Published in final edited form as:

J Mol Biol. 2006 November 10; 363(5): 931–944.

Mutational analysis of σ^{70} region 4 needed for appropriation by the bacteriophage T4 transcription factors AsiA and MotA

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Abstract

Transcriptional activation of T4 middle promoters requires σ^{70} -containing *E. coli* RNA polymerase, the T4 activator MotA, and the T4 co-activator AsiA. T4 middle promoters contain the σ^{70} –10 DNA element. However, these promoters lack the σ^{70} –35 element, having instead a MotA box centered at –30, which is bound by MotA. Previous work has indicated that AsiA and MotA interact with region 4 of σ^{70} , the C-terminal portion that normally contacts –35 DNA and the β -flap structure in core. AsiA binding prevents the σ^{70} / β -flap and σ^{70} /–35 DNA interactions, inhibiting transcription from promoters that require a –35 element. To test the importance of residues within σ^{70} region 4 for MotA and AsiA function, we investigated how σ^{70} region 4 mutants interact with AsiA, MotA, and the β -flap and function in transcription assays *in vitro*. We find that alanine substitutions at residues 584–588 (region 4.2) do not impair the interaction of region 4 with the β -flap or MotA, but they eliminate the interaction with AsiA and prevent AsiA inhibition and MotA/AsiA activation. In contrast, alanine substitutions at 551–552, 554–555 (region 4.1) eliminate the region 4/ β -flap interaction, significantly impair the AsiA/ σ^{70} interaction, and eliminate AsiA inhibition. However, the 4.1 mutant σ^{70} is still fully competent for activation if both MotA and AsiA are present. A previous NMR structure shows AsiA binding to σ^{70} region 4, dramatically distorting regions 4.1 and 4.2 and indirectly changing the conformation of the MotA interaction site at the σ^{70} C-terminus. Our analyses provide biochemical relevance for the σ^{70} residues identified in the structure, indicate that the interaction of AsiA with σ^{70} region 4.2 is crucial for activation, and support the idea that AsiA binding facilitates an interaction between MotA and the far C-terminus of σ^{70} .

Keywords

transcription; σ^{70} ; AsiA; MotA; polymerase

Introduction

Prokaryotic RNA polymerase holoenzyme is composed of a core (subunits β , β' , α_2 , ω) that has RNA synthesizing activity and a σ factor that imparts DNA specificity^{1,2}. The primary σ factor in *E. coli*, σ^{70} , recognizes promoters that are needed for transcription of general housekeeping genes and thus, is responsible for most of the transcription during exponential growth³. Sequence, structure, and biochemical studies indicate that σ^{70} shares four regions of similarity with primary σ proteins of other prokaryotes^{1,4}. When present in RNA polymerase holoenzyme ($E\sigma^{70}$), σ^{70} sets the start site for transcription by recognizing various DNA

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elements, and residues within each of the 4 regions have been shown to interact with sequences in promoter DNA ^{5; 6; 7; 8; 9}. The majority of *E. coli* promoters have -10 and -35 DNA elements, which are contacted by σ^{70} regions 2 and 4, respectively ^{8; 9}. Specific base recognition of the -35 sequences arises through a DNA-binding helix-turn-helix (H-T-H) motif in region 4.2 (H3-T-H4 in Fig. 1A). In addition, interactions between region 4 and core, particularly a structure called the β -flap, are required for proper positioning of regions 2 and 4 so they can contact the -10 and -35 elements simultaneously ^{10; 11; 12; 13} (Fig. 1A).

The presence of a good match to both the -10 and -35 canonical sequences is usually sufficient for σ^{70} to recognize a promoter without the aid of additional factors. However, activation factors can be needed when either sequence element deviates significantly from the consensus. Many activators in *E. coli* work through class I or class II activation mechanisms (reviewed in ^{14; 15}). Class I activators interact with residues in the C-terminal domain (CTD) of the α subunit of polymerase whereas a class II activator may also interact with residues in σ^{70} region 4. MotA/AsiA activation of bacteriophage T4 middle promoters arises through a different mechanism, called σ appropriation (reviewed in ¹⁶). MotA protein is a transcriptional activator ^{17; 18; 19}, whose CTD binds to a MotA box element, centered at -30 in T4 middle promoter DNA, and whose NTD interacts with the far C-terminal portion of σ^{70} , ²⁰ (Fig. 1A). AsiA is needed to co-activate transcription from T4 middle promoter ²¹. However, AsiA by itself is also an inhibitor of σ^{70} -dependent transcription from -10/-35 promoters ^{22; 23; 24}. Several studies have indicated that AsiA binds tightly to σ^{70} regions 4.1 and 4.2 ^{22; 23; 25; 26; 27; 28; 29; 30; 31; 32} (Fig. 1A). A recent structure of the AsiA/ σ^{70} region 4 complex implicated 17 σ^{70} residues as AsiA contacts and indicated that AsiA structurally remodels region 4, converting the DNA-binding H3-T-H4 to a continuous helix ³⁰. It has been suggested that this remodeling could also serve to facilitate the interactions of MotA with the MotA box and with the C-terminus of σ^{70} , ^{20; 30}.

Here we have performed mutational analyses to investigate the molecular interface used by σ^{70} with AsiA and MotA, and to ask whether there is biochemical support for σ^{70} residues that were identified in the σ^{70} region 4/AsiA structure. We demonstrate that substitutions of σ^{70} residues in regions 4.1 and 4.2 that interact with AsiA in the AsiA/ σ^{70} region 4 structure ³⁰ do indeed impair the interaction of AsiA with σ^{70} . We also find that substitutions within region 4.1 that significantly compromise the interaction of AsiA with σ^{70} impair the ability of AsiA to inhibit transcription. However, in the presence of both MotA and AsiA, polymerase containing the 4.1 substitutions remains fully competent for activation. In contrast, substitutions in σ^{70} region 4.2 that eliminate the AsiA/ σ^{70} interaction render polymerase immune both to AsiA inhibition and MotA/AsiA activation. Our work indicates that the interaction of AsiA with its contacts in σ^{70} region 4.2 is crucial for σ appropriation by MotA and AsiA and supports a model in which AsiA binding and remodeling of σ^{70} region 4 serve to facilitate the interaction of MotA with σ^{70} and with MotA box DNA.

Results

Rationale

Based on crystal structures using the primary σ factors of *T. aquaticus* and *T. thermophilus* and biochemical analyses with σ^{70} mutants (reviewed in ^{2; 33}), specific residues with σ^{70} region 4 are thought to be crucial for transcription from σ^{70} -dependent -10/-35 promoters, either because they contact the -35 DNA element or because they interact with the β -flap structure of core. The NMR structure of a complex of AsiA with σ^{70} region 4 ³⁰ shows that many of these same residues contact AsiA and thus, predicts that these residues may also be important for AsiA inhibition and/or MotA/AsiA activation. In addition, biochemical evidence ²⁰ has suggested that some of the residues within the far C-terminal portion of σ^{70} that normally interact with the β -flap may coincide with residues that contact MotA during MotA/AsiA

activation. (These various residues in regions 4.1 and 4.2 and the C-terminal portion of σ^{70} are indicated at the top of Fig. 1A.)

To investigate the functional significance of several of the σ^{70} residues that the structural work predicts could be important for AsiA and MotA function, we used σ^{70} proteins with mutations within regions 4.1 and 4.2 and the C-terminal portion of σ^{70} (listed in the bottom portion of Fig. 1A). Mutations in region 4.1 included one at F563 (F563Y), which was previously found in a 2-hybrid screen to be somewhat defective for an interaction with AsiA³⁴. However, this and another screen did not reveal an importance for other region 4.1 residues that contact AsiA in the AsiA/region 4 structure^{34; 35}. Thus, we constructed σ^{70} mutants with alanine substitutions within region 4.1 that should either affect (σ^{70} 551-552, 554-555A) or not affect (σ^{70} 557, 560A) the interaction of AsiA with region 4 based on the AsiA/region 4 structure. For region 4.2, we made a σ^{70} mutant that contained alanine substitutions at residues that directly interact with the -35 DNA element (σ^{70} 584-588A). Although residues within this portion of region 4.2 interact with AsiA in the structure, a previous analysis of region 4.2 substitutions had not revealed an importance for this portion of 4.2 for AsiA binding³⁶. Finally, we compared the functions of a fused σ^{70}/σ^{38} protein that contains multiple changes within the C-terminal portion of σ^{70} (σ^{70}/σ^{38} H5) with the function of a σ^{70}/σ^{38} fusion (σ^{70}/σ^{38} region 4) that contains multiple changes throughout region 4, including residues that are predicted to interact with AsiA, and with the function of the primary σ of pertussis ($\sigma^{\text{pertussis}}$) that despite multiple changes within region 4 from σ^{70} , retains all the predicted AsiA contact residues. The various σ^{70} mutants were then tested in assays to determine the effects of the σ^{70} mutations on AsiA inhibition, MotA/AsiA activation, and the interactions of σ^{70} region 4 with AsiA, MotA, and the β -flap.

Substitutions within σ^{70} region 4.1 that impair the interaction of σ^{70} with AsiA do not affect MotA/AsiA activation at the T4 middle promoter P_{uvsX}

During a T4 infection, transcription from most T4 middle promoters, such as P_{uvsX} (Fig. 1B), is fully dependent on AsiA and MotA. However, *in vitro* wild type polymerase can use P_{uvsX} in the absence of MotA and AsiA, presumably because of the presence of a perfect σ^{70} -10 element and partial matches to the σ^{70} -35 element and to the UP element sequence (for binding by the α subunit of core³⁷). Consequently, we used transcription from P_{uvsX} to monitor the effect of various σ^{70} mutations on transcription by polymerase alone and with AsiA and/or MotA (Fig. 2). As has been seen previously³⁸, polymerase containing wild type σ^{70} yields a moderate level of P_{uvsX} transcription *in vitro*, which is completely inhibited by the presence of AsiA. Addition of both AsiA and MotA yields a high level of activated transcription. In addition, in single round transcription assays and under certain conditions, such as the ones used here, we have found about a 2-fold increase in transcription in the presence of MotA without AsiA (Makela and Hinton, manuscript in preparation).

Like wild type polymerase, the polymerases reconstituted with any of the σ proteins having substitutions within region 4.1 (σ^{70} 551-552,554-555A, σ^{70} 557,560A, σ^{70} 555,557,560A, σ^{70} F563Y) were fully competent for MotA/AsiA activation at P_{uvsX} (Fig. 2). Thus, none of these mutations impaired σ appropriation at P_{uvsX} . In contrast, AsiA inhibition was not observed when using σ^{70} 551-552, 554-555A (Fig. 2A) and as has been reported previously³⁴, AsiA inhibition was impaired by σ^{70} F563Y (Fig. 2B). (Although the level of transcription with σ^{70} 551-552, 554-555A was extremely low, the results were highly reproducible.)

The ability of the σ^{70} mutants to interact with AsiA and MotA was investigated by two independent assays of protein-protein interactions. In the native gel assay, free σ was incubated with excess AsiA and/or MotA and the formation of protein-protein complexes was detected by a shift in σ migration. Previous work^{39; 40} has shown that either AsiA or MotA alone forms a complex with wild type σ^{70} and a new species is formed in the presence of all three,

indicative of a tertiary complex (Fig. 3). In the *E. coli* 2-hybrid assay, the interaction of σ^{70} region 4 with a bait protein, here AsiAK20A or the N-terminal half of MotA (MotA^{NTD}), which is known to contain the activation domain²⁰, was monitored by an increase in the level of activity of a reporter gene, *lacZ* (Fig. 4A). AsiA with the substitution K20A was used in this assay because previous work has shown that AsiAK20A results in a higher level of β -galactosidase activity when tested with σ^{70} region 4 than does wild type AsiA³⁴.

Interaction assays indicated that alanine substitutions at σ^{70} residues 555, 557, and 560 had little effect on the interaction of σ^{70} with AsiA or MotA. The pattern of complexes seen in the native gel with σ^{70} 555,560A was very similar to that seen with wild type σ^{70} (Fig. 3). 2-hybrid assays indicated that the σ^{70} 555, 557, 560A substitutions had no effect on the interaction with MotA^{NTD} (Fig. 4B, right) and only a slight decrease in the interaction with AsiAK20A (Fig. 4B, left). These results are consistent with the fact either σ^{70} 555, 557, 560A or σ^{70} 557, 560A yielded transcription patterns that were very similar to that of wild type σ^{70} (Fig. 2A).

Results obtained in the interaction assays with the other 4.1 mutants, σ^{70} 551-552, 554-555A and σ^{70} F563Y, were different. Previous results have shown that the σ^{70} F563Y substitution renders σ^{70} region 4 defective in its interaction with AsiAK20A in the 2-hybrid assay³⁴. We found that the 551-552, 554-555A substitutions also impaired the interaction of σ^{70} with AsiAK20A (Fig. 4C, left). In contrast, these substitutions had a more modest effect on the σ^{70} region 4/MotA^{NTD} interaction (Fig. 4C, right). In native gels, σ^{70} 551-552, 554-555 and σ^{70} F563Y were very slightly retarded when AsiA was added (Fig. 3). In the presence of MotA, a much slower migrating species was formed, consistent with the formation of a stable MotA/ σ^{70} complex. Addition of AsiA did not significantly change the position of this species, although a very slight shift (like that seen with AsiA alone) would not have been detected. We conclude that the 551-552, 554-555A and the F563Y substitutions result in a σ^{70} that is impaired in its interaction with AsiA but not with MotA. Consequently, σ^{70} mutant proteins having these substitutions are less susceptible to inhibition by AsiA because they are less able to interact with AsiA. However, the defect for AsiA binding does not impair MotA/AsiA activation. Thus, during transcription either the presence of MotA can improve the defective interaction of AsiA with σ^{70} 551-552, 554-555A or σ^{70} F563Y or an impaired interaction of AsiA with region 4.1 is still sufficient for AsiA to work as a co-activator.

We also used the two-hybrid assays to investigate the interactions of AsiAK20A and MotA^{NTD} with region 4.1 alone (Fig. 4D). We found that AsiAK20A interacts weakly with region 4.1 in the absence of region 4.2. Surprisingly, this interaction was not disrupted by the 551-552, 554-555A substitutions, suggesting that it might arise through different contacts than those used when AsiA interacts with all of region 4. In addition, we found that σ^{70} region 4.1 alone was not sufficient for an interaction with MotA^{NTD}.

Alanine substitutions at residues 584-588 within σ^{70} region 4.2 render σ^{70} defective for an interaction with AsiA and for AsiA inhibition and MotA/AsiA activation at P_{uvx}

The side chains of σ^{70} 4.2 residues R584, E585, and R588 are thought to interact directly with specific base determinants in the -35 sequences of host promoter DNA^{8; 35; 41; 42; 43}. Residues R584 and I587 were identified as contact residues for AsiA in the AsiA/ σ^{70} region 4 structure³⁰ (Fig. 1A), suggesting that AsiA prevents the binding of σ^{70} with the -35 element in part by directly blocking the σ^{70} region 4/DNA interaction. However, a previous analysis, investigating how single alanine substitutions affect the binding AsiA to σ^{70} region 4.2 peptides, had found that residues 584-588 were not important for the interaction of AsiA with region 4.2³⁶. To determine the importance of this portion of region 4.2 when present within all of region 4, we constructed σ^{70} with alanine substitutions at residues 584-588. In the 2-hybrid assay, these mutations eliminated the interaction of AsiAK20A with σ^{70} region 4 (Fig. 4C, left), indicating that one or more of residues 584-588 are indeed important for an interaction

with AsiA. However, we also found that these 4.2 contacts are not sufficient because the interaction of σ^{70} residues 569-613 (region 4.2 plus the C-terminal end of σ^{70}) with AsiA was low (Fig. 4E, left). Results with the native gel assay were difficult to interpret because σ^{70} 584-588A migrated very differently from wild type σ^{70} (Fig. 3). Nevertheless, the addition of AsiA did not significantly affect this migration, a result that was consistent with the lack of a detectable interaction between AsiA and σ^{70} 584-588A in the 2-hybrid assay.

In contrast to the results with AsiAK20A, the σ^{70} 584-588A did not impair the interaction of σ^{70} with MotA^{NTD}. In 2-hybrid assays with all of σ^{70} region 4 and MotA^{NTD}, the 584-588 alanine substitutions gave results that were similar to wild type region 4 (Fig. 4C, right). When using a portion of σ^{70} containing residues 569-613 (region 4.2 to the end of σ^{70}) (Fig. 4E, right), the presence of the substitutions even increased its interaction with MotA^{NTD}. In native gels, the migration of σ^{70} 584-588A was shifted in the presence of MotA without AsiA and the addition of AsiA did not change this migration (Fig. 3). The σ^{70} 584-588A/MotA complex migrated differently from the complex formed with wild type σ^{70} and MotA, again perhaps because the mutant σ alone migrated aberrantly.

In *in vitro* transcription assays, P_{UvsX} transcription by $E\sigma^{70}$ 584-588A was not inhibited by AsiA alone or activated by MotA/AsiA (Fig. 2A). (The 2-fold increase seen with MotA/AsiA is not significant because it is also observed with MotA alone). We conclude that alanine substitutions at σ^{70} residues 584-588 specifically impair the interaction of AsiA with σ^{70} region 4, and this impairment then eliminates AsiA inhibition and MotA/AsiA activation at P_{UvsX} .

Mutation of the far C-terminal region of σ^{70} significantly reduces MotA/AsiA activation

Previous work has indicated that the far C-terminal region of σ^{70} is needed for an interaction between MotA^{NTD} and σ^{70} ,²⁰. Specifically, in the 2-hybrid assay, a σ^{70}/σ^{38} H5 mutation, in which the last 17 residues of σ^{70} (Helix 5 (H5) in Fig. 1A) have been replaced with the corresponding residues of σ^{38} , eliminates the interaction of MotA^{NTD} with σ^{70} region 4, but decreases the interaction with AsiA by only about 50%²⁰. When we tested this mutant in transcription assays, we found that AsiA inhibition was normal and the modest increase in transcription observed with MotA alone was present, but MotA/AsiA activation was significantly reduced (Fig. 2B). This result supports the idea that during σ appropriation, the interaction of σ^{70} H5 with MotA is crucial for activation. However, the increase in transcription observed with MotA alone as well as the low level of MotA/AsiA activation that was observed with σ^{70}/σ^{38} H5 also suggest that either a very poor interaction of σ^{38} H5 with MotA is still somewhat productive for transcription or there is another interaction site for MotA, besides H5, that can promote transcription.

We also tested $\sigma^{70}\Delta 571-613$, which removes the far C-terminal region of σ^{70} as well as all of region 4.2. Previous work with a similar deletion ($\sigma^{70}\Delta 565-613$) had indicated that when only region 4.1 is present, AsiA alone significantly increases transcription from the T4 middle promoter P_{rIIB2} ⁴⁴. Thus, we wondered whether a similar result would be observed with P_{UvsX} . However, in this case, transcription was unaffected by the addition of AsiA or MotA, and there was only a small, but reproducible increase in transcription when both MotA and AsiA were present (Fig. 2A). As was discussed before, in 2-hybrid assays the interaction of region 4.1 alone with MotA^{NTD} was nearly background and the interaction with AsiA was significantly lower than that seen with all of region 4 (Fig. 4D). In native gels, the migration of $\sigma^{70}\Delta 571-613$ was unaffected by the addition of MotA, AsiA, or AsiA and MotA (Fig. 3). These results are consistent with the idea that the deletion of regions 4.2 and H5 significantly impairs the interaction of σ^{70} with either AsiA or MotA. However, the slight increase in transcription that is observed when both MotA and AsiA are present suggests that a weak interaction of AsiA with region 4.1 and an interaction of MotA with a region other than H5 can weakly promote transcription.

Replacement of σ^{70} region 4 with region 4 of σ^{38} eliminates interactions with AsiA and MotA while the primary σ of *B. pertussis* is competent for MotA/AsiA activation

As seen in Fig. 1A, region 4 of σ^{38} differs from region 4 of σ^{70} at many residues, including those that interact with AsiA in the AsiA/ σ^{70} structure and those that include the MotA site in H5. The addition of either AsiA alone or MotA and AsiA together had no significant effect on P_{UVS-X} transcription when using $E\sigma^{70}/\sigma^{38}$ region 4 (Fig. 2B), indicating that σ^{38} region 4 is not competent for AsiA inhibition or MotA/AsiA activation. Furthermore, the 2-fold increase in P_{UVS-X} transcription seen with MotA alone was also eliminated when using $E\sigma^{70}/\sigma^{38}$ region 4. When combined with the results with σ^{70}/σ^{38} H5 and $\sigma^{70}\Delta 571-613$, these results suggest that another interaction site for MotA may lie within region 4.1.

The primary σ of *B. pertussis* also varies from σ^{70} at many residues, including several in region 4 (Fig. 1). However, all of the residues that contact AsiA in the σ^{70} region 4/AsiA structure are identical in the *E. coli* and *B. pertussis* primary σ proteins. We found that $E\sigma^{pertussis}$ was significantly inhibited by AsiA (Fig. 2B), which is consistent with known AsiA/ σ^{70} contacts. In contrast, the far C-terminal region (H5) of the *pertussis* σ differs at several residues from that of σ^{70} . Despite these differences, we found that $E\sigma^{pertussis}$ was significantly activated by MotA/AsiA (Fig. 2B). This result suggests that none of the differences between $\sigma^{pertussis}$ and σ^{70} in H5 are crucial for the σ^{70} /MotA interaction.

Effect of σ^{70} mutations on the interaction of σ^{70} region 4 with the β -flap

Because many residues within region 4 are thought to interact with the β -flap^{10; 11; 12; 13} and AsiA is thought to prevent the σ^{70} / β -flap interaction³⁴, we investigated the effect of two of the σ^{70} region 4 mutants on the interaction of region 4 with the β -flap in the 2-hybrid assay. Previous work has shown that the interaction of region 4 with the β -flap can be monitored using this assay, provided that σ^{70} region 4 contains the substitution D581G¹³. D581G is thought to stabilize the H-T-H conformation of region 4.2 (H3-T-H4 in Fig. 1A) and does not affect the interaction of region 4 with the -35 DNA element^{13; 45}. In addition, previous work has also indicated that region 4 of σ^{38} , which contains a glycine at the position corresponding to σ^{70} D581, is fully competent for the interaction with the β -flap¹³. We found here that the alanine substitutions at 551-552, 554-555 eliminated the interaction of σ^{70} region 4 with the β -flap (Fig. 5A). This result is consistent with the very low level of transcription observed by polymerase containing the σ^{70} 551-552, 554-555A mutant in the absence of AsiA and MotA (Fig. 2A). However, this result was surprising since none of the substituted residues have been implicated in an interaction with the β -flap (Fig. 1A)^{45; 11; 12}. In contrast, the interaction of region 4 with the β -flap was actually increased by the alanine substitutions at 584-588 (Fig. 5B). We conclude that the significant reduction in transcription observed with polymerase containing σ^{70} 584-588A is not due to its inability to interact with the β -flap. Thus, it is likely to stem from the loss of the side chains at R584, E585, and R588, which are thought to interact directly with base determinants in the -35 DNA^{8; 35; 42; 43}.

In addition to the interaction between σ^{70} region 4 and the β -flap, contact between σ^{70} region 4.1 residues 555, 562, and 565 and another region of core, a portion of β' (residues 393 to 402), has been proposed^{10; 46}. However, our 2-hybrid assays using pCl-rpoC (357-430) and pBR α - σ^{70} or pBR α - σ^{70} D581G failed to detect any interaction (data not shown).

Discussion

Interface of σ^{70} region 4 with AsiA and MotA

T4 middle promoters are hybrid promoters, which contain an excellent match to the σ^{70} -10 element, but replace the σ^{70} -35 element with the MotA box sequence centered at -30 (reviewed in⁴⁷). The T4 MotA and AsiA proteins are needed to switch the sequence specificity

of polymerase for the upstream promoter DNA (reviewed in ¹⁶). For host promoters, polymerase recognition of a promoter sequence involves contact with the DNA but also the correct positioning of the DNA-binding domains within polymerase. In *E. coli*, recognition of -10/-35 promoters requires interaction between residues in σ^{70} region 2 and a -10 element and between residues in regions 4.1 and 4.2 and a -35 DNA element, respectively ^{1; 8; 9}. (Fig. 6A; highlighted in pink in Fig. 1A). Specific base determinants in the -35 DNA are contacted by 4.2 residues R584 ^{8; 35; 42}, E585 ^{8; 43}, and perhaps R588 ⁴¹, which are present in the second helix of H3-T-H4 (Fig. 1A). The interactions between region 4 and the β -flap are also needed to position region 4 properly relative to region 2, allowing simultaneous contact of both the -10 and -35 elements ^{10; 11; 12; 13; 48}. σ^{70} residues, which are needed for this contact, have been located within region 4.1 and at the C-terminus of σ^{70} , including H5 ^{11; 12; 34; 45} (highlighted in blue in Fig. 1A). Our work suggests that residues 551-552, 554-555 are also important. Alanine substitutions at these positions eliminate the interaction of σ^{70} with the β -flap in a 2-hybrid assay (Fig. 5A) and result in very low levels of transcription by polymerase alone (Fig. 2A). Thus, assuming that the interaction between the β -flap peptide and σ^{70} region 4 in the 2-hybrid assay reflects the interaction in holoenzyme, these results argue that residues 551-555 are involved in the σ^{70} / β -flap interaction or they affect the local conformation. The wild type level of activated P_{uvrX} transcription observed with σ^{70} 551-552, 554-555A in the presence of AsiA (Fig. 2A) and MotA eliminates the possibility that the mutations are deleterious simply because they result in a protein that is generally misfolded.

Previous work has indicated that in its interaction with AsiA, σ^{70} uses a broad interface that includes multiple residues in regions 4.1 and 4.2 (reviewed in ¹⁶). The interface revealed by the σ^{70} region 4/AsiA structure was quite surprising since it showed a previously unseen conformation of region 4, a dramatic reconfiguration that changes the DNA-binding H3-T-H4 to one continuous helix. Prior biochemical analyses that had determined specific AsiA residues needed for the AsiA/ σ^{70} interaction ^{31; 49} were in good agreement with the structure (30 reviewed in ¹⁶), providing support for the AsiA interface. The biochemical analyses here now provide support for several σ^{70} residues implicated by the structure. Alanine substitutions at σ^{70} residues that interact with AsiA in the structure [L551, R554 and E555 (σ^{70} 551-552, 554-555A mutant) or R584 and I587 (σ^{70} 584-588A mutant)] seriously impair AsiA/ σ^{70} binding (Fig. 4C) and AsiA inhibition (Fig. 2A). Substitution of F563, which is also predicted to interact with AsiA, also decreases the AsiA/region 4 interaction and AsiA inhibition (Fig. 2B and ³⁴). In contrast, σ^{70} mutants with substitutions of residues that are not predicted to interact (the σ^{70} 567, 560A and $\sigma^{pertussis}$ mutants) behave like wild type σ^{70} (Fig. 2A and B; Fig. 3). Previous work has also shown that a single alanine substitution at T552 is somewhat defective for the σ^{70} region 4/AsiA interaction and for AsiA inhibition ³⁵. Although T552 is not a contact point in the structure, it is adjacent to the implicated contact residue, L551.

The work here also indicates that the interaction of AsiA with region 4 does not appear to be a simple addition of its contacts with regions 4.1 and 4.2. AsiA does not interact with σ^{70} region 4.1 or region 4.2 alone in a yeast 2-hybrid assay ⁵⁰, and we have seen only a low level of interaction with either region alone in the *E. coli* 2-hybrid assay (Fig. 4D and E). In addition, we found that the effect of the 551-552, 554-555A mutations in the 2-hybrid with AsiA differed whether we used region 4.1 alone (Fig. 4D) or all of region 4 (Fig. 4C). These results might explain the differences between the AsiA contacts reported in the NMR structure of AsiA with all of region 4 ³⁰ and an NMR analysis performed using AsiA and separate σ^{70} peptides for region 4.1 (residues 540 to 565) and region 4.2 (residues 570 to 599) ²⁹.

Presently there is not a structure detailing the molecular interface between MotA and σ^{70} . Previous biochemical evidence has identified H5 as a region of contact for MotA ²⁰. We show here that MotA/AsiA activation is significantly reduced when using $E\sigma^{70}/\sigma^{38}H5$, but not when using $E\sigma^{pertussis}$ (Fig. 2B). These results suggest that L607, S609, F610, and/or L611, the H5

residues that are identical between the *E. coli* and the *pertussis* primary σ factors, may be contact residues for MotA. In addition, the weak transcription observed with AsiA and MotA even when using $\sigma^{70}\Delta 571-613$ (Fig. 2A), in which H5 is missing, or $\sigma^{70}/\sigma^{38}\text{H5}$ (Fig. 2B), in which σ^{70} H5 has been replaced with σ^{38} H5, suggests that it is region 4.1 that may contain additional contacts for MotA.

Model for AsiA/MotA activation at P_{uvsX}

Based on structural 30 and biochemical 20; 31 work, a model for how MotA and AsiA together activate transcription at P_{uvsX} has emerged. AsiA associates with polymerase by binding tightly to free σ^{70} , and then remaining stably bound after σ^{70} binds to core 24; 51; 52; 53. The dramatic conformational change of region 4 that is seen once AsiA is bound would then be incompatible with the interaction of σ^{70} with the -35 DNA and with the β -flap. Thus, AsiA alone acts as an inhibitor of transcription from promoters that require an interaction with the -35 DNA. Because AsiA prevents the interactions of σ^{70} with the β -flap and the -35 DNA, AsiA binding also makes σ^{70} H5 and the MotA box, centered at -30 , available for MotA. Thus, AsiA binding could facilitate MotA activation simply because of its inhibition function. However, our results with the $\sigma^{70}551-552$, $554-555\text{A}$ mutant suggest that eliminating σ^{70} interactions with the β -flap and with the -35 DNA is not sufficient for MotA activation at P_{uvsX} . When the $551-552$, $554-555\text{A}$ mutations were present, we did not detect an interaction between region 4 and the β -flap (Fig. 5A), and transcription was severely compromised (Fig. 2A). These results argue that when polymerase contains $\sigma^{70}551-552$, $554-555\text{A}$, the σ^{70}/β -flap interaction is disrupted and as a consequence, the σ^{70} region 4/ -35 DNA interaction is also lost. However, despite these defects, AsiA was still required for MotA activation (Fig. 2A). Our results suggest that AsiA provides an additional function for MotA apart from disrupting the region 4/ β -flap and region 4/DNA contacts. The AsiA-induced conformational change of H5, observed in the AsiA/ σ^{70} structure 30, would be an additional feature of AsiA binding that could be needed to facilitate MotA interaction with H5 and indirectly the interaction of MotA with MotA box DNA.

Previous work has shown that in the absence of MotA, AsiA alone increases *in vitro* transcription from the T4 middle promoter P_{HIB2} when using a σ^{70} that is truncated after region 4.1 at residue 565 44. This result suggested that MotA may function to remove the interaction of σ^{70} H5 with the β -flap, allowing the interaction of AsiA with σ^{70} region 4.1, which then activates transcription. Thus, when only region 4.1 is present, AsiA activates without MotA. However, we found that σ^{70} truncated just after region 4.1 ($\Delta 571-613$) did not interact with AsiA in the native gel assay (Fig. 3) and did not yield AsiA activation of P_{uvsX} in the absence (or presence) of MotA (Fig. 2A). Thus, we do not detect activation by AsiA alone at P_{uvsX} like that seen at P_{HIB2} . Further studies will be needed to test differences between various T4 middle promoters.

No other activators besides MotA are known to interact with σ^{70} H5. While certain class II activators have been shown to interact with σ^{70} region 4, they use the C-terminal half of H4 in region 4.2 54, 55; 56. However, H5 of σ^{38} may be involved in regulating the activity of $E\sigma^{38}$ at σ^{38} -dependent promoters needed to express genes required to cope with conditions of high salt 57, 58. A thorough understanding of how MotA interacts with σ^{70} H5 may shed light on how the C-terminal portion of σ factors can be used as a target for regulation.

Materials and Methods

DNA and Buffers

Plasmids were constructed using standard cloning procedures. Sequences of primers are available upon request. DNA sequencing (CBR-DNA Sequencing Facility of the University of Maryland, MWG Biotech, or UMDNJ (University of Medicine and Dentistry of New Jersey)

DNA Core facility) throughout the cloned regions confirmed the presence of only the desired sequences.

pDKT90, which contains P_{uvrX} , has been described 59. pBR α - σ^{70} , 60 and pBR α - σ^{70} D581G 61 direct transcription of an α - σ chimera gene, composed of residues 1-248 of α fused in frame to residues 528-613 of σ^{70} , with or without the substitution at D581G. In each case, the gene is under the control of tandem promoters *lpp* and isopropyl- β -D-thiogalactoside (IPTG)-inducible *lacUV5*. pBR α 60 encodes the N-terminal domain of wild type α (residues 1-248). pAC λ cI32 62 encodes residues 1-236 of the bacteriophage λ cI protein under the control of the IPTG-inducible *lacUV5* promoter. pAC Δ -35 λ cI-AsiAK20A 34 expresses a cI-AsiA fusion protein which contains the K20A substitution within the AsiA protein, and pCI-MotA^{NTD} 20 expresses a cI-MotA^{NTD} protein in which the N-terminal domain of *motA* (encoding residues 1 to 97, followed by a non-coded serine) has been fused in frame to cI in pAC λ cI32. The K20A substitution in AsiA was used because this substitution results in a higher level of β -galactosidase activity in the 2-hybrid assay 34. pAC λ cI- β -flap contains the *rpoB* gene encoding residues 858-946 of the β subunit of RNAP (the β -flap moiety) fused in frame to cI in pAC λ cI32 and has been described 13. pCI-rpoC (357-430), in which a portion of the *rpoC* gene encoding residues 357 to 430 of the β' subunit of RNAP is fused in frame to cI in pAC λ cI32 was constructed by first obtaining a PCR product containing this region of *rpoC* using chromosomal DNA from *E. coli* XLI-Blue (Stratagene), a primer that annealed to *rpoC* DNA encoding residues 357 to 362 preceded by a NotI site, a primer that annealed to *rpoC* DNA encoding residues 430 to 425 preceded by a BglII site, and Pfu polymerase (Stratagene). After digestion with BglII and NotI, the fragment was ligated with pAC λ cI32 that had been digested with BglII and NotI, resulting in pCI-rpoC (357-430).

pET σ^{fl} was obtained by inserting the EcoRI/HindIII fragment containing σ^{70} DNA from p σ^{fl} 63 into pET21(+) (Novagen) that had been digested with EcoRI and HindIII. The resulting plasmid expresses His₆-tagged σ^{70} protein, which has the sequence MRGSHHHHHHGSSGLVPRGSELGTRL fused to the start of σ^{70} . This σ^{70} also contains the substitutions V36L and N149D, but we have not found any consequence of these substitutions in numerous assays (data not shown). To make pET $\sigma^{584-588A}$ we used the Quikchange procedure (Stratagene) and primers that introduced codons for alanines at σ^{70} residues 584-588 as well as introduced a PstI site. The presence of the desired substitutions in the resulting plasmids were screened by PstI digestion and DNA sequencing. The ClaI/HindIII fragment containing the 3' end of σ^{70} DNA was then obtained from a plasmid with the desired change, and this fragment was inserted into pET σ^{fl} that had been digested with ClaI and HindIII, resulting in pET $\sigma^{584-588A}$. To construct pET $\sigma^{551-552,554-555A}$, we first deleted the DNA coding for σ^{70} residues 551-555 in pET σ^{fl} using the Quikchange procedure and primers that surrounded the DNA for residues 551-555, but lacked the 551-555 codons. Using the resulting deleted plasmid, we then used the Quikchange procedure with primers that surrounded the DNA for residues 551-555, but now had alanine codons for residues 551-555. After sequencing to identify a plasmid with the desired change, the XhoI fragment containing the 3' end of the σ^{70} gene was obtained and ligated to pET σ^{fl} that had been digested with XhoI, resulting in pET $\sigma^{551-552,554-555A}$. pET σ^{70}/σ^{38} region 4 encodes a σ^{70}/σ^{38} fusion protein in which σ^{70} residues 1-529 are followed by σ^{38} region 4 (residues 245-330). To construct this plasmid, we used the pBR α - σ^{38} , 60, in which DNA encoding region 4 of σ^{38} had been fused to DNA encoding the N-terminal domain of the α subunit of RNA polymerase. An XhoI site had also been placed at the α - σ junction in this plasmid. We performed PCR using pBR α - σ^{38} , a primer that annealed to the DNA at the α - σ^{38} junction, and a primer that annealed to the DNA at the end of the σ^{38} sequence and introduced a XhoI site. The resulting PCR product was digested with XhoI and ligated into pET σ^{fl} that had been digested with XhoI, resulting in pET σ^{70}/σ^{38} region 4. pET $\sigma^{70}/\sigma^{38}H5$ was constructed by ligating pET σ^{fl} that had been previously digested with ClaI and HindIII with the ClaI/HindIII fragment containing the σ^{38} sequences

from pBR α - σ^{70}/σ^{38} ; 20. pBR α - σ^{70}/σ^{38} is identical to pBR α - σ^{70} except that the σ^{70} sequences encoding amino acids 597-613 have been replaced with the comparable σ^{38} sequences (encoding amino acids 312-330). pET $\sigma^{\Delta 571-613}$ was constructed by ligating pET σ^{fl} that had been digested with ClaI and ScaI with the ClaI/ScaI fragment containing the 3' end of σ^{70} DNA from p σ^{1-570} , 20.

To construct pET15b-557A560A and pET15b-555A557A560A, we used a plasmid pET15b- σ^{70} , in which the *rpoD* gene is cloned between NdeI and EcoRI sites. We performed two-step PCR site-directed mutagenesis and obtained PCR products containing the corresponding mutations and BamHI and EcoRI sites at the ends of the each PCR fragment. As the pET15b- σ^{70} contains unique BamHI site in the *rpoD* gene, the plasmids pET15b-557A560A and pET15b-555A557A560A were obtained by inserting the corresponding BamHI/EcoRI fragments into the pET15b- σ^{70} that had been digested with BamHI and EcoRI. These plasmids produce N-terminally His₆-tagged σ^{70} with the indicated mutations.

To construct pBR α - $\sigma^{4.1}$ we obtained a PCR product with pET σ^{fl} and a primer that annealed to DNA encoding σ^{70} residues 528-534 preceded by an XhoI site and a primer that annealed to DNA encoding 562-568 followed by a BamHI site. After digestion of the product with BamHI and XhoI, the fragment was ligated with pBR α - σ^{70} that had also been digested with BamHI and XhoI. The resulting plasmid produces an α - $\sigma^{4.1}$ fusion protein, in which α residues 1-248 are followed by σ^{70} residues 528-568. pBR α - $\sigma^{4.2}$ produces an α - $\sigma^{4.2}$ fusion protein, in which α residues 1-248 are followed by the protein sequence LEL (brought in by the linker DNA) and then σ^{70} residues 569-613. It was constructed like pBR α - $\sigma^{4.1}$ except that the primers annealed to σ^{70} residues 569-574 and 608-613. pBR α - $\sigma^{4.2}$ 584-588A, pBR α - $\sigma^{4.1}$ 551-552, 554-555A, and pBR α - $\sigma^{4.2}$ 555,557,560A were constructed following similar procedures except that the PCR templates were pET σ^{70} 584-588A, pET σ^{70} 551-552, 554-555A, and pET15b-555A557A560A, respectively.

Protein buffer I contained 47 mM Tris-Cl (pH 7.9), 83 mM NaCl, 45% glycerol, 0.9 mM EDTA, 0.09 mM DTT, 0.009% Triton X-100, and 22 mM imidazole. Protein buffer II contained 5.2 mM Tris-Cl (pH 7.9), 72 mM Tris-acetate (pH 7.9), 26 mM NaCl, 5.2% glycerol, 0.2 mM EDTA, 0.3 mM DTT, 270 mM potassium glutamate, 7.2 mM magnesium acetate, and 180 μ g/ml BSA. DNA buffer contained 53 mM Tris-acetate (pH 7.9), 6 mM NaCl, 0.2% glycerol, 0.6 mM EDTA, 0.1 mM DTT, 200 mM potassium acetate, 5.3 mM magnesium acetate, 130 μ g/ml BSA, 0.48 mM each ATP, GTP, and CTP, and 24 μ M [α -³²P]UTP (6×10^4 dpm/pmol).

Proteins

The purification of wild type σ^{70} (not His₆-tagged), which was used for the native gel assays, has been described³⁹. Purification of the His₆-tagged proteins, σ^{70} , σ^{70} 551-552,554-555A, σ^{70}/σ^{38} H5, and $\sigma^{70}\Delta 571-613$, was done using BL21(DE3)/pLysE⁶⁴ cultures containing the desired pET σ plasmid as described⁶⁵ by denaturation of inclusion bodies containing the protein, Ni⁺⁺ resin affinity chromatography under denaturing conditions, and then a slow renaturation of the protein. His₆-tagged σ^{70}/σ^{38} region 4 was purified by a similar procedure except that after sonication of the cells, this protein was found in the $17,400 \times g$ supernatant rather than in inclusion bodies and consequently, it was subjected to Ni⁺⁺ resin chromatography under native conditions. 584-588A was purified by both procedures. Transcription assays His₆-tagged σ^{70} indicated that the His₆-tagged proteins σ^{70} 551-552, 554-555A, σ^{70} 584-588A, σ^{70}/σ^{38} region 4, $\sigma^{70}\Delta 571-613$, and the σ^{70} saturated core at a ratio of ~2:1 (data not shown). σ^{70} F563Y, $\sigma^{\text{pertussis}}$, and the σ^{70} used as the control for the σ^{70} F563Y and $\sigma^{\text{pertussis}}$ proteins (Fig. 5B) were gifts of Phil Boucher and Scott Stibitz (FDA). *E. coli* RNA polymerase core was purchased from Epicentre Technologies. MotA was purified as described³⁸. The purifications of N-terminally His₆-tagged AsiA, His₆AsiA 31, and C-terminally His₆-tagged

AsiA, AsiAHis₆⁴⁰ have been described. We have not observed any significant difference in activity between wild type AsiA, AsiAHis₆, and His₆AsiA.

To isolate the His₆-tagged proteins, σ^{70} ^{555, 557, 560A} and σ^{70} ^{557, 560A}, *E. coli* BL21(DE3) containing the appropriate plasmid was grown in LB broth with 100 μ g/ml ampicillin at 37° C to an OD₆₀₀ of 0.8-1.0, and synthesis of the mutant σ^{70} was induced by the addition of IPTG to 0.5 mM. After 6 more hours of growth, cells were harvested by centrifugation, resuspended in buffer A (20 mM Tris-HCl, pH 8.0; 500 mM NaCl; 5% glycerol; 2 mM imidazole, pH 8.0) and lysed by sonication. After centrifugation, the cleared lysate was loaded onto a 5 ml chelating Hi-trap Sepharose column attached to syringe. The column was then washed with buffer A containing 20 mM imidazole, and the proteins were eluted with 100 mM imidazole in the same buffer. The proteins were concentrated to 1–2 mg/ml by dialyzing against buffer B that contained 20 mM Tris-HCl, pH 8.0; 100 mM NaCl, 1 mM β -mercaptoethanol, 50 % glycerol, and stored at –20°C.

Two-hybrid assays

β -galactosidase assays were performed as described previously³¹ using cultures of *E. coli* KS1⁶⁶ containing the indicated plasmids except that cultures were grown for 3 hr in LB media supplemented with the appropriate antibiotics at the following concentrations: carbenicillin (50 μ g/ml), chloramphenicol (25 μ g/ml), and kanamycin (50 μ g/ml). β -galactosidase values represent the averages of duplicate assays. Error bars are shown in Fig. 4 and 5, but in some cases they are not seen because they are smaller than the symbols.

Native Protein Gels

Protein-protein complexes were separated from free proteins by gel electrophoresis on native, 6% polyacrylamide gels as described³⁹. Reactions were composed of 24 pmol of His₆AsiA in 2 μ l of a buffer containing 20 mM Tris-Cl (pH 7.9), 500 mM NaCl, 5% glycerol, and 100 mM imidazole; 4.6 pmol of σ^{70} in 2 μ l of a buffer containing 50 mM Tris-Cl (pH 8.0), 50 mM NaCl, 50% glycerol, 1 mM EDTA, 0.1 mM DTT, and 0.01% Triton X-100; and/or 17 pmol of MotA in 1 μ l of a buffer containing 20 mM Tris-Cl (pH 7.9), 270 mM NaCl, 10% glycerol, 1 mM EDTA, and 1 mM 2-mercaptoethanol. Reactions were incubated at 37° C for 10 min before electrophoresis. After electrophoresis, proteins were detected using SilverXpress® (Invitrogen).

In vitro transcriptions

Transcription reactions were assembled as indicated in the figure legends. Upon addition of rNTPs, reactions were incubated at 37° C for 20 sec, rifampicin (0.5 μ l of 300 ng/ μ l) was added, and then the reactions were incubated for an additional 7 min before being collected on dry ice. Gel load solution (1 $\times$ TBE, 7 M urea, 0.1% bromphenol blue, 0.1% xylene cyanol FF) was added at a volume of 5 times that of the reaction aliquots, and the solution was heated at 95° C for two minutes before electrophoresis on 4% polyacrylamide, 7 M urea denaturing gels run in 1/2 $\times$ TBE. After autoradiography, films were scanned using a Powerlook 2100XL densitometer and quantification was performed using Quantity One software from Bio-Rad, Inc.

Acknowledgements

We thank A. Makela for the construction of pET σ^{fl} and the purification of σ^{70}/σ^{38} H5. We thank A. Hochschild and B. Gregory for gifts of pAC λ CI- β -flap and pAC Δ -35 λ CI-AsiAK20A and P. Boucher and S. Stibitz for the gifts of $\sigma^{petussis}$ and σ^{70} F563Y. This research was supported in part by the Intramural Research Program of the NIH, National Institute of Diabetes and Digestive and Kidney Diseases (K.B., J.L., and D. H.) and NIH grant R01 GM59295 (L.M. and K.S.).

References

1. Gruber TM, Gross CA. Multiple sigma subunits and the partitioning of bacterial transcription space. *Annu Rev Microbiol* 2003;57:441–66. [PubMed: 14527287]
2. Murakami KS, Darst SA. Bacterial RNA polymerases: the whole story. *Curr Opin Struct Biol* 2003;13:31–9. [PubMed: 12581657]
3. Paget MS, Helmann JD. The sigma70 family of sigma factors. *Genome Biol* 2003;4:203. [PubMed: 12540296]
4. Lonetto M, Gribskov M, Gross CA. The sigma 70 family: sequence conservation and evolutionary relationships. *J Bacteriol* 1992;174:3843–9. [PubMed: 1597408]
5. Barne KA, Bown JA, Busby SJ, Minchin SD. Region 2.5 of the Escherichia coli RNA polymerase sigma70 subunit is responsible for the recognition of the 'extended-10' motif at promoters. *Embo J* 1997;16:4034–40. [PubMed: 9233812]
6. Haugen SP, Berkmen MB, Ross W, Gaal T, Ward C, Gourse RL. rRNA promoter regulation by nonoptimal binding of sigma region 1.2: an additional recognition element for RNA polymerase. *Cell* 2006;125:1069–82. [PubMed: 16777598]
7. Feklistov A, Barinova N, Sevostyanova A, Heyduk E, Bass I, Vvedenskaya I, Kuznedelov K, Merkiene E, Stavrovskaya E, Klimasauskas S, Nikiforov V, Heyduk T, Severinov K, Kulbachinskiy A. A Basal Promoter Element Recognized by Free RNA Polymerase sigma Subunit Determines Promoter Recognition by RNA Polymerase Holoenzyme. *Mol Cell* 2006;23:97–107. [PubMed: 16798040]
8. Campbell EA, Muzzin O, Chlenov M, Sun JL, Olson CA, Weinman O, Trester-Zedlitz ML, Darst SA. Structure of the bacterial RNA polymerase promoter specificity sigma subunit. *Mol Cell* 2002;9:527–39. [PubMed: 11931761]
9. Murakami KS, Masuda S, Campbell EA, Muzzin O, Darst SA. Structural basis of transcription initiation: an RNA polymerase holoenzyme-DNA complex. *Science* 2002;296:1285–90. [PubMed: 12016307]
10. Mekler V, Kortkhonja E, Mukhopadhyay J, Knight J, Revyakin A, Kapanidis AN, Niu W, Ebright YW, Levy R, Ebright RH. Structural organization of bacterial RNA polymerase holoenzyme and the RNA polymerase-promoter open complex. *Cell* 2002;108:599–614. [PubMed: 11893332]
11. Murakami KS, Masuda S, Darst SA. Structural basis of transcription initiation: RNA polymerase holoenzyme at 4 Å resolution. *Science* 2002;296:1280–4. [PubMed: 12016306]
12. Vassilyev DG, Sekine S, Laptenko O, Lee J, Vassilyeva MN, Borukhov S, Yokoyama S. Crystal structure of a bacterial RNA polymerase holoenzyme at 2.6 Å resolution. *Nature* 2002;417:712–9. [PubMed: 12000971]
13. Kuznedelov K, Minakhin L, Niedziela-Majka A, Dove SL, Rogulja D, Nickels BE, Hochschild A, Heyduk T, Severinov K. A role for interaction of the RNA polymerase flap domain with the sigma subunit in promoter recognition. *Science* 2002;295:855–7. [PubMed: 11823642]
14. Lawson CL, Swigon D, Murakami KS, Darst SA, Berman HM, Ebright RH. Catabolite activator protein: DNA binding and transcription activation. *Curr Opin Struct Biol* 2004;14:10–20. [PubMed: 15102444]
15. Barnard A, Wolfe A, Busby S. Regulation at complex bacterial promoters: how bacteria use different promoter organizations to produce different regulatory outcomes. *Curr Opin Microbiol* 2004;7:102–8. [PubMed: 15063844]
16. Hinton DM, Pande S, Wais N, Johnson XB, Vuthoori M, Makela A, Hook-Barnard I. Transcriptional takeover by sigma appropriation: remodelling of the sigma70 subunit of Escherichia coli RNA polymerase by the bacteriophage T4 activator MotA and co-activator AsiA. *Microbiology* 2005;151:1729–40. [PubMed: 15941982]
17. Mattson T, Richardson J, Goodin D. Mutant of bacteriophage T4D affecting expression of many early genes. *Nature* 1974;250:48–50. [PubMed: 4601455]
18. Hinton DM. Transcription from a bacteriophage T4 middle promoter using T4 motA protein and phage-modified RNA polymerase. *J Biol Chem* 1991;266:18034–44. [PubMed: 1917941]
19. Schmidt RP, Kreuzer KN. Purified MotA protein binds the -30 region of a bacteriophage T4 middle-mode promoter and activates transcription in vitro. *J Biol Chem* 1992;267:11399–407. [PubMed: 1597469]

20. Pande S, Makela A, Dove SL, Nickels BE, Hochschild A, Hinton DM. The bacteriophage T4 transcription activator MotA interacts with the far-C-terminal region of the sigma70 subunit of Escherichia coli RNA polymerase. *J Bacteriol* 2002;184:3957–64. [PubMed: 12081968]
21. Ouhammouch M, Adelman K, Harvey SR, Orsini G, Brody EN. Bacteriophage T4 MotA and AsiA proteins suffice to direct Escherichia coli RNA polymerase to initiate transcription at T4 middle promoters. *Proc Natl Acad Sci U S A* 1995;92:1451–5. [PubMed: 7877999]
22. Colland F, Orsini G, Brody EN, Buc H, Kolb A. The bacteriophage T4 AsiA protein: a molecular switch for sigma 70-dependent promoters. *Mol Microbiol* 1998;27:819–29. [PubMed: 9515707]
23. Pahari S, Chatterji D. Interaction of bacteriophage T4 AsiA protein with Escherichia coli sigma70 and its variant. *FEBS Lett* 1997;411:60–2. [PubMed: 9247142]
24. Severinova E, Severinov K, Darst SA. Inhibition of Escherichia coli RNA polymerase by bacteriophage T4 AsiA. *J Mol Biol* 1998;279:9–18. [PubMed: 9636696]
25. Severinova E, Severinov K, Fenyo D, Marr M, Brody EN, Roberts JW, Chait BT, Darst SA. Domain organization of the Escherichia coli RNA polymerase sigma 70 subunit. *J Mol Biol* 1996;263:637–47. [PubMed: 8947564]
26. Severinov K, Muir TW. Expressed protein ligation, a novel method for studying protein-protein interactions in transcription. *J Biol Chem* 1998;273:16205–9. [PubMed: 9632677]
27. Sharma UK, Ravishankar S, Shandil RK, Praveen PV, Balganes TS. Study of the interaction between bacteriophage T4 asiA and Escherichia coli sigma(70), using the yeast two-hybrid system: neutralization of asiA toxicity to E. coli cells by coexpression of a truncated sigma(70) fragment. *J Bacteriol* 1999;181:5855–9. [PubMed: 10482532]
28. Urbauer JL, Adelman K, Urbauer RJ, Simeonov MF, Gilmore JM, Zolkiewski M, Brody EN. Conserved regions 4.1 and 4.2 of sigma(70) constitute the recognition sites for the anti-sigma factor AsiA, and AsiA is a dimer free in solution. *J Biol Chem* 2001;276:41128–32. [PubMed: 11518715]
29. Simeonov MF, Bieber Urbauer RJ, Gilmore JM, Adelman K, Brody EN, Niedziela-Majka A, Minakhin L, Heyduk T, Urbauer JL. Characterization of the interactions between the bacteriophage T4 AsiA protein and RNA polymerase. *Biochemistry* 2003;42:7717–26. [PubMed: 12820881]
30. Lambert LJ, Wei Y, Schirf V, Demeler B, Werner MH. T4 AsiA blocks DNA recognition by remodeling sigma(70) region 4. *Embo J* 2004;23:2952–62. [PubMed: 15257291]
31. Pal D, Vuthoori M, Pande S, Wheeler D, Hinton DM. Analysis of regions within the bacteriophage T4 AsiA protein involved in its binding to the sigma70 subunit of E. coli RNA polymerase and its role as a transcriptional inhibitor and co-activator. *J Mol Biol* 2003;325:827–41. [PubMed: 12527294]
32. Stevens A, Rhoton JC. Characterization of an inhibitor causing potassium chloride sensitivity of an RNA polymerase from T4 phage-infected Escherichia coli. *Biochemistry* 1975;14:5074–9. [PubMed: 1103966]
33. Young BA, Gruber TM, Gross CA. Views of transcription initiation. *Cell* 2002;109:417–20. [PubMed: 12086598]
34. Gregory BD, Nickels BE, Garrity SJ, Severinova E, Minakhin L, Urbauer RJ, Urbauer JL, Heyduk T, Severinov K, Hochschild A. A regulator that inhibits transcription by targeting an intersubunit interaction of the RNA polymerase holoenzyme. *Proc Natl Acad Sci U S A* 2004;101:4554–9. [PubMed: 15070756]
35. Gregory BD, Nickels BE, Darst SA, Hochschild A. An altered-specificity DNA-binding mutant of *E. coli* σ^{70} facilitates the analysis of σ^{70} function in vivo. *Molec Microbiol*. 2005;in press
36. Minakhin L, Camarero JA, Holford M, Parker C, Muir TW, Severinov K. Mapping the molecular interface between the sigma(70) subunit of E. coli RNA polymerase and T4 AsiA. *J Mol Biol* 2001;306:631–42. [PubMed: 11243776]
37. Ross W, Ernst A, Gourse RL. Fine structure of E. coli RNA polymerase-promoter interactions: alpha subunit binding to the UP element minor groove. *Genes Dev* 2001;15:491–506. [PubMed: 11238372]
38. Hinton DM, March-Amegadzie R, Gerber JS, Sharma M. Bacteriophage T4 middle transcription system: T4-modified RNA polymerase; AsiA, a sigma 70 binding protein; and transcriptional activator MotA. *Methods Enzymol* 1996;274:43–57. [PubMed: 8902795]

39. Gerber JS, Hinton DM. An N-terminal mutation in the bacteriophage T4 *motA* gene yields a protein that binds DNA but is defective for activation of transcription. *J Bacteriol* 1996;178:6133–9. [PubMed: 8892810]
40. Pineda M, Gregory BD, Szczypinski B, Baxter KR, Hochschild A, Miller ES, Hinton DM. A family of anti-sigma70 proteins in T4-type phages and bacteria that are similar to AsiA, a Transcription inhibitor and co-activator of bacteriophage T4. *J Mol Biol* 2004;344:1183–97. [PubMed: 15561138]
41. Gardella T, Moyle H, Susskind MM. A mutant *Escherichia coli* sigma 70 subunit of RNA polymerase with altered promoter specificity. *J Mol Biol* 1989;206:579–90. [PubMed: 2661827]
42. Siegele DA, Hu JC, Walter WA, Gross CA. Altered promoter recognition by mutant forms of the sigma 70 subunit of *Escherichia coli* RNA polymerase. *J Mol Biol* 1989;206:591–603. [PubMed: 2661828]
43. Keener J, Nomura M. Dominant lethal phenotype of a mutation in the -35 recognition region of *Escherichia coli* sigma 70. *Proc Natl Acad Sci U S A* 1993;90:1751–5. [PubMed: 7680477]
44. Minakhin L, Niedziela-Majka A, Kuznedelov K, Adelman K, Urbauer JL, Heyduk T, Severinov K. Interaction of T4 AsiA with its target sites in the RNA polymerase sigma70 subunit leads to distinct and opposite effects on transcription. *J Mol Biol* 2003;326:679–90. [PubMed: 12581632]
45. Nickels BE, Garrity SJ, Mekler V, Minakhin L, Severinov K, Ebright RH, Hochschild A. The interaction between sigma70 and the beta-flap of *Escherichia coli* RNA polymerase inhibits extension of nascent RNA during early elongation. *Proc Natl Acad Sci U S A* 2005;102:4488–93. [PubMed: 15761057]
46. Sharp MM, Chan CL, Lu CZ, Marr MT, Nechaev S, Merritt EW, Severinov K, Roberts JW, Gross CA. The interface of sigma with core RNA polymerase is extensive, conserved, and functionally specialized. *Genes Dev* 1999;13:3015–26. [PubMed: 10580008]
47. Miller ES, Kutter E, Mosig G, Arisaka F, Kunisawa T, Ruger W. Bacteriophage T4 genome. *Microbiol Mol Biol Rev* 2003;67:86–156. [PubMed: 12626685]
48. Geszvain K, Gruber TM, Mooney RA, Gross CA, Landick R. A hydrophobic patch on the flap-tip helix of *E.coli* RNA polymerase mediates sigma(70) region 4 function. *J Mol Biol* 2004;343:569–87. [PubMed: 15465046]
49. Sharma UK, Praveen PV, Balganesht TS. Mutational analysis of bacteriophage T4 AsiA: involvement of N- and C-terminal regions in binding to sigma(70) of *Escherichia coli* in vivo. *Gene* 2002;295:125–34. [PubMed: 12242019]
50. Sharma UK, Chatterji D. Both regions 4.1 and 4.2 of *E. coli* sigma(70) are together required for binding to bacteriophage T4 AsiA in vivo. *Gene*. 2006
51. Hinton DM, Vuthoori S. Efficient inhibition of *Escherichia coli* RNA polymerase by the bacteriophage T4 AsiA protein requires that AsiA binds first to free sigma70. *J Mol Biol* 2000;304:731–9. [PubMed: 11188759]
52. Hinton DM, Vuthoori S, Mulamba R. The Bacteriophage T4 Inhibitor and Coactivator AsiA Inhibits *Escherichia coli* RNA Polymerase More Rapidly in the Absence of {sigma}70 Region 1.1: Evidence that Region 1.1 Stabilizes the Interaction between {sigma}70 and Core. *J Bacteriol* 2006;188:1279–85. [PubMed: 16452409]
53. Stevens A. New small polypeptides associated with DNA-dependent RNA polymerase of *Escherichia coli* after infection with bacteriophage T4. *Proc Natl Acad Sci U S A* 1972;69:603–7. [PubMed: 4551978]
54. Rhodius VA, Busby SJ. Transcription activation by the *Escherichia coli* cyclic AMP receptor protein: determinants within activating region 3. *J Mol Biol* 2000;299:295–310. [PubMed: 10860739]
55. Lonetto MA, Rhodius V, Lamberg K, Kiley P, Busby S, Gross C. Identification of a contact site for different transcription activators in region 4 of the *Escherichia coli* RNA polymerase sigma70 subunit. *J Mol Biol* 1998;284:1353–65. [PubMed: 9878355]
56. Landini P, Busby SJ. The *Escherichia coli* Ada protein can interact with two distinct determinants in the sigma70 subunit of RNA polymerase according to promoter architecture: identification of the target of Ada activation at the *alkA* promoter. *J Bacteriol* 1999;181:1524–9. [PubMed: 10049384]
57. Rosenthal AZ, Hu M, Gralla JD. Osmolyte-induced transcription: -35 region elements and recognition by sigma38 (*rpoS*). *Mol Microbiol* 2006;59:1052–61. [PubMed: 16420371]

58. Ohnuma M, Fujita N, Ishihama A, Tanaka K, Takahashi H. A carboxy-terminal 16-amino-acid region of sigma(38) of *Escherichia coli* is important for transcription under high-salt conditions and sigma activities in vivo. *J Bacteriol* 2000;182:4628–31. [PubMed: 10913098]
59. March-Amegadzie R, Hinton DM. The bacteriophage T4 middle promoter PuvsX: analysis of regions important for binding of the T4 transcriptional activator MotA and for activation of transcription. *Mol Microbiol* 1995;15:649–60. [PubMed: 7783637]
60. Dove SL, Huang FW, Hochschild A. Mechanism for a transcriptional activator that works at the isomerization step. *Proc Natl Acad Sci U S A* 2000;97:13215–20. [PubMed: 11087868]
61. Nickels BE, Dove SL, Murakami KS, Darst SA, Hochschild A. Protein-protein and protein-DNA interactions of sigma70 region 4 involved in transcription activation by lambda cI. *J Mol Biol* 2002;324:17–34. [PubMed: 12421556]
62. Hu JC, Kornacker MG, Hochschild A. *Escherichia coli* one- and two-hybrid systems for the analysis and identification of protein-protein interactions. *Methods* 2000;20:80–94. [PubMed: 10610807]
63. Wilson C, Dombroski AJ. Region 1 of sigma70 is required for efficient isomerization and initiation of transcription by *Escherichia coli* RNA polymerase. *J Mol Biol* 1997;267:60–74. [PubMed: 9096207]
64. Studier FW. Use of bacteriophage T7 lysozyme to improve an inducible T7 expression system. *J Mol Biol* 1991;219:37–44. [PubMed: 2023259]
65. Vuthoori S, Bowers CW, McCracken A, Dombroski AJ, Hinton DM. Domain 1.1 of the sigma(70) subunit of *Escherichia coli* RNA polymerase modulates the formation of stable polymerase/promoter complexes. *J Mol Biol* 2001;309:561–72. [PubMed: 11397080]
66. Dove SL, Joung JK, Hochschild A. Activation of prokaryotic transcription through arbitrary protein-protein contacts. *Nature* 1997;386:627–30. [PubMed: 9121589]

A.

B.

Fig. 1.

Region 4 of wild type σ^{70} and mutant σ proteins and the T4 middle promoter P_{uvSx} . A. The C-terminal amino acid sequence of σ^{70} is shown from residues 540 to 613. Residues in pink contact the -35 element of *E. coli* promoter DNA⁸ while residues in blue contact the β -flap tip^{11; 12; 13; 45}. Every tenth residue is marked with a small vertical line. Above the sequence is shown the locations of regions 4.1 and 4.2¹ and the positions of the α -helices H1, H2, and H5 and the helix-turn-helix H3-T-H4 structures observed in region 4 of the primary σ of *T. aquaticus* complexed with -35 element DNA⁸. Residues that contact AsiA in the AsiA/ σ^{70} region 4 structure³⁰ are designated with an asterisk and the region of the MotA contact site²⁰ is indicated. Below are shown the region 4 sequences of the mutant σ proteins, with residues that differ from σ^{70} in red. B. The sequence of P_{uvSx} from -61 to +9 is shown with the start of transcription indicated as +1. The locations of the σ^{70} -10 element and the MotA box are indicated. Sequences within P_{uvSx} that match the canonical polymerase elements σ^{70} -10 element (TATAAT); σ^{70} extended -10 element (-15T, -14G); σ^{70} -35 element (TTGACA); and α UP element (AAAa/ta/tTa/tTTTTnnAAAA) are in red.

Fig. 2.

Transcriptional activities of polymerases reconstituted with mutant σ proteins using P_{uvsvX} *in vitro* in the presence of AsiA and/or MotA. The indicated σ (0.2 pmol) and AsiAHis₆ (3 pmol) were incubated in 1.8 μ l of protein buffer I for 10 min at 37° C. A solution containing 0.05 pmol of core in 1.1 μ l of protein buffer II was then added, and the resulting solution was incubated for an additional 10 min at 37° C. Transcription was initiated by adding this solution to 2.1 μ l of DNA buffer containing 0.01 pmol DNA. After 37° C for 20 sec, rifampicin (150 ng) was added to limit transcription to a single round. For each indicated σ , [the amount of P_{uvsvX} RNA observed]/[the amount of P_{uvsvX} RNA observed with σ^{70} polymerase in the absence of MotA and AsiA] is shown using polymerase alone, polymerase with MotA, polymerase

with AsiA, and polymerase with AsiA and MotA. Values were determined from two or more transcriptions. Error is indicated by darker shaded area. In some cases where the level of transcription was low, 10-fold values ($10 \times$ values) are also shown. In these cases, zero is the bottom of the enclosed box.

Fig. 3.

σ/AsiA , σ/MotA , and $\text{AsiA}/\sigma/\text{MotA}$ complexes in native protein gels. The indicated σ was incubated with excess His_6AsiA and/or MotA and then subjected to electrophoresis through native 6 % acrylamide gels as described in Materials and Methods. The positions of σ , the σ/AsiA complex, the σ/MotA complex, and the $\text{AsiA}/\sigma/\text{MotA}$ complex are shown. The positions of free MotA and AsiA are also marked. Excess free MotA protein is the dark band that migrates near the top of the gel in the lanes with added MotA . The excess free AsiA is usually not seen because AsiA is a small protein (10 kDa) and stains poorly.

Fig. 4. 2-hybrid assay and the effect of σ^{70} substitutions on the interaction of σ^{70} region 4 with the AsiAK20A and MotA^{NTD}. (A) Cartoon shows the test promoter, which has a λ operator (O_R) located 62 bp upstream from the initiation site of the *lac* core promoter. The presence of the α - σ chimera protein, expressed by a pBR α - σ plasmid, and the cI-bait fusion protein, expressed by a pCI-bait plasmid, can result in an increase in *lacZ* transcription and subsequent β -galactosidase activity if there is an interaction between the σ present in the chimera and the bait protein. The addition of IPTG induces expression of both the α - σ chimera and the cI-bait fusion protein. B-E. Effect of σ^{70} mutations on the interaction of σ^{70} region 4 with AsiAK20A (left panels) and MotA^{NTD} (right panels). Cultures contained the pCI-AsiAK20A or pCI-

MotA^{NTD} plasmid and either the control plasmid, pBR α that lacks σ^{70} region 4, or as indicated, a pBR α - σ^{70} plasmid that has σ^{70} region 4 (σ^{70} 4), σ^{70} region 4.1 (σ^{70} 4.1), σ^{70} region 4.2 (σ^{70} 4.2) with or without the indicated σ^{70} substitutions. Graphs show β -galactosidase activity (in Miller units) versus the concentration of IPTG present in the cultures.

Fig. 5.

The σ^{70} substitutions 551-552,554-555A (A) eliminate the interaction of the β -flap with σ^{70} region 4 whereas substitutions 584-588A (B) do not impair the interaction of σ^{70} region 4 with the β -flap. Cultures contained a pBR α - σ^{70} plasmid, which had σ^{70} region 4 (σ^{70} 4) with the indicated substitutions, and either the control plasmid, pAC λ CI32, the pCI plasmid that lacks a fused bait protein, or the pCI- β -flap plasmid (β -flap). Graphs show β -galactosidase activity (in Miller units) versus the concentration of IPTG present in the cultures.

Fig. 6.

Interaction of σ^{70} region 4 with -35 element and the β -flap versus its interaction with AsiA and MotA. Structures of (A) *T. aquaticus* σ region 4 (residues corresponding to *E. coli* σ^{70} residues 546-612) with the -35 region of promoter DNA⁸ (PDB: 1KU7) and of (B) *E. coli* σ^{70} region 4 (residues 546-613) with AsiA (residues 2-90)³⁰ (PDB: 1TLH). σ^{70} region 4 is shown in gold, AsiA is shown in green, and the DNA is shown in magenta. Alpha helices H2, H3, H4, and H5 in σ^{70} (see Fig. 1A) are labeled. Spacefill structures of σ^{70} residues 551, 554, and 555 in region 4.1 and residues 584 and 587 in region 4.2, which are predicted to interact with AsiA in the structure and whose substitution impairs the σ^{70} /AsiA interaction, are shown in red in (B). The portion of σ^{70} region 4 that interacts with the β -flap is indicated by the blue oval in (A). H5 of σ^{70} that interacts with MotA is indicated by the black rectangle in (B). Note that the binding of AsiA to σ^{70} region 4 converts H3-T-H4 of σ^{70} , which normally interacts with the -35 sequences of promoter DNA, into one continuous helix.