). (*C*) Substitutions G907K and I905A/F906A in the β-flap compromise AsiA-dependent transcription inhibition in vitro. Results of single-round in vitro transcription assays performed using RNAP holoenzyme reconstituted with the indicated core enzyme in the absence or presence of increasing concentrations (50 or 100 nM) of wild-type AsiA protein. Radiolabeled transcripts and quantification from 1 representative experiment are shown (see Fig. S4*B* for averages and SDs of 3 independent experiments).

these substitutions also affect the σ^{70} region 4/β-flap interaction (Fig. S3 and ref. 31), reconstituted RNAP holoenzymes bearing these substitutions can initiate transcription from −10/−35 promoters in vitro (31).

Disrupting the AsiA/β-Flap Interaction Compromises AsiA-Mediated Transcription Inhibition. We performed in vitro transcription assays to assess the effect of disrupting the AsiA/β-flap interaction on the ability of AsiA to inhibit transcription from a −10/−35 promoter (T7A2). We found that weakening the AsiA/β-flap interaction with substitution N74D in AsiA substantially reduced AsiA-mediated transcription inhibition (Fig. 4*A*). The N74D substitution did not affect the ability of AsiA to form a stable complex with wild-type σ^{70}, indicating that the reduced ability of AsiA N74D to inhibit transcription is not attributable to altered protein stability (Fig. 4*B*). Weakening the AsiA/β-flap interaction with the β-flap substitutions (G907K or I905A and F906A) also reduced AsiA-mediated transcription inhibition (Fig. 4*C*). Taken together, the results in Fig. 4 establish that weakening the AsiA/β-flap interaction compromises the ability of AsiA to inhibit σ^{70}-dependent transcription.

Discussion

Here, we demonstrate that AsiA interacts with the β-flap (Fig. 1), AsiA can interact simultaneously with the β-flap and σ^{70} region 4 (Fig. 2), and weakening the AsiA/β-flap interaction compromises AsiA-dependent transcription inhibition (Figs. 3 and 4). We propose that the interaction of AsiA with the β-flap helps to stabilize the AsiA-containing RNAP holoenzyme and that, when complexed with the RNAP holoenzyme, AsiA makes simultaneous contact with the β-flap and σ^{70} region 4 (Fig. 5*A*).

The Role of the AsiA/β-Flap Interaction. The possibility that AsiA interacts directly with the β-flap has been raised previously, based on the observation that the β-flap–tip helix shares amino acid similarity with portions of σ^{70} region 4 that interact with AsiA (22). However, our finding that AsiA can interact simultaneously with σ^{70} region 4 and the β-flap indicates that AsiA must present distinct binding sites for σ^{70} region 4 and the β-flap. The significance of the amino acid similarity between the β-flap–tip helix and σ^{70} region 4 is therefore uncertain.

In prior work we demonstrated that the ability of AsiA to stably associate with the RNAP holoenzyme and inhibit σ^{70}-dependent transcription depends on the strength of the σ^{70} region 4/β-flap interaction (23). Thus, we showed that substitutions in σ^{70} region 4 that weaken the σ^{70} region 4/β-flap interaction facilitate AsiA-dependent transcription inhibition and substitutions that strengthen the σ^{70} region 4/β-flap interaction have the opposite effect. These findings provide an explanation for an apparent anomaly in our data here. Specifically, 2-hybrid analysis indicated that β-flap substitutions G907K and I905A/F906A weakened the AsiA/β-flap interaction to a greater extent than did AsiA substitution N74D (Fig. 3). Nevertheless, we found that the N74D substitution compromised AsiA-dependent transcription inhibition to a greater extent than did the β-flap substitutions (Fig. 4). The likely explanation is that the β-flap substitutions weaken not only the AsiA/β-flap interaction, but also the σ^{70} region 4/β-flap interaction (Fig. S3 and ref. 31), thereby exerting opposing effects on the ability of AsiA to associate with the RNAP holoenzyme.

Anti-σ factors typically function by occluding core-binding determinants in their cognate σ factors. Among the structurally characterized anti-σ factors, all except AsiA interact with 2 or more structural domains of σ simultaneously (15). In contrast, our results indicate that AsiA interacts with 1 structural domain of σ^{70} (region 4) and 1 structural domain of RNAP core enzyme (the β-flap). This difference may reflect the fact that AsiA has an additional function that requires its presence as a stable component of the RNAP holoenzyme: to serve as a coactivator of T4 middle gene transcription (33). T4 middle promoters, which are recognized by the σ^{70}-containing RNAP holoenzyme, bear a near-consensus −10 element and a binding site for the T4-encoded transcription activator MotA, centered at position −30 (34–36). Activation of T4 middle gene transcription, which depends on the AsiA-containing RNAP holoenzyme, requires an interaction between DNA-bound MotA and σ^{70} region 4 (37). Genetic analysis of the MotA/σ^{70} region 4 interaction suggests that MotA interacts with determinants of σ^{70} that would be occluded when σ^{70} region 4 is bound to the β-flap (37); thus it has been proposed that the role of AsiA as a coactivator of middle gene transcription is to expose this otherwise occluded surface of σ^{70} region 4 so it is accessible to MotA (33, 38).

The β-Flap as a Principal Target Site for Regulatory Proteins. The β-flap plays important roles during all stages of the transcription cycle: initiation, elongation, and termination. As mentioned above, during initiation, the interaction between the β-flap and σ region 4 facilitates promoter binding (12). Furthermore, structural work indicates that the β-flap largely defines the RNA exit channel and the nascent RNA emerges from underneath the β-flap (39–41). Consequently, the β-flap can influence transcription pausing and termination through interactions with the nascent RNA (42–45).

The diverse functional roles played by the β-flap during the transcription cycle make the β-flap a plausible target for regulatory factors. However, to date, only a few examples of regulatory proteins that target the β-flap have been described, including the bacteriophage T4-encoded coactivator of late-gene transcription

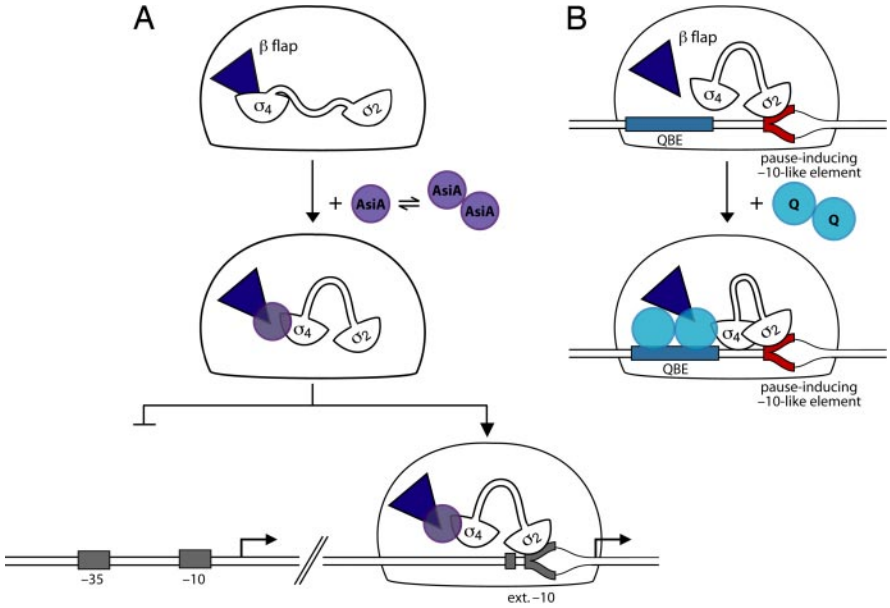

Fig. 5. Interactions with σ^{70} region 4 and the β-flap stabilize the association of both AsiA and λQ with the RNAP holoenzyme. (*A*) AsiA, which forms a binary complex with σ^{70} (not depicted) prior to the formation of the AsiA-containing RNAP holoenzyme (56), is shown interacting with σ^{70} region 4 and the β-flap in the context of the RNAP holoenzyme. The AsiA-containing holoenzyme is unable to use −10/−35 promoters, but can initiate transcription from extended −10 promoters. (*B*) λQ engages the RNAP holoenzyme during early elongation at the bacteriophage λ late promoter, $P_{R'}$. After transcription initiates at $P_{R'}$, the RNAP holoenzyme pauses when σ^{70} region 2 encounters a pause-inducing sequence that resembles a promoter −10 element (*Upper*). When bound to its DNA recognition site (the QBE), λQ establishes contact with both σ^{70} region 4 and the β-flap (*Lower*). These protein–protein interactions facilitate the stable association of λQ with the paused elongation complex.

gp33 (46) and the lambdoid phage-encoded Q antiterminator proteins (31). Gp33 functions in the context of a holoenzyme containing the phage-encoded σ factor, gp55, a truncated member of the σ^{70} family that bears weak homology to σ region 2 (47). When bound to the β-flap, gp33 serves as a structural analogue to σ region 4, linking RNAP to the DNA indirectly, via an interaction with the sliding clamp protein gp45 (the activator of late-gene transcription) (46). In contrast to gp33, which affects transcription initiation, the Q antiterminator proteins associate with RNAP during transcription elongation and confer on the enzyme the ability to read through transcription terminators (48). Our finding that AsiA, another regulator of transcription initiation, also targets the β-flap provides support for an emerging view that the β-flap can be targeted by regulatory proteins during multiple stages of the transcription cycle.

The mechanistic requirement for AsiA to interact with both σ^{70} region 4 and the β-flap bears a striking resemblance to events that facilitate the stable association of the bacteriophage λ Q protein with RNAP (Fig. 5). Whereas AsiA engages the RNAP holoenzyme before promoter binding, the λQ protein engages the RNAP holoenzyme during a σ^{70}-dependent early elongation pause (48); nevertheless, in both cases, the stable association of the regulator with RNAP evidently depends on interactions with both σ^{70} region 4 (49) and the β-flap (31). Simultaneous association with a domain of σ and a domain of the core enzyme may be a general strategy used by regulators that target a specific form of the RNAP holoenzyme.

Methods

Strains and Plasmids. A complete list of strains and plasmids is provided in Tables S1 and S2.

Proteins. Purification of wild-type and mutant AsiA proteins bearing an N-terminal hexahistidine tag is described in *SI Text*. Wild-type and mutant σ^{70} proteins bearing an N-terminal hexahistidine tag were purified from BL21(DE3) cells transformed with plasmid pLNH12His$_6$-σ^{70} or its His$_6$-σ^{70} mutant derivatives [procedure as described (50)]. *E. coli* RNAP core enzyme used in Fig. 3 was purchased from Epicentre. Wild-type and mutant RNAP core enzymes [prepared as described (31)] were gifts from P. Deighan (Harvard Medical School, Boston, MA).

β-Galactosidase Assays. *LacZ* expression was determined from β-galactosidase assays performed with microtiter plates and a microtiter plate reader [procedure as described (51)]. In experiments performed in the presence of increasing concentrations of IPTG, assays were conducted 3 times in duplicate on separate occasions with similar results. Values represent averages from 1 experiment; duplicate measurements differed by <5%. In experiments performed in the presence of a single IPTG concentration, values represent the averages of 3 independent measurements (with SDs).

AsiA/σ^{70} Binding Assays. Binding assays were performed essentially as described (52). AsiA (80 pmol) and σ^{70} (14 pmol) were incubated alone or in combination at 37 °C for 5 min before electrophoresis through a 4–15% native polyacrylamide gel (BioRad) in Tris-glycine buffer [30 mM Tris·HCl (pH 8.0), 192 mM glycine] at 100 V for 90 min. Proteins were visualized by Coomassie blue staining.

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