

ExsA Recruits RNA Polymerase to an Extended -10 Promoter by Contacting Region 4.2 of Sigma-70[∇]

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ExsA is a member of the AraC family of transcriptional activators and is required for expression of the *Pseudomonas aeruginosa* type III secretion system (T3SS). ExsA-dependent promoters consist of two binding sites for monomeric ExsA located approximately 50 bp upstream of the transcription start sites. Binding to both sites is required for recruitment of σ^{70} -RNA polymerase (RNAP) to the promoter. ExsA-dependent promoters also contain putative -35 hexamers that closely match the σ^{70} consensus but are atypically spaced 21 or 22 bp from the -10 hexamer. Because several nucleotides located within the putative -35 region are required for ExsA binding, it is unclear whether the putative -35 region makes an additional contribution to transcription initiation. In the present study we demonstrate that the putative -35 hexamer is dispensable for ExsA-independent transcription from the P_{exsC} promoter and that deletion of σ^{70} region 4.2, which contacts the -35 hexamer, has no effect on ExsA-independent transcription from P_{exsC} . Region 4.2 of σ^{70} , however, is required for ExsA-dependent activation of the P_{exsC} and P_{exsD} promoters. Genetic data suggest that ExsA directly contacts region 4.2 of σ^{70} , and several amino acids were found to contribute to the interaction. *In vitro* transcription assays demonstrate that an extended -10 element located in the P_{exsC} promoter is important for overall promoter activity. Our collective data suggest a model in which ExsA compensates for the lack of a -35 hexamer by interacting with region 4.2 of σ^{70} to recruit RNAP to the promoter.

Pseudomonas aeruginosa is an opportunistic human pathogen that causes a variety of acute and chronic infections in immunocompromised individuals (52, 53). A primary determinant of *P. aeruginosa* virulence is a type III secretion system (T3SS) (24, 70). The T3SS consists of a macromolecular conduit through which effector toxins are translocated into eukaryotic host cells (70). The translocated toxins promote tissue destruction and evasion of the host immune response (3, 55, 69). Mutants lacking a functional T3SS have reduced virulence in both tissue culture and animal infection models (2, 33).

The central regulator of T3SS gene expression is ExsA (25, 67, 68). ExsA directly binds to 10 different promoters and activates transcription of the core genes required for assembly and function of the T3SS (12, 64). ExsA belongs to the family of AraC/XylS transcriptional regulators. AraC family members generally consist of an amino-terminal ligand interaction domain and two carboxy-terminal helix-turn-helix DNA-binding motifs (14). AraC regulators are often classified by the type of ligand that regulates their activity. ExsA belongs to a small subset of AraC regulators that respond to protein ligands and control T3SS gene expression (50). Representatives of this subfamily are also found in *Vibrio parahaemolyticus* (ExsA), *Shigella flexneri* (MxiE), and *Salmonella enterica* (InvF) (17, 25, 42, 71). ExsA-dependent transcription in *P. aeruginosa* is antagonized by ExsD, which functions as an antiactivator by inhibiting the DNA-binding activity and self-association properties of ExsA (13, 43, 61). Similarly, the ExsA homolog in *Vibrio parahaemolyticus* is required for T3SS1 gene expression, and

the ExsD homolog is thought to antagonize ExsA activity (71). Transcriptional activation by *S. flexneri* MxiE is also antagonized by a protein ligand (OspD1), but the inhibitory mechanism has not been established (48). In contrast, transcriptional activation by the *S. enterica* regulator InvF is positively regulated by the SicA coactivator through a direct binding interaction (18). *S. flexneri* IpgC, which copurifies with MxiE, may also function as a coactivator (49). In summary, modulation of activator function by protein ligands can occur in a positive or negative fashion and may be a common theme for AraC family members that regulate T3SS gene expression.

ExsA-dependent promoters in *P. aeruginosa* consist of two adjacent binding sites for monomeric ExsA. Binding site 1 completely overlaps a putative σ^{70} -RNA polymerase (RNAP) -35 recognition hexamer, while binding site 2 extends upstream and includes an adenine-rich region (12). ExsA binds via a monomer assembly pathway in which ExsA bound to site 1 recruits a second ExsA monomer to binding site 2 (12, 14). Like most AraC family members, ExsA is dependent on σ^{70} for transcriptional activation (64), and ExsA-dependent promoters contain apparent σ^{70} -RNAP hexamers that closely resemble the *P. aeruginosa* consensus sequences (TTGACA and TATAAT for the -35 and -10 sites, respectively) (12, 34). The placement of the -10 hexamers and transcription start sites has been established for several ExsA-dependent promoters by potassium permanganate footprinting experiments and 5' rapid amplification of cDNA ends (RACE) mapping, respectively (64, 67). These experiments indicated that σ^{70} -dependent transcription originates from the same start sites in the presence and absence of ExsA (64). The apparent -35 and -10 hexamers of ExsA-dependent promoters are spaced 4 to 5 bp farther apart than the 17 bp typical of most σ^{70} -dependent promoters. Increased spacing has not been reported for any

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AraC family regulators but is seen for Spo0A, a transcriptional activator of the sporulation regulon in *Bacillus subtilis* (45). Spo0A activates promoters with extended spacing (20 to 22 bp) between near-consensus σ^A -RNAP (the σ^{70} homolog) -35 and -10 hexamers (8, 39, 57). The current model suggests that Spo0A activates transcription by repositioning RNAP prebound to the -35 site such that σ^A region 2 can interact with the -10 hexamer to initiate open complex formation (39). Despite the apparent similarity to the extended spacing of Spo0A-dependent promoters, genetic and biochemical experiments suggest an entirely different mechanism for ExsA-dependent activation. A kinetic analysis of abortive transcript production from the P_{exsC} and P_{exsD} promoters reveals that the primary function of ExsA is to recruit σ^{70} -RNAP to promoter DNA (64). Additionally, ExsA-dependent promoters in which the spacing between the -35 and -10 hexamers has been reduced to 16 or 17 bp are severely reduced in expression. These data suggest that ExsA, unlike Spo0A, does not function by compensating for increased promoter spacing and raises the question as to whether the -35 -like elements of ExsA-dependent promoters represent actual σ^{70} -RNAP contact points.

Transcriptional activators typically promote transcription through specific contacts with the α and/or σ^{70} subunit of RNAP (41). The specific RNAP contacts made by these proteins are thought to depend largely on the location of the activator promoter-binding site relative to the -35 hexamer. Class I promoters usually contain an activator DNA-binding site located ≥ 20 bp upstream of the -35 hexamer (22). The available data suggest that activation of a class I promoter is typically mediated by specific contacts between the activator protein and the carboxy-terminal domain of the RNAP α subunit (α -CTD) (22). In contrast, class II promoters usually contain an activator DNA-binding site positioned in close proximity to or overlapping the -35 hexamer. Activation of a class II promoter is thought to occur by contacts with the σ^{70} subunit or both the σ^{70} and α subunits of RNAP (51, 56). Activation by AraC family members often involves interactions with region 4.2 of σ^{70} RNAP. This region contains a DNA-binding domain that recognizes the -35 hexamer. The carboxy-terminal end of region 4.2 also interacts with a diverse group of class II transcriptional activators (20). For example the AraC family regulators RhaR and RhaS, which are involved in the metabolism of rhamnose, contact several amino acids in region 4.2 of σ^{70} , and this interaction is required for transcriptional activation (7, 66).

In this study we characterized the interaction between ExsA and the σ^{70} subunit. Our data indicate that ExsA functions as a class II transcriptional activator at the P_{exsC} and P_{exsD} promoters, does not require the α subunit of RNAP, and instead contacts several amino acids in region 4.2 of σ^{70} . We also provide evidence that the -35 -like element of the P_{exsC} promoter is not an authentic RNAP recognition hexamer for ExsA-independent or -dependent transcription and demonstrate that ExsA-independent transcription at the P_{exsC} promoter requires an extended -10 promoter element.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are summarized in Table 1. *Escherichia coli* strains were

maintained on LB agar plates containing the following antibiotics/chemicals as necessary: gentamicin (15 $\mu\text{g/ml}$), ampicillin (50 or 100 $\mu\text{g/ml}$), tetracycline (10 $\mu\text{g/ml}$), kanamycin (50 $\mu\text{g/ml}$), and indole-3-acrylic acid (0.5 mM). *P. aeruginosa* strains were maintained on Vogel-Bonner minimal medium (65) with antibiotics as indicated (carbenicillin [300 $\mu\text{g/ml}$] and tetracycline [50 $\mu\text{g/ml}$]). For the LuxR experiments bacteria were grown in the presence of 200 nM *N*-(3-oxo-hexanoyl)-L-homoserine lactone (Sigma, St. Louis, MO). To assay for ExsA-dependent gene expression in the presence of mutant and wild-type RNAP subunits, *E. coli* strains from LB agar plates grown overnight were inoculated into 10 ml of LB to an optical density at 600 nm (OD_{600}) of 0.1 and grown with vigorous aeration at 30°C until the OD_{600} reached 0.6. β -Galactosidase assays were performed as previously described (19), and the reported values are the averages from at least three independent experiments.

Plasmid construction and promoter mutagenesis. Plasmid construction is summarized in Table 2, and the primers used are listed in Table 3.

The $P_{\text{exsC-lacZ}}$, $P_{\text{exsD-lacZ}}$, and $P_{\text{exoT-lacZ}}$ transcriptional reporters were generated by PCR amplification of the promoters and cloning into the KpnI/EcoRI (P_{exsC} / P_{exsD}) or SalI/EcoRI (P_{exoT}) sites of the λ integration plasmid pAH125 (28). The $P_{\text{luxI-lacZ}}$ translational fusion reporter was generated by cloning the AatII/EcoRI restriction fragment from plasmid pluxI-lacZ (63) into plasmid pAH125. The resulting plasmids were integrated at the λ attachment site of *E. coli* strains GS162 and/or GA2071 by an electroporation method as described previously (28).

The constitutive ExsA expression plasmid p2UY21 was created through the following series of subcloning steps. ExsA expression plasmid pEB102 was created by PCR amplifying the *exsA* gene from *P. aeruginosa* strain PA103 using NdeI/SacI-containing primers and cloning the resulting fragment into plasmid pUY30 (62). Plasmid p2UY21 was created by cloning the 210-bp ApoI fragment from plasmid pMCTX- $P_{\text{lacUV5mut-lacZ}}$ (described below) into the MfeI/EcoRI sites of plasmid pEB102. Plasmid pMCTX- $P_{\text{lacUV5mut-lacZ}}$ was created by annealing complementary oligonucleotides (5'-AGCTTAGGCTTATCATTATGCTTCCGGCTCGTATAATGTGTG-3' and 5'-AATTCACACATTATACGAGCCGGAAGCATAAAGTGATAAGCCTA-3') and cloning the resulting fragment into the HindIII-EcoRI sites of plasmid pMini-CTX-lacZ (5). Constitutive LuxR expression plasmid p2UY21-*luxR* was created by cloning the NdeI-SacI fragment from pMU102 (63) into plasmid p2UY21. The pGEX*rpoD*(K593A,R596A,R599A) triple mutant σ^{70} expression plasmid and P_{exsC} promoter point mutant transcription templates were generated by QuikChange site-directed mutagenesis (Stratagene).

The carboxy-terminal hexahistidine-tagged α subunit expression vector pET24-*rpoA*_{His} was created by PCR amplifying the *rpoA* gene from *P. aeruginosa* strain PA103 lacking its native stop codon by using NdeI-NotI-containing primers and cloning the resulting fragment into pET-24a (Novagen). The *P. aeruginosa* β and β' subunit expression vectors (pET24-*rpoB* and pET24-*rpoC*, respectively) were created by PCR amplification of *rpoB* or *rpoC* from *P. aeruginosa* strain PA103 by using NdeI-NotI containing primers and cloning the resulting fragment into pET-24a. The carboxy-terminal hexahistidine-tagged σ^{70} expression vector (pET23-*rpoD*_{His}) was created by PCR amplification of the *rpoD* gene lacking its native stop codon from *P. aeruginosa* strain PA103 using primers incorporating NdeI-HindIII restriction sites and cloning the resulting fragment into pET-23b (Novagen). Point mutations in *rpoD* were introduced by QuikChange site-directed mutagenesis (Stratagene).

Purification of *P. aeruginosa* RNAP core enzyme, σ^{70} and holoenzyme reconstitution. ExsA was purified as previously described under native conditions as an amino-terminal decahistidine-tagged fusion protein (12). Individual *P. aeruginosa* RNAP subunits were purified as described previously (60) with modifications. *E. coli* Tuner(DE3) carrying pET24-*rpoA*_{His} was grown at 37°C in 50 ml of Luria broth containing 50 $\mu\text{g/ml}$ kanamycin to an OD_{600} of 0.7, at which time IPTG (isopropyl- β -D-thiogalactopyranoside) (1 mM) was added and the culture was incubated for an additional 3 h at 37°C. Bacteria were harvested by centrifugation and suspended in 4 ml of buffer A (20 mM Tris-HCl [pH 7.9], 500 mM NaCl, and 5 mM imidazole). Cells were lysed via sonication on ice, and unbroken cells were removed by centrifugation (15 min, 16,000 \times g, 4°C). Solid ammonium sulfate (60% of saturation) was added, and samples were allowed to precipitate for 15 min at 4°C with agitation. The precipitate was collected by centrifugation (20 min, 16,000 \times g, 4°C) and resuspended in 10 ml of buffer B (20 mM Tris-HCl [pH 7.9], 6 M guanidine HCl, and 500 mM NaCl) containing 5 mM imidazole. Prior to Ni²⁺ affinity chromatography (see below), the material was subjected to ultracentrifugation (30 min, 100,000 \times g, 4°C) to remove particulates.

E. coli Tuner(DE3) carrying pET23-*rpoD*_{His} was grown at 37°C in 200 ml LB containing 200 $\mu\text{g/ml}$ ampicillin to an OD_{600} of 0.5, at which time IPTG (1 mM) was added and the culture was incubated for an additional 3 h at 37°C. Bacteria were harvested by centrifugation and suspended in 5 ml buffer B containing 5

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics or purpose	Reference or source
<i>Pseudomonas aeruginosa</i> strain PA103	Wild-type parental strain	26
<i>Escherichia coli</i> strains		
DH5 α	<i>recA</i> cloning strain	29
GS162	Wild-type strain carrying $\Delta lacU169$	58
SA1751	Thermoinducible Int expression from the cryptic prophage for minicircle recombination	16
GA2071	<i>rpoD</i> suppression strain	40
BL21(DE3) Tuner	Protein purification	Novagen
BW25141	Maintenance of <i>pir</i> -dependent plasmids	28
Plasmids		
pREii α	<i>rpoA</i> expression vector	9
pGS490	<i>rpoA</i> expression vector with a stop codon at 239	36
pJN105	Arabinose-inducible expression vector	47
pUY30	Arabinose-inducible expression vector	62
pMini-CTX- <i>lacZ</i>	Vector for single-copy integration of <i>lacZ</i> reporters onto the <i>P. aeruginosa</i> chromosomal <i>attB</i> site	31
pMCTX- <i>P_{lacUV5mut-lacZ}</i>	Transcriptional fusion of the <i>P_{lacUV5mut-lacZ}</i> promoter to <i>lacZ</i>	This study
p2UY21- <i>exsA</i>	Plasmid that constitutively expresses <i>exsA</i>	This study
p2UY21- <i>luxR</i>	Plasmid that constitutively expresses <i>luxR</i>	This study
pMU102	<i>luxR</i> expression vector	63
pAH125	Vector for single-copy integration of <i>lacZ</i> reporters onto the <i>E. coli</i> λ attachment site	28
<i>luxI-lacZ</i>	Translational fusion of the <i>P_{luxI}</i> promoter to <i>lacZ</i>	63
pAH125- <i>P_{luxI-lacZ}</i>	Translational fusion of the <i>P_{luxI}</i> promoter to <i>lacZ</i>	This study
pAH125- <i>P_{exsC-lacZ}</i>	Transcriptional fusion of the <i>P_{exsC}</i> promoter to <i>lacZ</i>	This study
pAH125- <i>P_{exsD-lacZ}</i>	Transcriptional fusion of the <i>P_{exsD}</i> promoter to <i>lacZ</i>	This study
pAH125- <i>P_{exoT-lacZ}</i>	Transcriptional fusion of the <i>P_{exoT}</i> promoter to <i>lacZ</i>	This study
pGEX- <i>rpoD</i> and its derivatives	Plasmid that constitutively expresses <i>rpoD</i> or one of 16 alanine point mutations	40
pGEX- <i>rpoD</i> ^(K593A,R596A,R599A)	<i>rpoD</i> expression plasmid carrying the K593A, R596A, and R599A mutations	This study
pET-23b	Protein expression vector that includes a carboxy-terminal His ₆ tag	Novagen
pET23- <i>rpoD</i> _{His} and its derivatives	RpoD expression vector with a carboxy-terminal His ₆ tag	This study
pET-24a	Protein expression vector that includes a carboxy-terminal His ₆ tag	Novagen
pET24- <i>rpoA</i> _{His}	CTD _{His6} -tagged RpoA expression vector	This study
pET24- <i>rpoB</i>	Untagged RpoB expression vector	This study
pET24- <i>rpoC</i>	Untagged RpoC expression vector	This study
pOM90	<i>In vitro</i> transcription template	54
pOM90- <i>P_{exsC}</i>	<i>In vitro</i> transcription template containing the <i>P_{exsC}</i> promoter	This study
pOM90- <i>P_{exsD}</i>	<i>In vitro</i> transcription template containing the <i>P_{exsD}</i> promoter	64
pSA508- <i>P_{exsC}</i> and its derivatives	<i>P_{exsC}</i> template vector yielding minicircle pMCP _{exsC}	64; this study
pTRCHIS-b	Source of <i>P_{trc}</i> promoter	Invitrogen
pOM90- <i>P_{trc(250)}</i>	<i>In vitro</i> transcription template containing the <i>P_{trc}</i> promoter	This study
pOM90- <i>P_{trc(180)}</i>	<i>In vitro</i> transcription template containing the <i>P_{trc}</i> promoter	This study
pOM90- <i>P_{RE#}</i>	<i>In vitro</i> transcription template containing the <i>P_{RE#}</i> promoter	This study
pET23- <i>rpoD</i> (1-574)	RpoD expression vector lacking region 4.2	This study

mM imidazole. Cells were lysed via sonication on ice, and unbroken cells were removed by centrifugation (15 min, 38,000 \times g, 4°C).

The α and σ subunits were denatured and solubilized with guanidine as described above and purified under denaturing conditions by Ni²⁺ affinity chromatography. Lysates were applied to a 1-ml HisTrap column (GE Healthcare) previously equilibrated with buffer B containing 5 mM imidazole, washed with 10 ml buffer B containing 30 mM imidazole, and developed with a 10-ml linear imidazole gradient (30 to 500 mM) in buffer B. The elution peaks were established by SDS-PAGE. The purified α subunit was stored on ice for immediate use in core RNAP reconstitution. Purified σ^{70} was dialyzed overnight against buffer E (50 mM Tris-HCl [pH 7.9], 200 mM KCl, 10 mM MgCl₂, 10 μ M ZnCl₂, 1 mM EDTA, 5 mM 2-mercaptoethanol, and 20% [vol/vol] glycerol) at 4°C, subjected to ultracentrifugation (30 min, 100,000 \times g, 4°C), and stored in 50% glycerol at -20°C.

The β and β' RNAP subunits were purified from *E. coli* inclusion bodies. *E. coli* Tuner(DE3) carrying either pET24-*rpoB* or pET24-*rpoC* was grown at 37°C in 1 liter of LB containing 50 μ g/ml kanamycin to an OD₆₀₀ of 0.5, at which time IPTG (1 mM) was added and the culture was incubated for an additional 3 h at

37°C. Bacteria were harvested by centrifugation and suspended in 16 ml of buffer C (40 mM Tris-HCl [pH 7.9], 300 mM KCl, 10 mM EDTA, 1 mM dithiothreitol [DTT], and 1 \times protease inhibitor cocktail [Roche]) containing 0.2 mg/ml lysozyme and 0.2% (wt/vol) sodium deoxycholate. The bacteria were incubated on ice for 20 min and lysed by sonication. Inclusion bodies were collected by centrifugation (30 min, 38,000 \times g, 4°C) and washed with 16 ml buffer C containing 0.2% *n*-octyl- β -D-glucoside. Inclusion bodies were sonicated and centrifuged as described above, followed by a final wash with 16 ml buffer C. Washed inclusion bodies were solubilized in 2 ml of buffer D (50 mM Tris-HCl [pH 7.9], 6 M guanidine-HCl, 10 mM MgCl₂, 10 μ M ZnCl₂, 1 mM EDTA, 10 mM DTT, and 10% [vol/vol] glycerol) and incubated at 25°C for 10 min. The resulting material was subjected to ultracentrifugation (30 min, 100,000 \times g, 4°C), and the soluble fraction was stored on ice for immediate use in core enzyme reconstitution.

RNAP core enzyme was reconstituted by mixing 0.3 mg purified α subunit, 1.5 mg purified β subunit, and 3 mg β' subunit in buffer D (2 ml) and dialyzing twice against 500 ml of buffer E at 4°C with constant stirring. The resulting material was subjected to ultracentrifugation (30 min, 100,000 \times g, 4°C), and the soluble

TABLE 2. Construction of plasmids used in this study

Figure	Product	Primer pair	Cloning vector
1B	p2UY21-LuxR	44122038-44122037	p2UY21
1, 2	pAH125-P _{exsC}	39530603-49188917	pAH125
2	pGEX- <i>rpoD</i> (K593A, R596A, R599A)	48669731-48669730	pGEX- <i>rpoD</i>
3-6	RpoD _{His}	43812190-43812191	pET-23b
3	RpoD (K597A)	46001014-46001013	pET23RpoD _{His}
3	RpoD (R603A)	47437714-47437715	pET23RpoD _{His}
3	RpoD (D616A)	47437713-47437712	pET23RpoD _{His}
3	RpoD (K597A,R603A)	46001014-46001013	pET23RpoD _{His} (R603A)
3	RpoD(K597A, R600A, R603A)	48432036-48432035	pET23RpoD _{His} (K597A,R603)
3-6	RpoA _{HisCTD}	46775590-46775589	pET-24a
3-6	RpoB	46775588-46775587	pET-24a
3-6	RpoC	47100507-46775585	pET-24a
3	pOM90-P _{exsC}	35048925-35048926	pOM90
4	P _{exsC} (G41T)	43648443-43648442	pSA508-P _{exsC}
4	P _{exsC} (T40A)	48552525-48552524	pSA508-P _{exsC}
4	P _{exsC} (G39C)	48552527-48552526	pSA508-P _{exsC}
4	P _{exsC} (A38T)	43648441-43648440	pSA508-P _{exsC}
4	P _{exsC} (C37G)	43648439-43648438	pSA508-P _{exsC}
4	P _{exsC} (A36T)	48552529-48552528	pSA508-P _{exsC}
4	P _{exsC} (A33G)	43648437-43648436	pSA508-P _{exsC}
4-6	P _{exsC} (TG)	48552531-48552530	pSA508-P _{exsC}
4-6	P _{exsC} (T8G)	43579324-43579323	pSA508-P _{exsC}
6	pOM90-P _{trc(250)}	25444818-25444816	pOM90
6	pOM90-P _{trc(179)}	25444818-25444814	pOM90
6	pOM90-P _{RE#}	48495914-48495913	pOM90
6	RpoD (1-574)	43812190-48495915	pET-23b

fraction was applied to a 1-ml HiTrap heparin HP column (GE Healthcare) equilibrated with buffer E. The column was washed with 10 ml buffer E containing 0.4 M KCl and developed with a 10-ml linear KCl gradient (0.4 to 2 M) in buffer E. The elution peaks were analyzed by SDS-PAGE, and pure fractions containing stoichiometric core RNAP ($\alpha_2\beta\beta'$) were dialyzed against 1 liter buffer E containing 50% glycerol and stored at -20°C .

RNAP holoenzyme was reconstituted by mixing core RNAP (500 nM) and σ^{70} (1 μM) in 35 μl $1\times$ transcription buffer (40 mM Tris-HCl [pH 7.5], 50 mM KCl, 10 mM MgCl_2 , 1 mM DTT, 0.1% Tween 20, and 0.5 mg/ml bovine serum albumin [BSA]) for 30 min at 25°C . The resulting holoenzyme (1 μl) was then used in a 20- μl transcription reaction mixture.

In vitro transcription assays. Supercoiled transcription templates containing the P_{exsC} and P_{exsD} promoters were described previously (14, 64). The pOM90-P_{exsC} template was generated by PCR amplifying the P_{exsC} promoter (nucleotides [nt] -207 to +192 relative to the transcriptional start site) and cloning as an EcoRI fragment into pOM90 (54). The resulting template contains a fusion of the P_{exsC} promoter to the *rpoC* transcriptional terminator (*rpoC_{ter}*) on pOM90 and directs synthesis of a 261-nt transcript. The pOM90-P_{trc180} and pOM90-P_{trc250} templates were generated by PCR amplifying the P_{trc} promoter (nucleotides -61 to +109/179 relative to the transcriptional start site) from pTRCHIS-b (Invitrogen) and cloning as an EcoRI fragment into pOM90. The pOM90-P_{trc180} and pOM90-P_{trc250} templates fuse the P_{trc} promoter to *rpoC_{ter}*, resulting in 180- and 250-base transcripts, respectively. Finally, the pOM90-P_{RE#} template was generated by annealing complementary oligonucleotides, and the resulting BamHI-EcoRI fragment was cloned into pOM90. The pOM90-P_{RE#} template fuses the synthetic P_{RE#} promoter to the *rrnB* T1 terminator and results in a 135-base transcript.

Single-round transcription assays (20- μl final volume) were performed by incubating ExsA_{His} (35 nM) with transcription templates (2 nM) at 25°C in $1\times$ transcription buffer (40 mM Tris-HCl [pH 7.5], 50 mM KCl, 10 mM MgCl_2 , 1 mM DTT, 0.1% Tween 20, and 0.5 mg/ml BSA) containing the initiating nucleotides ATP and GTP (0.75 mM). After 10 min, 25 nM reconstituted *P. aeruginosa* RNAP holoenzyme was added, and open complexes were allowed to form for 1 min at 25°C in the presence of ExsA_{His} or for 20 min at 25°C in the absence of ExsA_{His}. Elongation was allowed to proceed by the addition of the remaining nucleotides (0.25 mM ATP/GTP/CTP, 0.75 mM UTP, and 2.5 μCi [α - ^{32}P]CTP) in $1\times$ transcription buffer containing heparin (final concentration, 50 $\mu\text{g}/\text{ml}$). Reactions were stopped after 5 min at 25°C by the addition of 20 μl stop buffer (98% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene

cyanol). Samples were heated at 95°C for 5 min and immediately incubated on ice before electrophoresis on 5% denaturing polyacrylamide gels.

RESULTS

The carboxy-terminal domain of the RNAP α subunit is not required for ExsA-dependent transcriptional activation. Since ExsA activates transcription primarily through recruitment of RNAP (64) and many transcriptional activators that recruit do so by contacting the carboxy-terminal domain of the RNAP α subunit (α -CTD), we tested the hypothesis that ExsA uses a similar mechanism. Previous studies have shown that ExsA activates transcription *in vitro* to similar levels using RNAP from either *P. aeruginosa* or *E. coli* (64). To demonstrate that ExsA can activate transcription from the P_{exsC}, P_{exsD}, and P_{exoT} promoters in *E. coli*, ExsA was expressed from a plasmid (p2UY21-*exsA*) under the transcriptional control of a constitutive α -CTD-independent promoter. ExsA-dependent transcription was measured from transcriptional reporters consisting of ExsA-dependent promoters (P_{exsC}, P_{exsD}, and P_{exoT}) fused to *lacZ* and integrated at the *E. coli* λ phage attachment site. Significant ExsA-dependent activation of all three promoters was observed relative to a control plasmid (Fig. 1A), demonstrating that ExsA is sufficient to activate transcription from P_{exsC}, P_{exsD}, and P_{exoT}, as was previously shown for P_{exsC} in *E. coli* (61).

To determine the role of the α -CTD, we used an established *E. coli* assay in which the native α subunit (α -wt) or α lacking the C-terminal 239 amino acids (α - Δ CTD) was expressed from a plasmid such that its cellular concentration exceeded that of native α subunit expressed from the chromosome. This approach was necessary because deletion of the α subunit CTD is

TABLE 3 . Primers used in this study

Primer identification no.	Sequence
44122038	CATGGCCATATGAAAAACATAAATGCCGAC
44122037	CATGGCGAGCTCTTAATTTTTAAAGTATGG
39530603	GCGACGCGGTACCATGAAGGACGTCCTGCAGC TCATCC
49188917	TGATGAATTCGCTCCTAAAGCTCAGCGC ATGC
48669731	CAGATCGAAGCGGCGGCGCTGGCCAAACTGG CTCACCCGAGCCGT
48669730	ACGGCTCGGGTGAGCCAGTTTGGCCAGCGCCG CCGCTTCGATCG
43812190	CCGAGCCATATGTCCGAAAAGCGCAA
43812191	GGCAGGAAGCTTCTCGTCGAGGAAGGAGCG
46001014	CAGATCGAAGCCGCGGCGTTGCGCAAG
46001013	CTTGCGCAACGCCGCGCTTCGATCTG
47437714	TCGCGACGGATGGGCCAGTTGCGCAA
47437715	TTGCGCAAGCTGGCCATCCGTCGCGA
47437713	CGCTCCTTCTCGCCGAGAAGCTTGCG
47437712	CGCAAGCTTCTCGGCGAGGAAGGAGCG
48432036	GCCGCGGCGTTGGCCAAAGCTGGCCCAT
48432035	ATGGGCCAGCTTGCCAAACGCCGCGGC
46775590	GCCACCCATATGCAGAGTTCGGTAAATGAGTT
46775589	GCTACGCGGCCGCGGCGCAGTGGCCTTGTC GTCTTTCTTA
46775588	GCCACCCATATGGCTTACTCATACACTGAGAA AAAACG
46775587	GCCTACGCGGCCGCGGCTTATTCGGTTTCCAGT TCGATGTCG
47100507	GCCACCCATATGAAAGACTTGCTTAATCTGT TGAA
46775585	GCCTACGCGGCCGCGGCTTAGTTACCGCTCGA GTTACGCGCTT
35048925	ATACTGGAATTCTGCGGTTCCCCCCC
35048926	ACGAATGAATCCCACATCGGCCTCCAGCAAC
43648443	AAGAAAAGTCTCTCATTGACAAAAGCGATGC
43648442	GCATCGCTTTTGTCAATGAGAGACTTTTCTT
48552525	AAAGTCTCTCAGAGACAAAAGCGAG
48552524	CTCGCTTTTGTCTCTGAGAGACTTT
48552527	AAGTCTCTCAGTCACAAAAGCGAGG
48552526	CCTCGCTTTTGTGACTGAGAGACTT
43648441	AAAAGTCTCTCAGTGTCAAAAAGCGATGCATA
43648440	TATGCATCGCTTTTGACACTGAGAGACTTTT
43648439	AAAGTCTCTCAGTGTGAAAAGCGATGCATAG
43648438	CTATGCATCGCTTTTCTCACTGAGAGACTTT
48552529	TCTCTCAGTGACTAAAAGCGAGGCAT
48552528	ATGCCTCGCTTTAGTCACTGAGAGA
43648437	TCTCTCAGTGCACAAAGGCGATGCATAGCCCG
43648436	CGGGCTATGCATCGCCTTTGCTCACTGAGAGA
48552531	GGCATAGCCCGGACCTAGCATGCGCT
48552530	AGCGCATGCTAGGTCCGGGCTATGCC
43579324	AGCCCGGTGCTAGCAGGCGCTGAGCTTTAGG
43579323	CCTAAAGCTCAGCGCTGCTAGCACCCTGGCT
25444818	CTGCGAATTCACCGTTTCTGGCAAATATTC
25444816	CCGCGAATTCGGTTTTATCTCCTTATTTA ATCG
25444814	CTATGAATTCGAGTGCCACACAGATTTT
48495914	GATCCTCGTTGCGTTTGTGTTGACGAGCTCTAT GTTATAATTTCTAAGCTTG
48495913	AATTCAAGCTTAGGAAATTATAACATAGAGTC GTGCAAAACAAACGCAACGAG
48495915	GGCAGGAAGCTTCGACTGCATGGTGGAGTC

lethal in *E. coli* (37). ExsA-dependent transcription following overexpression of α - Δ CTD was plotted as a percentage of the activation observed with overexpressed α -wt. As a control, we also measured LuxR-dependent activation of a $P_{luxI-lacZ}$ tran-

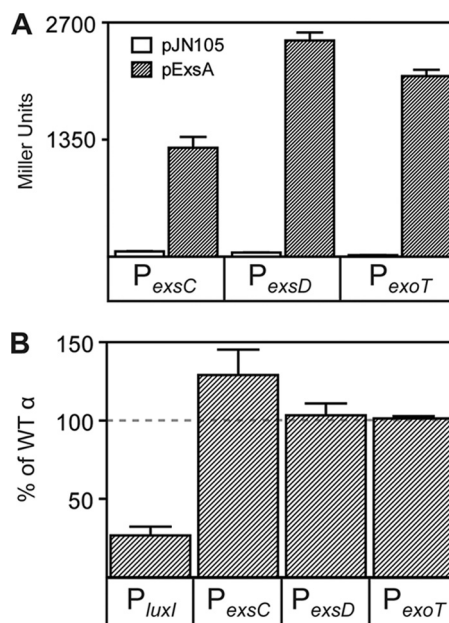


FIG. 1. The RNAP α -CTD is not required for ExsA-dependent activation of transcription. (A) *E. coli* strain GS162 carrying the indicated transcriptional reporters ($P_{exsC-lacZ}$, $P_{exsD-lacZ}$, or $P_{exoT-lacZ}$) was transformed with a vector control (pJN105) or a constitutive ExsA expression plasmid (p2UY21, labeled pExsA in the figure). The resulting strains were grown in LB to an OD_{600} of 0.6 and assayed for β -galactosidase activity (reported in Miller units). (B) *E. coli* GS162 carrying a $P_{luxI-lacZ}$ reporter and a LuxR expression plasmid (p2UY21-*luxR*) and the reporter strains from panel A were transformed with a plasmid expressing the native α or α - Δ CTD subunit. The resulting strains were grown in LB to an OD_{600} of 0.6 and assayed for β -galactosidase activity. The reporter activities obtained in cells expressing α - Δ CTD were normalized to the same strain expressing native (WT) α and reported as the percentage of native activity. The results represent the averages for three independent experiments, and error bars represent the standard errors of the means.

scriptional fusion (1). LuxR is an activator known to require the α -CTD (59). ExsA-dependent activation of the $P_{exsC-lacZ}$, $P_{exsD-lacZ}$, and $P_{exoT-lacZ}$ reporters in the presence of α - Δ CTD was $\geq 100\%$ of that seen with α -wt, indicating that ExsA does not require the α -CTD for transcriptional activation (Fig. 1B). Curiously, activation from the P_{exsC} promoter in the presence of α - Δ CTD was 125% of the wild-type value, suggesting that the α -CTD might have an inhibitory function at this promoter. In contrast, activation of the $P_{luxI-lacZ}$ reporter was reduced to $\sim 33\%$ of the wild-type value in the presence of α - Δ CTD.

ExsA-dependent transcription in *E. coli* is dependent on specific amino acids within region 2 of σ^{70} domain 4. A number of class II transcriptional activators interact with a basic amino acid region (region 2) of σ^{70} domain 4. Since the ExsA-binding sites overlap a near-consensus -35 RNAP recognition hexamer, we hypothesized that ExsA recruits RNAP through an interaction with region 4.2 of the σ^{70} subunit. Lonetto et al. generated an *rpoD* plasmid expression library containing alanine point mutations in 16 nonessential positions of region 4.2 to test for activator-specific defects in gene expression (40). Those experiments were performed in *E. coli* strain GA2071 where expression of chromosomal *rpoD* is tightly repressed. To measure ExsA activity, the $P_{exsC-lacZ}$ and $P_{exsD-lacZ}$ transcrip-

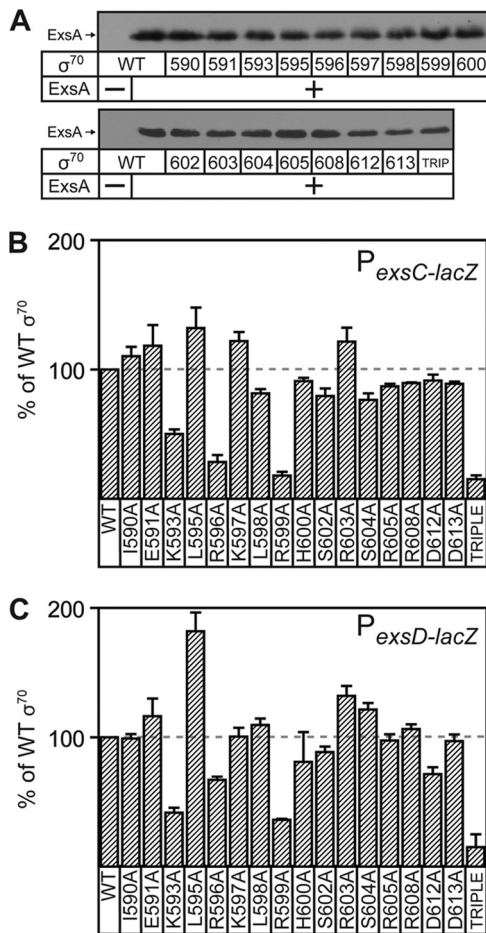


FIG. 2. ExsA-dependent transcription requires several amino acids in region 4.2 of *E. coli* σ^{70} . (A) ExsA immunoblots demonstrating that steady-state expression levels are similar in each of the strains used below. (B and C) *E. coli* strain GA2071 (tightly suppressed for native σ^{70} expression) carrying the $P_{exsC-lacZ}$ (B) or $P_{exsD-lacZ}$ (C) transcriptional reporter and the p2UY21 ExsA expression plasmid was transformed with a wild-type σ^{70} expression plasmid or an σ^{70} expression plasmid carrying the indicated mutations in region 4.2. The resulting strains were grown in LB to an OD₆₀₀ of 0.6 and assayed for β -galactosidase activity. The reported values (percentage of activity in the presence of wild-type σ^{70} subunit) are the averages from three independent experiments, and error bars represent the standard errors of the means.

tional reporters were introduced at the λ phage attachment site of *E. coli* strain GA2071, and the resulting strains were transformed with a plasmid expressing wild-type RpoD or the RpoD point mutants. Given the tendency for reversion of *rpoD* point mutants to the native sequence (40), the integrity of each expression plasmid was verified by nucleotide sequence analysis after introduction into strain GA2071. ExsA was constitutively expressed from plasmid p2UY21. Since the RpoD mutants in this library do not affect activator-independent transcription, ExsA expression levels were similar in each of the tested strains (Fig. 2A) (40). ExsA-dependent expression from the P_{exsC} and P_{exsD} reporters in the presence of RpoD mutants was plotted relative to that in the presence of wild-type RpoD (Fig. 2B and C). The most drastic effect on ExsA-dependent activation of the $P_{exsC-lacZ}$ reporter resulted with

the K593A, R596A, and R599A substitutions, which exhibited 50%, 29%, and 25% of the activity seen with native RpoD, respectively (Fig. 2B). Similarly, $P_{exsD-lacZ}$ reporter activity was also impaired by the K593A, R596A, and R599A substitutions to 42%, 67%, and 36% of the native RpoD levels (Fig. 2C).

The effects observed from the single amino acid substitutions were modest (2- to 3-fold) and likely reflect the fact that each of the individual positions represents only a portion of the ExsA- σ^{70} interaction site. We predicted that ExsA-dependent transcription might result from synergistic interactions with each of the three amino acid positions. This proved to be true, as the activity of the P_{exsC} and P_{exsD} reporters in the presence of a triple RpoD mutant (K593A, R596A, R599A) was only 15% of the wild-type activity in both cases (Fig. 2B and C). We did note that strain GA2071 expressing the RpoD triple mutant exhibited a 2-fold growth defect yet had ExsA levels equivalent to those of GA2071 expressing native RpoD (Fig. 2A).

ExsA-dependent transcription *in vitro* is dependent on *P. aeruginosa* σ^{70} region 4.2. To further characterize the role of σ^{70} region 4.2, the mutations from *E. coli* *rpoD* (K593A, R596A, and R599A) that affect ExsA-dependent transcription *in vivo* were introduced into *P. aeruginosa* *rpoD*. Native and mutant forms of *P. aeruginosa* RpoD were expressed in *E. coli* and purified under denaturing conditions by Ni²⁺ affinity chromatography. Core RNAP was generated by expressing the *P. aeruginosa* α , β , and β' subunits in *E. coli*, purifying the individual purified components (Fig. 3A), and reconstituting σ -saturated RNAP holoenzyme with either native RpoD, RpoD-K593A, RpoD-R596A, RpoD-R599A, or the triple RpoD mutant. RNAP holoenzyme activity was normalized between the different RpoD-reconstituted polymerases by comparing the production of single-round *in vitro* transcription products from the P_{trc} promoter. Transcription from the P_{trc} promoter was not affected by the K593A, K596A, or K599A mutation in region 4.2 of σ^{70} (40).

Reconstituted RNAP holoenzymes were then assayed for ExsA-dependent transcription *in vitro* using supercoiled plasmid templates containing the P_{exsC} and P_{exsD} promoters fused to the *rpoC_{ter}* terminator. Each of the templates generates a 261-nucleotide, terminated transcript. As expected, terminated transcripts were not observed with core RNAP alone. We initially tested the individual σ^{70} mutants (K597A, R600A, and R603A) for ExsA-dependent activation of the P_{exsC} or P_{exsD} promoters but found that none had an activation defect greater than 50% of native RpoD (data not shown). This result was not surprising given that a similar observation was made when testing the individual σ^{70} mutants for activation in *E. coli* (Fig. 2B and C). In contrast, the triple RpoD mutant produced far less *exsC* and *exsD* transcripts than did native RpoD (Fig. 3B and C). These combined data indicate that σ^{70} region 4.2 is required for ExsA-dependent activation of the P_{exsC} and P_{exsD} promoters both *in vivo* and *in vitro*.

The near-consensus -35 sequence at the P_{exsC} promoter is not required for ExsA-independent transcription. We previously demonstrated that the P_{exsC} promoter has low basal activity in the absence of ExsA (64). To determine whether the putative -35 sequence is required for ExsA-independent promoter activity, we generated P_{exsC} transcription templates containing point mutations at each of the -35 nucleotide positions. Each of the nucleotide substitutions, with the exception

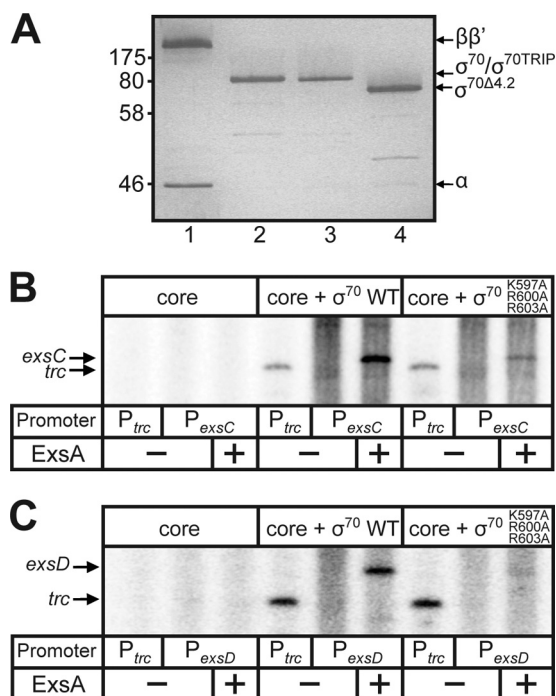


FIG. 3. ExsA-dependent *in vitro* transcription is dependent on region 4.2 of σ⁷⁰ from *P. aeruginosa*. (A) Silver-stained SDS-polyacrylamide gel of purified and reconstituted core polymerase subunits α, β, and β' (lane 1), native σ⁷⁰ (lane 2); σ⁷⁰ carrying the K597A, R596A, and R599A amino acid substitutions (lane 3); and σ⁷⁰ lacking region 4.2 (lane 4). (B and C) Single-round *in vitro* transcription assays. ExsA_{His} (35 nM) was incubated with 2 nM supercoiled P_{exsC} or P_{exsD} promoter template (pOM90-P_{exsC} or pOM90-P_{exsD}) at 25°C in the presence of rATP and rGTP. After 10 min, *P. aeruginosa* core RNAP, σ⁷⁰-RNAP, or σ⁷⁰ (K597A/R596A/R599A)-RNAP was added (25 nM each); and the activity of σ-saturated enzymes was normalized with P_{trc}, and the reaction mixture was incubated for 1 min at 25°C. Heparin and substrate nucleotides (including 2.5 μCi [α-³²P]CTP) were immediately added, and the reaction mixture was incubated for 5 min at 25°C. Reactions were terminated, and the resulting products were electrophoresed on a 5% denaturing polyacrylamide-urea gel and subjected to phosphorimaging. The ExsA-dependent terminated transcripts (261 nt) from the P_{exsC} or P_{exsD} promoter and the runoff transcripts (250 or 180 nt) from the P_{trc} promoter are indicated.

of G41T, was divergent from the σ⁷⁰ consensus (Fig. 4A). The mutant promoters were assayed for ExsA-independent transcript levels and compared to the native P_{exsC} promoter and to a negative control containing a single point mutation (T8G) in the established -10 Pribnow box (Fig. 4A). To account for subtle differences in template concentration and purity, the P_{exsC} transcripts were normalized to a constitutive transcript generated from a promoter located on the plasmid backbone (64). Whereas the negative control (T8G) lacking a functional -10 hexamer exhibited a 50-fold decrease in transcription compared to P_{exsC} (Fig. 4), the remaining point mutants had little (less than 2-fold) or no effect on ExsA-independent transcription (Fig. 4B and C). These data indicate that the putative -35 hexamer is not important for ExsA-independent transcription at the P_{exsC} promoter.

Although a near-consensus, but improperly spaced, -35 sequence is present at the P_{exsC} promoter, it is possible that a weak, unrecognizable -35 hexamer with a poor match to the

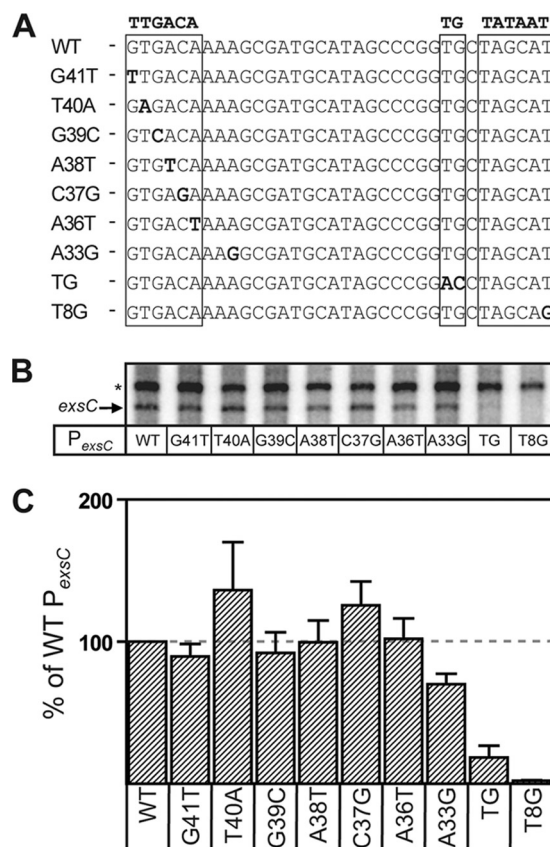


FIG. 4. The near-consensus -35 hexamer in the P_{exsC} promoter is not required for ExsA-independent transcription. (A) Diagram showing the mutant P_{exsC} promoter derivatives used in this experiment. The -35, extended -10, and -10 elements are boxed, and the individual point mutations are in bold. (B) Single-round *in vitro* transcription assays showing ExsA-independent transcription from P_{exsC} derivatives containing -35 (G41T, T40A, G39C, A38T, C37G, A36T, and A33G), extended -10 (TG), and -10 (T8G) point mutations. Reactions were performed as described in the legend to Fig. 3, except open complexes were allowed to form for 20 min in the absence of ExsA. (C) Quantification of the *in vitro* transcription data shown in panel B. The amount of exsC transcript produced in each experiment was normalized to an ExsA-independent transcript (64) produced from a weak promoter on the minicircle backbone. The reported values are the averages from three independent experiments, and error bars represent the standard errors of the means.

σ⁷⁰ consensus is present and optimally spaced (16/17 bp) from the -10 hexamer. Potential -35 hexamers spaced at either 16 or 17 bp would have the sequence AAAGCG or AAAAGC, respectively (matches to consensus are underlined). To test this hypothesis, we constructed a single point mutation in the P_{exsC} promoter (A33G) such that the potential -35 hexamer spaced 16 bp (AAGGCG) from the -10 hexamer more closely resembles the -35 consensus sequence and the potential -35 hexamer spaced at 17 bp (AAAGGC) would be a weaker match to the consensus. The A33G mutation had no significant effect (<2-fold) compared to native P_{exsC}. These combined data suggest that the putative -35 sequence is not important for ExsA-independent transcription.

The P_{exsC} promoter sequence located immediately upstream of the -10 box resembles an extended -10 promoter (Fig.

4A). Extended -10 promoters contain the sequence TGxTA TAAT and can function in either the presence or absence of a -35 hexamer (4, 44). To determine whether the P_{exsC} promoter contains an extended -10 element, we mutated the consensus TG sequence to AC (here referred to as $P_{\text{exsC-TG}}$). As expected, the mutant $P_{\text{exsC-TG}}$ promoter had a significant reduction in ExsA-independent transcription (5-fold) compared to the native P_{exsC} promoter (Fig. 4B and C). These combined data suggest that the P_{exsC} promoter lacks a -35 hexamer and that an extended -10 element may provide basal promoter activity.

The extended -10 element is important for ExsA-independent and -dependent P_{exsC} promoter activity. Since ExsA-independent activity of the P_{exsC} promoter requires an apparent extended -10 sequence, we asked whether ExsA-dependent activation had a similar requirement using *in vitro* transcription assays. $P_{\text{exsC-TG}}$ promoter activity was reduced 3-fold in the presence of ExsA, demonstrating that the extended -10 element affects P_{exsC} to similar extents in the presence and absence of ExsA (Fig. 5A and B). In contrast, the T8G mutation ablates both ExsA-dependent and -independent promoter activity. Note that ExsA-independent transcripts were not observed under these conditions due to the short RNAP incubation time (1 min) required to detect ExsA-dependent open complex formation in the linear range (Fig. 5A and data not shown). To rule out the trivial explanation that the DNA-binding activity of ExsA is affected by the TG mutation, we employed electrophoretic mobility shift assays (EMSAs) and found no significant difference in the binding affinity of ExsA for the $P_{\text{exsC-TG}}$ and native P_{exsC} promoters or in formation of shift complexes 1 and 2 (Fig. 5C).

Region 4.2 of σ^{70} is required for ExsA-dependent but not ExsA-independent transcription. Region 4.2 of σ^{70} recognizes the -35 hexamer and is essential for recognition of most bacterial promoters (15). Region 4.2 is also a common target for AraC family transcriptional activators. We have provided evidence that ExsA interacts with this region and that the putative -35 sequence is not a determinant for RNAP recruitment at the P_{exsC} promoter. Based on these data, we hypothesized that the P_{exsC} extended -10 element compensates for the lack of a functional -35 hexamer. To test this idea, we employed *in vitro* transcription assays utilizing RNAP holoenzyme reconstituted with σ^{70} lacking the carboxy-terminal 43 amino acids, $\sigma^{70\Delta 4.2}$, which encompasses region 4.2. Whereas deletion of region 4.2 renders promoters that are dependent upon -35 hexamers nonfunctional, the same deletion has little effect on transcription initiation and elongation from extended -10 promoters (38). The following promoters were used as controls for this experiment: (i) P_{trc} , which contains a strong -35 hexamer and requires region 4.2 of σ^{70} , and (ii) $P_{\text{RE\#}}$, a synthetic promoter which lacks a -35 hexamer and does not require region 4.2 but is dependent upon an extended -10 element (10, 38) (Fig. 6A). Although $\sigma^{70\Delta 4.2}$ has slightly reduced affinity for core RNAP enzyme (38), holoenzyme reconstituted with $\sigma^{70\Delta 4.2}$ (here referred to as RNAP- $\sigma^{\Delta 4.2}$) and native RNAP holoenzyme generated similar levels of transcript from the $P_{\text{RE\#}}$ promoter (Fig. 6B). In contrast, RNAP- $\sigma^{70\Delta 4.2}$ generated significantly less transcript from the P_{trc} promoter than did RNAP- σ^{70} (Fig. 6B). Consistent with our hypothesis that the P_{exsC} promoter lacks a functional -35 hexamer, RNAP- $\sigma^{70\Delta 4.2}$

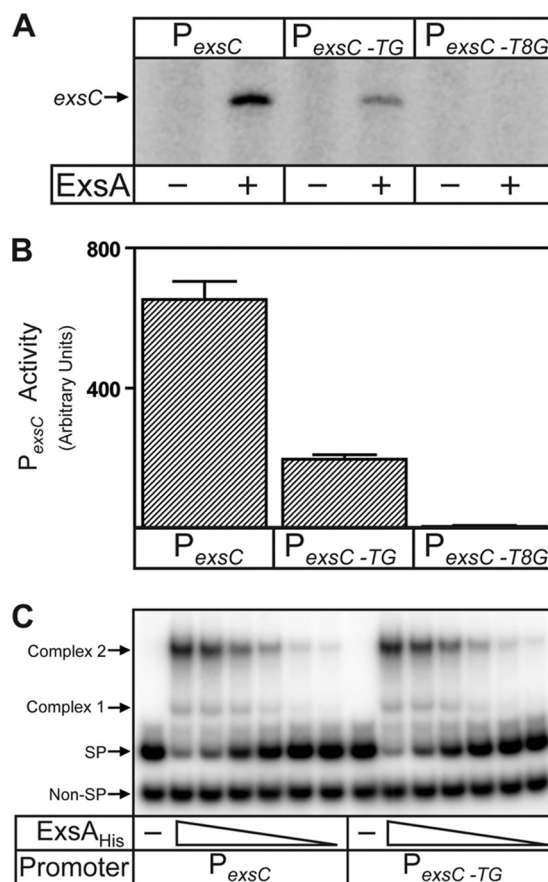


FIG. 5. The extended -10 element within the P_{exsC} promoter is important for overall promoter activity independent of ExsA function. (A and B) Single-round *in vitro* transcription assays and quantification of the corresponding transcripts from the P_{exsC} , $P_{\text{exsC-TG}}$, and $P_{\text{exsC-T8G}}$ promoters. Experiments were performed as described in the legend to Fig. 3, allowing 1 min for open complex formation in both the absence and presence of ExsA. The reported values (arbitrary densitometry units) are the averages from three independent experiments, and error bars represent the standard errors of the mean. (C) Electrophoretic mobility shift assays (EMSAs) of the P_{exsC} and $P_{\text{exsC-TG}}$ promoter probes. Specific (SP) and nonspecific (Non-SP) probes (0.25 nM each) were incubated in the absence of ExsA_{His} (–) or with increasing concentrations of ExsA_{His} (1 to 36 nM; 2-fold dilutions) for 15 min, followed by electrophoresis and phosphorimaging. ExsA_{His}-dependent shift products 1 and 2 are indicated.

and RNAP- σ^{70} generated similar levels of P_{exsC} transcript in the absence of ExsA (Fig. 6B). In addition, the $P_{\text{exsC-TG}}$ and $P_{\text{exsC-T8G}}$ mutants were essentially devoid of RNAP- $\sigma^{70\Delta 4.2}$ -dependent activity. Finally, we tested whether ExsA-dependent transcripts were produced from the P_{exsC} promoter using RNAP- $\sigma^{70\Delta 4.2}$. Although ExsA-dependent transcription was drastically reduced with RNAP- $\sigma^{70\Delta 4.2}$, a detectable transcript was made when reactions were allowed to proceed for 1 min for open complex formation. These same conditions do not support the detection of transcription in the absence of ExsA using native RNAP- σ^{70} holoenzyme (Fig. 5A). It is unclear whether the weak ExsA-dependent transcription in the absence of region 4.2 represents additional contacts between ExsA and σ^{70} outside region 4.2 or additional contacts between ExsA and other RNAP subunits.

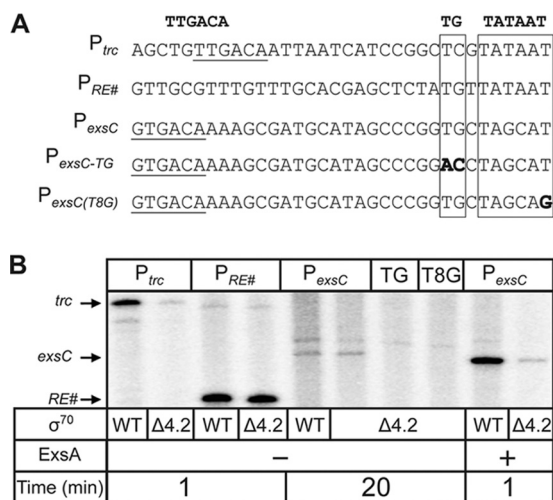


FIG. 6. Region 4.2 of σ^{70} is not required for ExsA-independent transcription of the P_{exsC} promoter. (A) Diagram of transcription templates used in this experiment. The -35 regions (underlined), extended -10 elements (boxed), -10 elements (boxed), and point mutations (bold) are indicated. (B) Single-round *in vitro* transcription assays were performed with σ^{70} and $\sigma^{70\Delta 4.2}$ reconstituted RNAP holoenzymes normalized for specific activity using the P_{RE#} extended -10 promoter (lanes 3 and 4). Reactions were performed as described in the legend to Fig. 3, and open complexes were allowed to form for 1 min (lanes 1 to 4, 9, and 10) or 20 min (lanes 5 to 8) as indicated.

DISCUSSION

In the present study we find that recruitment of RNAP by ExsA does not require the CTD of the RNAP α subunit, a common target for AraC family regulators. Although these studies were performed with *E. coli* we believe the findings would be identical for *P. aeruginosa*. Data supporting this claim include the following: (i) ExsA activates transcription from T3SS promoters *in vitro* to similar extents with RNAP (normalized for specific activity using an α -CTD-independent promoter) from either *E. coli* or *P. aeruginosa* (64). (ii) the carboxy-terminal 90 amino acids of the α subunits from *E. coli* and *P. aeruginosa* share 86% identity, and (iii) heterologous activators known to require the α -CTD, including LuxR from *Vibrio fischeri* (used in this study), can efficiently activate *E. coli* RNAP (59). For these reasons, we believe that the involvement of the α -CTD in ExsA-dependent activation would have been detected in our experiments.

Interestingly, ExsA-dependent transcription from the P_{exsC} promoter was slightly elevated (125%) following expression of α - Δ CTD compared to the full-length α subunit (Fig. 1B). A possible explanation for this finding is that the α -CTD may bind the P_{exsC} promoter and antagonize ExsA function. In this scenario, the α -CTD-P_{exsC} promoter interaction might sterically hinder the DNA-binding activity of ExsA or its ability to contact RNA polymerase. We did not test whether ExsA interacts with the amino-terminal domain (NTD), since Egan et al. have shown that an extremely diverse group of AraC family members do not require this domain for transcriptional activation (23).

Using a plasmid-based mutant *rpoD* expression library, we found that ExsA requires the K593, R596, and R599 amino acids of σ^{70} for full activation of the P_{exsC} and P_{exsD} promoters

(Fig. 2). These specific residues are some of the most frequently observed contact points for AraC family members and unrelated transcriptional regulators (20, 40). In fact, an alignment of ExsA with the AraC family members RhaS and MelR reveals a conserved aspartate residue known to interact with R599 of σ^{70} (27, 66). Whether this aspartate or other conserved positions are important for the interaction of ExsA with σ^{70} will be the subject of future studies. Although ExsA-dependent activation defects of greater than 2-fold were not routinely observed with a single point mutation in *rpoD*, expression of the chromosomal *rpoD* gene is only suppressed in these experiments, and leaky expression of *rpoD* may result in higher levels of ExsA-dependent activation that would bias the data toward transcriptional activation defects smaller than those observed. Furthermore, the literature suggests that RNAP-activator interaction regions most likely consist of several amino acid contacts (40). Consistent with this, we find that the σ^{70} triple mutant (K593A, R596A, R599A) showed a cumulative 6-fold effect on ExsA-dependent transcription *in vitro* and *in vivo* (Fig. 2 and 3). It is possible that ExsA may interact with amino acids in region 4.2 that we did not test, other regions in σ^{70} , and/or different RNAP subunits. The 16 amino acids in the mutant *rpoD* expression library were selected because these positions are reported to have little effect on activator-independent transcription (40). Some amino acids in region 4.2 were omitted from this library because alanine substitution resulted in unstable protein or because they are required for interaction with the -35 hexamer (40). It is therefore possible that other amino acids are also important for the interaction with ExsA. Finally, the finding that a σ^{70} derivative lacking region 4.2 ($\sigma^{\Delta 4.2}$) is still capable of weak ExsA-dependent activation supports the hypothesis that ExsA interacts with several regions of σ^{70} and/or multiple RNAP subunits (Fig. 6B).

Although the mechanism of transcription activation is known for only a small number of AraC family activators, most activate transcription by facilitating both closed and open complex formation (11). Activators that facilitate both closed and open complex formation do so by contacting the α -CTD and σ^{70} region 4.2, respectively (11). It is therefore curious that ExsA requires region 4.2 of σ^{70} and functions primarily to recruit RNAP. A possible explanation for this finding is that the ExsA- σ^{70} region 4.2 interaction affects the rate of isomerization to an open complex. This explanation seems unlikely, however, as disruption of the ExsA- σ^{70} region 4.2 interaction results in at least a 5-fold defect in activation, whereas ExsA is known to only marginally affect (2-fold) the rate of isomerization to an open complex. We believe a more likely explanation is that ExsA interaction with σ^{70} region 4.2 results primarily in the recruitment of RNAP. This is in contrast to the reported activity of the well-characterized cI protein of phage lambda, which increases the isomerization rate at the P_{RM} promoter by contacting σ^{70} region 4.2 (21, 30). The cI example is somewhat paradoxical, since it has been well established that in the absence of a transcriptional activator, σ^{70} region 4.2 normally interacts directly with DNA at the -35 position to facilitate the initial binding of RNAP to the promoter (15). In fact, the observation that ExsA recruits RNAP through contacts with σ^{70} region 4.2 seems to better support the known function of region 4.2. We believe the most likely explanation for these

discrepancies is that protein-protein interactions with σ^{70} region 4.2 can affect both closed and open complex formation. In support of this claim, a single point mutation (R596H) in σ^{70} region 4.2 changes the mechanism of cI activation to an enhancement of closed complex formation while having almost no effect on the rate of isomerization to an open complex (21). This finding may indicate that the specific contacts between transcriptional activators and σ^{70} region 4.2 do not determine whether closed or open complex formation is enhanced. In fact, Dove et al. have suggested that the promoter sequence and location of the activator-binding site may play the most important part in determining the mechanism of transcriptional activation by an activator (21). Further studies analyzing the structure of activator-RNAP complexes are needed to address this curious observation.

We provide evidence that the putative -35 hexamer in the P_{exsC} promoter is not sufficient for ExsA-independent expression. This is consistent with a previous study demonstrating that the -35 hexamer from P_{exsD} , although a close match to the σ^{70} consensus, is also not used as an RNAP recognition site (12, 64). To further characterize the role of the P_{exsC} -35 region, we generated point mutations at every position in the -35 site, and the resulting mutations had no significant effect (<2 -fold) on ExsA-independent transcription, while control mutations in the -10 hexamer resulted in undetectable levels of transcript (Fig. 4). An explanation for this result is that an authentic -35 hexamer is located at a more favorable position (16 or 17 bp relative to the -10 sequence) but has few matches to the consensus sequence. We tested this hypothesis by creating a single point mutation in P_{exsC} (A33G), which should significantly increase or decrease ExsA-independent activation if the -35 hexamer is positioned 16 or 17 bp from the -10 hexamer, respectively (46). No significant effect was observed with this mutant, suggesting that a -35 hexamer is not required for ExsA-independent transcription at the P_{exsC} promoter. Unfortunately, we were unable to assess the role of the -35 hexamer with respect to ExsA-dependent transcription, since mutations in the -35 region are known to disrupt ExsA binding to site 1 (12).

Consistent with the hypothesis that a -35 hexamer is not required for ExsA-independent transcription from the P_{exsC} promoter, we identified a putative extended -10 element (38). A point mutation within this element resulted in a significant reduction in both ExsA-dependent and ExsA-independent transcription (Fig. 4 and 5). EMSA experiments demonstrated that the extended -10 mutation had no effect on ExsA binding to the promoter (Fig. 5C). These data indicate that the P_{exsC} promoter contains an extended -10 promoter that might partially compensate for the lack of a functional -35 hexamer. Since *exsA* expression is autoregulated through the P_{exsC} promoter, it is tempting to speculate that the extended -10 element is important in maintaining a basal level of the *exsCEBA* transcript. The fact that the extended -10 element is required for maximal P_{exsC} promoter activity, however, prevented us from directly testing this hypothesis. Nevertheless, 5' RACE promoter-mapping experiments demonstrate that *exsCEBA* transcript is detectable in an *exsA* mutant (64), suggesting that P_{exsC} exhibits some level of basal activity. We propose a model in which ExsA recruits RNA polymerase to an extended -10 promoter (P_{exsC}) by contacting σ^{70} region 4.2. Interestingly, the

residual transcription from P_{exsC} seen with $\sigma^{70\Delta 4.2}$ -RNAP was shown to be ExsA dependent (compare Fig. 5A and 6B), further suggesting that an additional region of σ^{70} or perhaps an RNAP subunit other than α and σ^{70} may be involved in ExsA-dependent transcriptional activation, as has been suggested for other AraC regulators (6, 23, 32, 35).

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