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Mechanism of Transcriptional Activation by *Pseudomonas aeruginosa* ExsA▽†

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ExsA is a transcriptional activator of the *Pseudomonas aeruginosa* type III secretion system (T3SS). The T3SS consists of >40 genes organized within 10 transcriptional units, each of which is controlled by the transcriptional activator ExsA. ExsA-dependent promoters contain two adjacent ExsA binding sites that when occupied protect the −30 to −70 region from DNase I cleavage. The promoters also possess regions bearing strong resemblance to the consensus −10 and −35 regions of σ^{70}-dependent promoters. The spacing distance between the putative −10 and −35 regions of ExsA-dependent promoters, however, is increased by 4 to 5 bp compared to that in typical σ^{70}-dependent promoters. In the present study, we demonstrate that ExsA-dependent transcriptional activation requires a 21- or 22-bp spacer length between the −10 and −35 regions. Despite the atypical spacing in this region, in vitro transcription assays using σ^{70}-saturated RNA polymerase holoenzyme (RNAP-σ^{70}) confirm that ExsA-dependent promoters are indeed σ^{70} dependent. Potassium permanganate footprinting experiments indicate that ExsA facilitates an early step in transcriptional initiation. Although RNAP-σ^{70} binds to the promoters with low affinity in the absence of ExsA, the activator stimulates transcription by enhancing recruitment of RNAP-σ^{70} to the P_{exsC} and P_{exsD} promoters. Abortive initiation assays confirm that ExsA enhances the equilibrium binding constant for RNAP while having only a modest effect on the isomerization rate constant.

Pseudomonas aeruginosa is an opportunistic pathogen of humans, causing acute and chronic infections in immunocompromised individuals (43, 44). A primary determinant of *P. aeruginosa* virulence is a type III secretion system (T3SS) (16, 55). The T3SS promotes tissue destruction and phagocytic avoidance through the action of several toxins that are translocated into eukaryotic host cells (4, 45). Mutants lacking a functional T3SS are attenuated for virulence in both tissue culture and animal infection models (3, 30).

The primary regulator of T3SS gene expression is ExsA (17, 53, 54). ExsA controls T3SS gene expression by directly binding to and activating transcription from all T3SS promoters (6, 31). ExsA is a member of the large family of AraC/XylS transcriptional regulators (17). The domain structure of these proteins generally consists of a conserved 100-amino-acid helix-turn-helix DNA binding domain located at the carboxy terminus and an amino-terminal dimerization and/or ligand binding domain (15). One way in which AraC/XylS family members can be distinguished from one another is by the type of bound ligand; ligands include sugars, small metabolites, urea, aromatic compounds, and proteins (20, 42). Family members responsive to protein ligands currently constitute a small group of AraC/XylS activators that regulate T3SS gene expression (42). Representatives of this subfamily are found in *P. aeruginosa* (ExsA), *Salmonella enterica* (InvF), and *Shigella flexneri* (MxiE) (8, 11, 12). Transcriptional activation by *P. aeruginosa* ExsA is antagonized by ExsD through a direct binding interaction (38). ExsD functions as an antiactivator by inhibiting the DNA binding activity of ExsA (48). Similarly, transcriptional activation by MxiE is antagonized by the OspD1 antiactivator through a direct binding interaction (40). In contrast, transcriptional activation by *S. enterica* InvF and *S. flexneri* MxiE is dependent upon protein coactivators that directly bind to their respective activators (11, 41).

Transcriptional start sites for several ExsA-dependent promoters have been mapped by primer extension (53, 54). The transcriptional start sites for the P_{exsD}, P_{exoS}, and P_{orf1} promoters are favorably positioned downstream from near-consensus −10 (TATAAT) and −35 (TTGACA) recognition hexamers typical of σ^{70}-dependent promoters in both *Escherichia coli* and *P. aeruginosa* (14, 26). Atypical, however, is the apparent increase in spacing (21 to 22 bp) between the −10 and −35 elements of ExsA-dependent promoters compared to the optimal spacing of 17 bp for typical σ^{70}-dependent promoters (6). Whether these near-consensus promoter sequences of ExsA-dependent promoters truly serve as recognition hexamers for σ^{70}-saturated RNA polymerase holoenzyme (RNAP-σ^{70}) is not known.

The DNA binding properties of ExsA have been characterized through genetic and biochemical studies (6, 7, 31). Purified ExsA is monomeric in solution and specifically binds to T3SS promoters with apparent equilibrium constants in the low nanomolar range (1 to 5 nM). Two distinct ExsA-promoter probe complexes are detected by electrophoretic mobility shift assays. Whereas the higher-mobility complex represents one ExsA monomer bound to the promoter probe, the lower-mobility complex represents two bound ExsA molecules (6). An alignment of all 10 ExsA-dependent promoters identified an ExsA consensus binding site that is centered around highly conserved guanine and cytosine nucleotides at the −47 and −45 positions, respec-

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tively (6). Further determinants for ExsA binding include a conserved adenine-rich region centered at the −51 position and several highly conserved nucleotides within the −35 region. The available data suggest that there are two distinct ExsA binding sites, one overlapping the putative −35 region (site 1) and a second consisting of the adenine-rich region (site 2) (6). In support of this, DNase I footprinting assays reveal an ExsA-dependent region of protection that begins at the −35 region and extends upstream to the −70 position relative to the transcriptional start site (6, 31). This region of protection is similar to that of other AraC family transcriptional activators such as RhaR and RhaS, both of which bind to two adjacent promoter sites (51). Furthermore, nucleotide substitutions within the adenine-rich, −47G/−45C, and −35 regions result in a significant decrease in ExsA-dependent transcriptional activation (6). The same substitutions, however, have variable effects on DNA binding. Whereas substitutions in the −47G/−45C or −35-like region significantly impair ExsA binding to sites 1 and 2, substitutions in the A-rich region inhibit binding only to site 2. These data suggest that two adjacent ExsA binding sites are required for full transcriptional activation and that binding of monomeric ExsA to site 1 is required for binding of the second ExsA molecule to site 2. Occupation of site 2 is dependent upon the amino terminus of ExsA (7), which likely includes a multimerization domain, typical of most AraC family members (15). This sequential monomer binding route of promoter complex assembly is a common protein-DNA interaction scheme that permits kinetic discrimination of specific and nonspecific DNA sequences (35).

Transcriptional activators generally function by recruiting RNAP to nonstandard promoters and/or facilitating isomerization to an open complex. In this study we characterized the mechanism of transcriptional activation by ExsA. Genetic data demonstrate that the extended spacing between the −10 and −35 regions of T3SS promoters is an essential determinant for ExsA-dependent activation. Whereas RNAP-σ^{70} binds to T3SS promoters inefficiently in the absence of ExsA, our data demonstrate that ExsA facilitates transcription by recruiting RNAP-σ^{70} to the P_{exsC} and P_{exsD} promoters, where it then forms an active open complex.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are summarized in Table S1 in the supplemental material. *Escherichia coli* strains were maintained on L-agar plates containing the following antibiotics as necessary: gentamicin, 15 μg/ml; ampicillin, 50 or 100 μg/ml; tetracycline, 10 μg/ml; kanamycin, 50 μg/ml; and spectinomycin, 50 μg/ml. *P. aeruginosa* strains were maintained on Vogel Bonner minimal medium (49) with the following antibiotics as indicated: gentamicin, 100 μg/ml; carbenicillin, 300 μg/ml; and tetracycline, 50 μg/ml. To assay for T3SS gene expression *P. aeruginosa* strains were grown with vigorous aeration at 30°C in tryptic soy broth supplemented with 100 mM monosodium glutamate, 1% glycerol, and 2 mM EGTA as indicated. β-Galactosidase assays were performed as previously described, and the reported values are the averages of those from at least three independent experiments (13).

Plasmid construction and promoter mutagenesis. The primer sequences used to generate PCR products and the vectors into which each product was cloned are provided in Tables S2 and S3 in the supplemental material, respectively. Addition or deletion mutations in the P_{exoT} and P_{exsD} promoters were constructed using a two-step PCR. In the first step, megaprimers were generated by PCR using a forward primer incorporating the mutation and a common reverse primer. The megaprimers were gel purified (IBI Scientific, Peosta, IA) and used in a subsequent PCR with a common forward primer. The final PCR products were cloned as HindIII/EcoRI restriction fragments into mini-CTX-*lacZ* and integrated onto the PA103 chromosome as previously described (27). The P_{tacI} constructs were generated by cloning annealed complementary oligonucleotides with KpnI/HindIII ends into mini-CTX-*lacZ*. Point mutations in the P_{tacI} promoter were introduced by QuikChange site-directed mutagenesis (Stratagene). To limit β-galactosidase toxicity, *E. coli* subcloning strains were transformed with the $LacI^q$-overexpressing plasmid pMS421 (21).

Purification of $ExsA_{His}$ and RNAP. $ExsA_{His}$ was purified by metal affinity chromatography and shown to possess DNA binding activity by electrophoretic mobility shift assay as previously described (6). RNAP was purified from *P. aeruginosa* strain AK1012 (lacking expression of lipopolysaccharide O antigen) (33) as previously described (9) with the following modifications. Overnight cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.1 into 5 liters of tryptic soy broth and grown with shaking at 37°C. At an OD_{600} of 1.0, cells were harvested by centrifugation (10 min, 6,000 × *g*, 4°C), washed with 500 ml of 0.85% NaCl, collected by centrifugation, and resuspended in 60 ml purification buffer (20 mM Tris-HCl [pH 8.0], 0.05 mM EDTA, 1.7 mM phenylmethylsulfonyl fluoride, 0.3 mM dithiothreitol [DTT], and 5% glycerol) containing 0.1 M NaCl. Cells were lysed via passage through a French pressure cell, and unbroken cells were removed by centrifugation (30 min, 35,000 × *g*, 4°C). Polyethyleneimine (0.5% [wt/vol] final concentration; Sigma) was added to the soluble fraction and incubated at 4°C for 30 min with constant stirring. The precipitate was collected by centrifugation (30 min, 35,000 × *g*, 4°C) and washed with purification buffer containing 0.25 M NaCl using a Dounce homogenizer. Following centrifugation, RNAP was extracted with purification buffer containing 0.8 M NaCl. Insoluble material was removed by centrifugation, and solid ammonium sulfate (30% final concentration) was added to the soluble fraction and incubated at 4°C for 2 h with constant stirring. Insoluble material was removed by centrifugation, and ammonium sulfate (60% final concentration) was added to the soluble fraction and allowed to precipitate as described above. The precipitate was collected by centrifugation, homogenized in 11 ml of purification buffer containing 0.1 M NaCl, and dialyzed for 18 h at 4°C against 2 liters of purification buffer containing 0.1 M NaCl. Prior to heparin column chromatography, the material was subjected to ultracentrifugation (100,000 × *g* for 30 min at 4°C) to ensure solubility. Soluble material was loaded onto a 5-ml heparin column and developed with a linear elution gradient (0.1 to 1 M NaCl), and peak fractions (based on polymerase activity) were pooled. The heparin column procedure was repeated a second time, followed by a final purification using Superdex-300 gel filtration chromatography. Purified polymerase was stored at −20°C in purification buffer containing 0.1 M NaCl and 50% glycerol. The specific activity of RNAP was determined as described previously (2). One unit of RNAP activity is defined as the amount of enzyme required to incorporate 1 pmol of UMP into acid-precipitable material in 20 min. Protein samples were denatured in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer and electrophoresed on 15% acrylamide denaturing gels. Gels were analyzed by Coomassie blue and silver staining methods.

Transcription templates. Supercoiled plasmid templates containing the P_{exsD} promoter fused to the *rpoC* terminator were generated and purified as described previously (7). Supercoiled minicircle templates were created by cloning PCR-generated P_{exsC} and P_{exsD} promoters (nucleotides [nt] −238 to +192 relative to the transcriptional start site) as SacI/KpnI fragments into pSA508 (10). The resulting plasmids add 29 additional bases upstream of the *rpoC* transcriptional terminator to generate 221-base transcripts from each promoter. Minicircle purification was as described previously (10) with the following modifications. T3SS promoter-containing pSA508 derivatives were introduced by transformation into *E. coli* strain SA1751 [λint^+ *xis439* *c*I857 $(cro\text{-}chlA)_{\Delta H1}$]. Transformants were grown in 800 ml of LB containing ampicillin (50 μg/ml) to an OD_{600} of 0.8 at 30°C, heat shocked at 42°C for 15 min, returned to 30°C in an ice-water bath, and grown at 30°C for an additional 30 min. Cells were harvested by centrifugation, and plasmid DNA was isolated with the Fast-Ion plasmid maxi kit (IBI Scientific, Peosta, IA). Transcription templates were subjected to agarose gel electrophoresis and visualized by methylene blue staining. Supercoiled DNA was excised and gel purified, and analytical samples were examined by agarose gel electrophoresis to confirm that the preparations were largely free of nicked template.

In vitro transcription assays. Single-round transcription assays (20-μl final volume) were performed by incubating $ExsA_{His}$ (35 nM) with supercoiled transcription templates (2 nM) at 25°C in 1× transcription buffer (40 nM Tris-HCl [pH 7.5], 50 mM KCl, 10 mM $MgCl_2$, 0.01% Tween 20, and 1 mM DTT) containing the initiating nucleotides ATP and GTP (0.75 mM) for the P_{exsD} and P_{exsC} promoters, respectively. After 10 min, 25 nM *E. coli* RNAP holoenzyme (Epicentre, Madison, WI) or *P. aeruginosa* RNAP holoenzyme was added, and

open complexes were allowed to form for 5 min at 30°C. Elongation was allowed to proceed by the addition of the remaining unlabeled nucleotides (0.75 mM each, including 5 μCi [α-^{32}P]CTP) in 1× transcription buffer containing heparin (50-μg/ml final concentration). Reactions were stopped after 10 min at 30°C by the addition of stop buffer (20 μl) (98% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol). Samples were heated to 95°C for 5 min and electrophoresed immediately on 5% denaturing urea-polyacrylamide gels.

Transcriptional start site identification. RNA was isolated from mid-log-phase (OD_{600} of 0.8) bacterial cells using RNAprotect reagent and an RNA mini isolation kit (Qiagen, Valencia, CA). Purified mRNA (200 ng) and gene-specific, antisense primers (positioned ~500 bases downstream of the translational start sites) were used in reverse transcription reactions to generate cDNA for the P_{exsD} and P_{exsC} promoters with the SuperScript III first-strand synthesis system (Invitrogen). Reverse transcriptase (RT) reactions were allowed to proceed at 55°C for 30 min and were terminated by incubation at 70°C for 15 min. cDNA was purified using the MinElute PCR purification kit (Qiagen). The 5′ ends of the resulting cDNAs were identified using the PCR-based method of 5′ rapid amplification of cDNA ends (RACE) (46); 10 independent clones for each promoter were sequenced to confirm the start site.

Abortive initiation assays. The steady-state properties of the abortive initiation assay have been described previously (39). Abortive initiation assays were performed with P_{exsD} or P_{exsC} supercoiled minicircle templates (2 nM) in the presence and absence of $ExsA_{His}$ (35 nM) in 1× transcription buffer. The substrates for the abortive initiation reactions were as follows: for the P_{exsD} promoter, 1 mM ATP, 1 mM UTP, and 0.33 μCi [α-^{32}P]UTP to form pppApApApUpU and pppApApUpU; for the P_{exsC} promoter, 1 mM GTP, 1 mM CTP, 1 mM UTP, and 0.33 μCi [α-^{32}P]UTP to form pppGpCpUpUpU and pppCpUpUpU. Reaction mixtures including $ExsA_{His}$ were incubated in 1× transcription buffer with template DNA for 10 min at 25°C prior to nucleotide/RNAP addition. To measure the lag time to open complex formation (τ), two separate reactions were performed for each of the seven RNAP concentrations tested (25, 28.6, 33.3, 40, 50, 66.7, and 100 nM). The first set of reaction mixtures contained template, $ExsA_{His}$ (35 nM as indicated), and substrate nucleotides in 1× transcription buffer, and the reaction was initiated by the addition of RNAP. The second set of reaction mixtures contained template, $ExsA_{His}$ (35 nM), and RNAP in 1× transcription buffer and were preincubated for 60 min at 30°C. Transcription was initiated by the addition of nucleotides. Both sets of reactions were allowed to proceed at 30°C. Samples were taken at various time points (1 to 120 min), the reactions were terminated with stop buffer, and the products were electrophoresed on denaturing 25% polyacrylamide gels. Gels were subjected to phosphorimaging and densitometry. The rate of abortive synthesis was calculated for reactions initiated with nucleotides by linear regression analysis (least squares). Curves plotted for reactions initiated by RNAP addition were analyzed by drawing a line through the curve but parallel to the reaction initiated by nucleotide addition. τ_{obs} was also obtained for these curves by linear regression analysis by solely using values 3 times greater than the initial estimate for τ_{obs}, as described previously (39), yielding results comparable to those with the first method. GraphPad Prism (GraphPad Software, Inc) was used to plot abortive initiation data and evaluate τ_{obs}.

Potassium permanganate footprinting. Supercoiled minicircles carrying P_{exsC} or P_{exsD} were used as templates for the potassium permanganate footprinting reactions (57). Reaction mixtures containing $ExsA_{His}$ were incubated for 10 min at 25°C to allow DNA binding in 1× potassium permanganate reaction buffer (40 mM Tris-HCl pH [7.5], 25 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1% Tween 20, and 100 ng/ml bovine serum albumin). RNAP was added to the indicated concentrations, and open complex formation was allowed to proceed for 3 min at 25°C. Potassium permanganate (Sigma-Aldrich, St. Louis, MO) was immediately added (10 mM final concentration) and allowed to modify DNA for 1 min at 25°C. Reactions were stopped with termination buffer (0.5 M potassium acetate [pH 7.0], 1.5 M 2-mercaptoethanol, 5 mM EDTA), and products were purified with a PCR column purification kit (IBI Scientific) and eluted into 30 μl elution buffer (10 mM Tris-HCl [pH 8.5]). Modification by potassium permanganate was detected by primer extension. Primers (50 pmol) were end labeled with 50 μCi [γ-^{32}P]ATP (Perkin-Elmer) and 10 U polynucleotide kinase (New England Biolabs, Ipswich, MA) as instructed by the manufacturer. Primer extension reactions (10 μl) were performed on the potassium permanganate-modified plasmids with 1 mM deoxynucleoside triphosphates, 0.5 μl end-labeled primer, and 1.25 units sequencing-grade *Taq* DNA polymerase (Promega, Madison, WI) in 1× sequencing buffer (50 mM Tris-HCl [pH 9.0], 2 mM $MgCl_2$) under the following conditions: 1 cycle of 2 min at 94°C and 30 cycles of 0.5 min at 94°C, 0.5 min at 55°C, and 1 min at 72°C. Stop buffer (10 μl) was immediately added to each reaction mixture to terminate DNA synthesis. Dideoxy sequencing reactions for A and T were generated using the same thermocycling program and the following reaction components: 5 fmol P_{exsC}/P_{exsD} minicircle, 0.5 pmol labeled primer, 1.25 units sequencing-grade *Taq* DNA polymerase, 500 μM termination nucleotide (ddATP or ddTTP), and 20 μM elongation nucleotides (dCTP, 7-deaza-dGTP, dATP, and dTTP). Sequencing reactions were terminated with an equal volume of stop buffer and run alongside primer extension reactions on denaturing 6% polyacrylamide gels. Gels were dried and visualized by phosphorimaging, and analysis was performed with MultiGauge v3.0 software (Fujifilm).

RESULTS

The spacing between the −10 and −35 regions is critical for ExsA-dependent activation. Each of the ExsA-dependent promoters used in this study (P_{exoT}, P_{exsD}, and P_{exsC}) contains hexamers that match the consensus −10 and −35 regions of σ^{70}-dependent promoters at 4/6 or 5/6 of the nucleotide positions (Fig. 1A; see Fig. 2A and 4). Nevertheless, it is not known whether the −10 and/or −35 region is truly recognized by RNAP-σ^{70}. One reason for this uncertainty is that the −10 and −35 regions of ExsA-dependent promoters are separated by 21 or 22 bp, whereas optimal spacing for σ^{70}-dependent promoters is 17 bp (2, 24, 26, 50). One interpretation of these data is that the increased spacing between the −10 and −35 regions prevents transcription in the absence of ExsA and that ExsA functions by overcoming the increased spacing. We predicted that if this was true, reducing the spacing of ExsA-dependent promoters to 17 bp would reconstitute a functional σ^{70}-dependent promoter and eliminate the requirement for ExsA. To test this hypothesis, a series of *lacZ* transcriptional reporters was constructed in which the spacing between the −10 and −35 regions of P_{exoT} was incrementally increased (22 to 24 bp) or decreased (16 to 20 bp) from the native spacing of 21 bp by inserting or deleting nucleotides at the −23 position relative to the start of transcription (Fig. 1A). The resulting reporters were integrated at a neutral chromosomal attachment site in wild-type PA103 and an *exsA* mutant (18). Strains were cultured under permissive (Ca^{2+}-limiting) conditions for T3SS gene expression and assayed for β-galactosidase activity. Whereas expression of the native $P_{exoT\text{-}lacZ}$ reporter is *exsA* dependent, reporter derivatives in which the spacing was increased to 23 to 24 bp or decreased to 16 to 20 bp were largely inactive in either the absence or presence of *exsA* (Fig. 1B). The reporter having a spacing of 22 bp ($P_{exoT\text{-}22\text{-}lacZ}$), however, retained *exsA* dependence, although activity was reduced ~3-fold compared to the native $P_{exoT\text{-}lacZ}$ promoter. This finding is consistent with the fact that all ExsA-dependent promoters have a spacing of 21 or 22 bp (6) and suggests that the spacing distance between the putative −10 and −35 elements is critical for ExsA-dependent activation of T3SS promoters.

The lack of activity for the $P_{exoT\text{-}17\text{-}lacZ}$ reporter was somewhat surprising, as we expected that reducing the spacing to the σ^{70}-dependent consensus of 17 bp might result in constitutive, ExsA-independent expression. We considered the possibility that the nucleotide sequence surrounding the −23 position is important for promoter activity. To test this possibility, we changed the nucleotides flanking the −23 position of the native $P_{exoT\text{-}lacZ}$ reporter to its complementary sequence while at the same time maintaining the spacing and G+C ratio of the native promoter (Fig. 1A). The resulting reporter ($P_{exoT\text{-}C\text{-}lacZ}$)

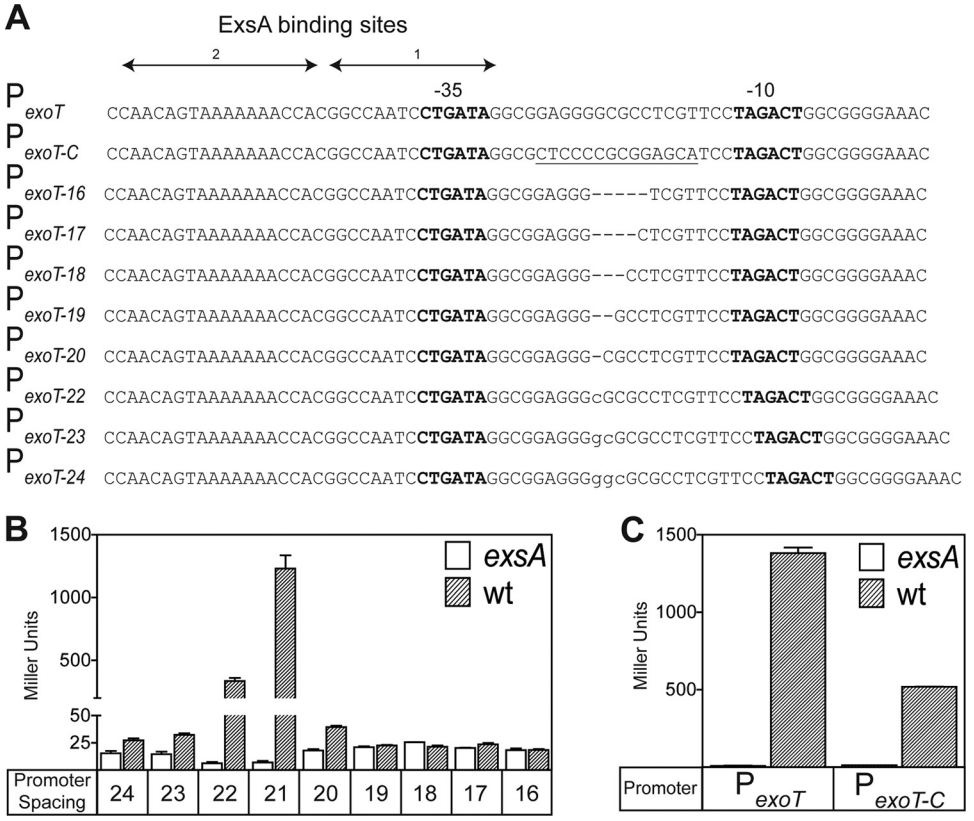

FIG. 1. The spacing distance between the −10 and −35 regions is critical for ExsA-dependent activation of the P_{exoT} promoter. (A) Diagram of the native and mutant $P_{exoT\text{-}lacZ}$ reporters. The $P_{exoT16\text{-}lacZ}$-$P_{exoT24\text{-}lacZ}$ reporter constructs carry incremental 1-bp insertions (lowercase) or deletions (dash) between the putative −10 and −35 elements of the native P_{exoT} promoter as indicated. A 14-bp segment of the native P_{exoT} reporter was replaced with the complementary sequence (underlined), resulting in the $P_{exoT\text{-}C\text{-}lacZ}$ reporter. The −10 and −35 regions are indicated in bold. ExsA binding sites 1 and 2 are indicated by solid arrows. (B and C) Wild-type (wt) PA103 and the *exsA*::Ω mutant carrying the indicated reporters were cultured under inducing conditions for T3SS gene expression and assayed for β-galactosidase activity. The reported values (Miller units) are the averages from three independent experiments, and error bars represent the standard errors of the means.

retained significant ExsA-dependent activity compared to the native P_{exoT} reporter (Fig. 1C). This finding suggests that nucleotides adjacent to the −23 position are not essential for ExsA-dependent transcription and is consistent with the idea that the spacing distance between the putative −10 and −35 regions is a primary determinant of ExsA-dependent activation.

Another trivial explanation for the lack of constitutive $P_{exoT\text{-}17\text{-}lacZ}$ reporter activity is that the putative −10 and −35 hexamers of the P_{exoT} promoter are inadequate for σ^{70}-dependent activation. To test this possibility we used the P_{exsD} promoter, since both the −10 and −35 regions match the σ^{70}-dependent consensus sites at five of six positions. Deletions (4 bp) were separately introduced into the $P_{exsD\text{-}lacZ}$ reporter at three different locations, in each case reducing the spacing between the −10 and −35 regions from 21 bp to 17 bp (Fig. 2A). Compared to the native $P_{exsD\text{-}lacZ}$ construct, however, none of the modified reporters ($P_{exsD\text{-}17A\text{-}lacZ}$, $P_{exsD\text{-}17B\text{-}lacZ}$, and $P_{exsD\text{-}17C\text{-}lacZ}$) had significant activity in either the absence or presence of *exsA* (Fig. 2B). To examine this further, the 21-bp spacer region from the $P_{exsD\text{-}lacZ}$ reporter was replaced with the 16-bp spacer region from the constitutive P_{tacI} promoter (Fig. 2A). The P_{tacI} promoter is active in *P. aeruginosa* (Fig. 2D) and has previously been studied both in vivo and in vitro (19). The P_{exsD}-P_{tacI} hybrid reporter ($P_{exsD\text{-}tacI16}$), however, had very little activity in either the absence or presence of *exsA* (Fig. 2C). Finally, hybrid reporters were constructed by replacing the −10 and/or −35 region of the $P_{tacI\text{-}lacZ}$ reporter with the corresponding elements from the P_{exsD} promoter (Fig. 2A). Whereas the native $P_{tacI\text{-}lacZ}$ reporter had significant activity in the absence of *exsA*, the hybrid $P_{tacI\text{-}lacZ}$ reporters containing the −10 and/or −35 region from the P_{exsD} promoter demonstrated a 7- to 14-fold reduction in activity (Fig. 2D). These combined data indicate that even when properly spaced, the putative −10 and −35 hexamers of P_{exsD} are suboptimal for σ^{70}-dependent transcription and suggest that ExsA may function by recruiting RNAP to the promoter.

ExsA activates transcription from T3SS promoters in vitro. To examine the mechanism of ExsA-dependent transcriptional activation, we developed a single-round in vitro transcription assay using $ExsA_{His}$ purified from *E. coli* (Fig. 3A, lane 1) and RNAP-σ^{70} holoenzyme purified from *P. aeruginosa* (lane 2) or RNAP-σ^{70} holoenzyme from *E. coli* (obtained commercially). The specific activity of RNAP-σ^{70} isolated from *P. aeruginosa* was three- to fourfold lower than that of *E. coli* RNAP-σ^{70} (data not shown). The initial transcription template consisted of supercoiled plasmid DNA carrying the P_{exsD} promoter fused to a strong transcriptional terminator ($rpoC_{ter}$). The plasmid

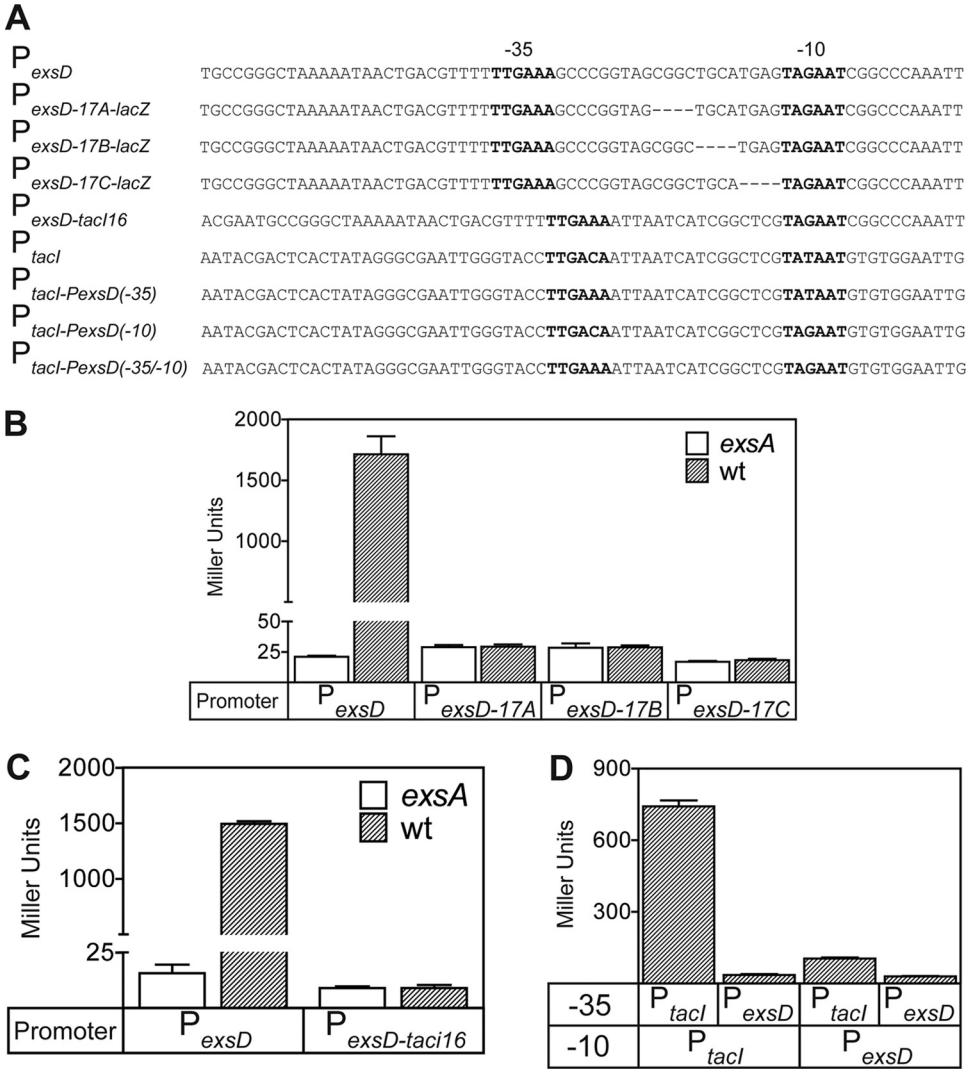

FIG. 2. Spacing requirements of the P_{exsD} promoter and the role of the −10 and −35 regions. (A) Diagram of the native P_{exsD} promoter and mutant derivatives, P_{tacI}, and hybrid promoters derived from both P_{exsD} and P_{tacI}. The $P_{exsD\text{-}17A\text{-}lacZ}$, $P_{exsD\text{-}17B\text{-}lacZ}$, and $P_{exsD\text{-}17C\text{-}lacZ}$ reporters contain the indicated 4-bp deletions (dashes) between the −10 and −35 regions. To construct $P_{exsD\text{-}tacI16\text{-}lacZ}$, the native 21-bp spacer region from P_{exsD} was replaced with the 16-bp spacer from P_{tacI}. The $P_{tacI\text{-}PexsD(-35)\text{-}lacZ}$, $P_{tacI\text{-}PexsD(-10)\text{-}lacZ}$, and $P_{tacI\text{-}PexsD(-35/-10)\text{-}lacZ}$ reporters were constructed by replacing the −10 and/or −35 regions of P_{tacI} with the corresponding regions of P_{exsD}. The putative −10 and −35 regions are indicated in bold. (B to D) Wild-type PA103 and the *exsA*::Ω mutant carrying the indicated reporters were cultured under T3SS-inducing conditions and assayed for β-galactosidase activity. The reported values represent the averages from three independent experiments, and error bars indicate the standard errors of the means.

template was preincubated with $ExsA_{His}$, RNAP-σ^{70}, and the initiating nucleotide for transcription (ATP, as determined below) for 15 min. Ribonucleotides (including [α-^{32}P]CTP) were then added in the presence of heparin (to prevent RNAP-σ^{70} from reinitiating), and transcripts were allowed to elongate for 10 min. RNAP-σ^{70} from either *E. coli* or *P. aeruginosa* generated the expected terminated transcript of 261 nt in an ExsA-dependent manner (Fig. 3B). Since the *E. coli* RNAP holoenzyme used for these studies is σ^{70} saturated and the *P. aeruginosa* RNAP holoenzyme isolated from log-phase cells is presumed to be largely σ^{70} saturated (Fig. 3A, lane 2), we conclude that ExsA-dependent promoters are σ^{70} dependent.

ExsA-independent transcription was not observed at high RNAP concentrations (200 nM) and at incubation times of as long as 2 h (data not shown). Because the transcription templates used in these experiments are self-replicating, supercoiled plasmids (~5 kb in length), we hypothesized that in the absence of ExsA, strong constitutive plasmid promoters might outcompete the P_{exsD} or P_{exsC} promoter for RNAP occupancy. A similar result was previously observed for the *gal* promoters in *E. coli* and was addressed by constructing small supercoiled plasmid templates called minicircles (10). To generate P_{exsC} and P_{exsD} minicircles, the promoters were cloned upstream of the *rpoC* transcriptional terminator in the parental vector pSA508 (3.4 kb). The minicircles excise in vivo from the parental plasmid as supercoiled plasmids through a temperature-dependent recombination event. The resulting P_{exsC} and P_{exsD} minicircles (~0.83 kb) consist solely of the cloned promoter

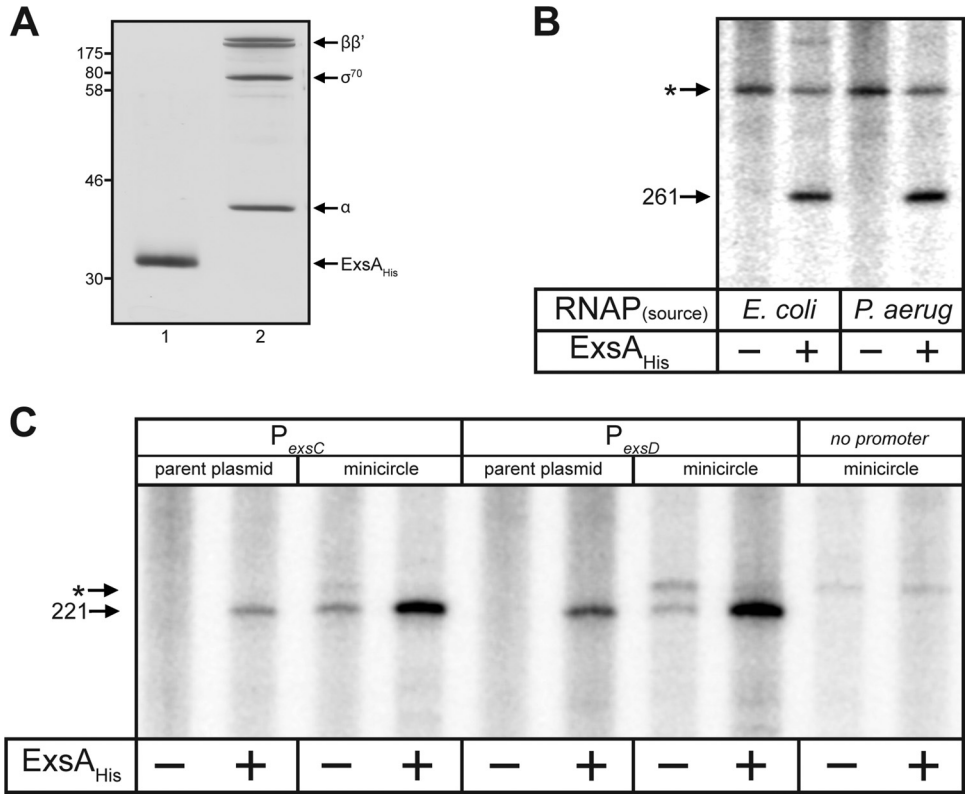

FIG. 3. Purified $ExsA_{His}$ activates transcription in vitro. (A) Silver-stained gel of $ExsA_{His}$ purified from *E. coli* (lane 1) and σ^{70}-RNAP purified from *P. aeruginosa* (lane 2). Molecular mass standards (in kDa) are indicated on the left. (B) Single-round in vitro transcription assays. $ExsA_{His}$ (35 nM) was incubated with 2 nM supercoiled P_{exsD} promoter template (pOM90-P_{exsD}) at 25°C in the presence of rATP. After 10 min, RNAP from *E. coli* or *P. aeruginosa* (25 nM of each, normalized to specific activity of *E. coli* RNAP) was added, and the reaction mixture was incubated for 5 min at 30°C. Heparin and the remaining ribonucleotides (including 5 μCi [α^{32}P]CTP) were immediately added, and the reaction mixture was incubated for 10 min at 30°C. Reactions were terminated, and the resulting products were electrophoresed on a 5% polyacrylamide-urea gel and subjected to phosphorimaging. The ExsA-dependent terminated transcript (261 nt) from the P_{exsD} promoter is indicated. A transcript originating from an undetermined plasmid promoter is indicated with an asterisk. (C) Single-round in vitro transcription assays were performed as described above using the parental pSA508-P_{exsC}/pSA508-P_{exsD} plasmid templates or minicircles derived from the parental plasmids. The terminated transcript (221 nt) from these templates is indicated. A transcript originating from a weak plasmid promoter is indicated with an asterisk.

fragments (0.43 kb), the *rpoC* transcriptional terminator, and residual plasmid sequences. Similar to the findings presented in Fig. 3B, the larger parental P_{exsC} or P_{exsD} plasmids were permissive for ExsA-dependent transcription, while ExsA-independent transcripts were undetectable (Fig. 3C). Minicircle templates derived from the parental plasmids, however, supported both ExsA-dependent and -independent transcription, although the amount of terminated transcript in the absence of ExsA was significantly reduced for both the P_{exsC} and P_{exsD} minicircle templates. Detection of ExsA-independent transcription from the minicircle templates is consistent with the possibility that strong promoters on the parental plasmid outcompete the P_{exsC} and P_{exsD} promoters for RNAP-σ^{70} occupancy and suggests that RNAP-σ^{70} binds to T3SS promoters poorly in the absence of ExsA.

Transcription from the P_{exsC} and P_{exsD} promoters initiates 8 to 9 nt downstream of near-consensus −10 TATA boxes. Transcriptional start sites for several ExsA-dependent promoters were previously mapped by primer extension (53, 54). As expected of σ^{70}-dependent promoters, the P_{exsD}, P_{exoS}, and P_{orf1} promoters initiate transcription 7 to 9 bp downstream of the −10 TATA box (Fig. 4). The P_{exsC} start site, however, mapped to two adjacent nucleotides located ~50 bases upstream of the putative −10 region. To resolve this apparent discrepancy, 5′ RACE and abortive initiation assays were used to reexamine the transcriptional start sites for the P_{exsC} and P_{exsD} promoters.

For the RACE assays, mRNA was isolated from wild-type PA103 and an *exsA* mutant grown under inducing conditions for T3SS gene expression (i.e., with EGTA). The mRNA was reverse transcribed into cDNA using gene-specific primers (*exsC* or *exsD*) and cloned into a plasmid vector for sequencing. At least 10 clones were sequenced for each promoter/RNA sample. Consistent with the previous primer extension data, the 5′ end of the *exsD* mRNA mapped to nucleotides located 7 to 9 bases downstream of the −10 region in both the wild type and the *exsA* mutant (Fig. 4). The 5′ end of the *exsC* mRNA, however, mapped to two distinct regions located 50 bp upstream and 8 to 9 bp downstream of the −10 region. Whereas the position of the former site is consistent with the previous primer extension data, the location of the latter site is nearly identical to the P_{exsD}, P_{exoS}, and P_{orf1} promoters with respect to the putative −10/−35 regions (Fig. 4). Subsequent studies (described below) indicate that the P_{exsC} promoter initiates transcription at the second site.

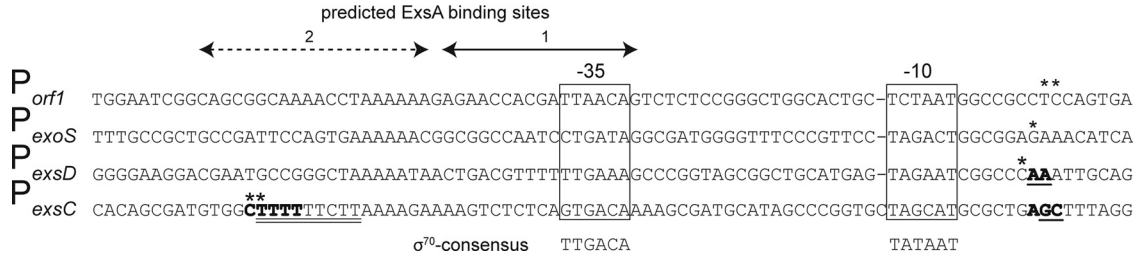

FIG. 4. Diagram of the P_{orf1}, P_{exoS}, P_{exsD}, and P_{exsC} promoters. The putative −10 and −35 regions are boxed. Transcription start sites previously mapped by primer extension (53, 54) are indicated with an asterisk. The start sites mapped in this study by RACE analysis and by abortive initiation assays are in bold or underlined, respectively. A putative transcriptional terminator from a transcript located upstream of the P_{exsC} promoter is indicated by the double underline.

An inherent limitation of 5′ RACE analysis is that the exact starting nucleotide cannot always be determined. To more precisely map the start sites, we analyzed abortive transcription products. Abortive RNA synthesis is thought to occur at all promoters and results from RNAP that initiates transcription but fails to clear the promoter and randomly aborts transcription within 15 to 20 nt (32). By starving an in vitro transcription reaction for one or more nucleotides and incorporating specific radiolabeled nucleotides, the length of the abortive transcripts can reveal the exact transcriptional start site. The abortive initiation assay mixtures for the P_{exsC} promoter contained GTP, CTP, and radiolabeled UTP. Under these conditions, the putative start site located 50 bp upstream of the −10 region would generate a 10- to 12-nt transcript before terminating at an adenine, while the start site located 8 to 9 bp downstream of the −10 region would generate a 4- to 5-nt product. In the presence of $ExsA_{His}$, two abortive transcripts were generated (Fig. 5A, lane 4). The shorter product was nearly identical in size to the abortive transcript (4 nt, pppAAUU) from the well-characterized P_{l8UV5} promoter when provided only ATP and radiolabeled UTP (data not shown). These data are consistent with the aborted P_{exsC} transcripts representing pppCUUU (4 nt) and pppGCUUU (5 nt) and indicate that P_{exsC} transcription initiates from the G and C nucleotides located 8 and 9 nt downstream of the −10 region (Fig. 4). The fact that the same products were not detected when the transcription reaction mixtures lacked UTP further supports this conclusion (Fig. 5A, lanes 1 and 2). The pppCUUU and pppGCUUU abortive transcripts were also were generated in the absence of $ExsA_{His}$, albeit to a much lesser extent and only after an extended incubation period (Fig. 5A, lane 6). The failure to detect the 10- to 12-nt product from the site located 50 bp upstream of the −10 region suggests that this start site mapped by primer extension and RACE may be an artifact.

Abortive initiation assays for the P_{exsD} promoter that were limited to ATP and radiolabeled UTP generated two ExsA-dependent products (pppAAUU and pppAAAUU) (Fig. 5B, lanes 1 and 2), the shorter of which is identical in size to the aborted transcript generated by the P_{l8UV5} promoter (data not shown). This finding is consistent with transcription initiating at the adenine nucleotides located 7 to 8 bp downstream of the −10 region (Fig. 4). To confirm this finding, the assays were repeated in the presence of ATP, UTP, and radiolabeled GTP, where only the terminal nucleotide would be labeled. As expected, the resulting abortive products (pppAAUUG and ppp AAAUUG) were 1 nt longer (Fig. 5B, lane 2 versus lane 4) and ExsA dependent (lane 3 versus lane 4).

The ability to measure ExsA-independent abortive products can provide information regarding the mechanism of transcriptional activation by ExsA (discussed below). Unfortunately, extended incubation of RNAP-σ^{70} with the P_{exsD} promoter in the absence of ExsA resulted in the appearance of background bands, which raised questions as to whether the abortive transcripts were truly arising from the P_{exsD} promoter. To determine whether the aborted products were indeed derived from P_{exsD}, a mutant promoter ($P_{exsD+GG}$) in which two additional guanine nucleotides were added between nt +6 and +7 was generated. Compared to the wild-type P_{exsD} promoter, the products generated from the $P_{exsD+GG}$ promoter (pppAA UUGGG and pppAAAUUGGG) were 2 bases greater in length (Fig. 5C, lane 2 versus lane 3) and, importantly, were clearly detected in the absence of $ExsA_{His}$ if the incubation time was extended to 240 min.

ExsA promotes an early step in transcriptional initiation. The most common rate-limiting steps during transcriptional initiation are closed and open complex formation (32). Closed complexes result from the binding of RNAP to the promoter; transition of the closed complex to an open complex involves unwinding of the −10 region of the promoter to single-stranded DNA (ssDNA). Open complex formation provides RNAP access to the template strand and is required for subsequent elongation of the transcript (32). The open complex can be detected with the DNA modification reagent potassium permanganate, which preferentially oxidizes pyrimidine bases in ssDNA (1). To determine whether ExsA is required for the initial steps in initiation of P_{exsC} and P_{exsD} transcription, the minicircles carrying these promoters were incubated with $ExsA_{His}$ and/or RNAP-σ^{70} and then subjected to potassium permanganate modification. Modified minicircles were then used as templates in primer extension reactions with DNA polymerase and radiolabeled primers. DNA polymerase terminates transcription at bases oxidized by potassium permanganate, and the resulting enrichment of the terminated fragments indicates the regions of ssDNA. Incubation of the P_{exsC} and P_{exsD} minicircles with both $ExsA_{His}$ and a low concentration of RNAP-σ^{70} (1.5 nM) resulted in strong permanganate modification of the −10 regions within each promoter (Fig. 6A and B). Weaker modification of the same regions could also be seen with RNAP-σ^{70} alone. These findings demonstrate that

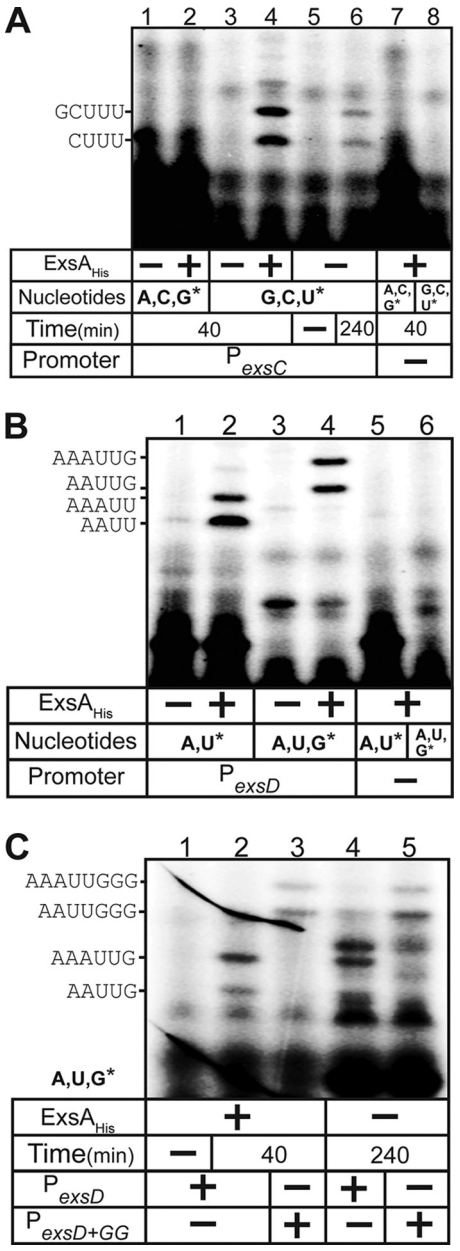

FIG. 5. Abortive initiation assays for the P_{exsC} and P_{exsD} promoters. Reactions using the P_{exsC} (A) or P_{exsD} (B and C) minicircle templates were allowed to proceed for 40 min or 240 min, as indicated, with RNAP and substrate nucleotide sets (asterisks indicate labeled nucleotides) in the absence or presence of $ExsA_{His}$. Reactions were terminated, and the products were electrophoresed through a 25% denaturing polyacrylamide gel and visualized by phosphorimaging. Control reactions using a supercoiled minicircle template lacking T3SS promoters were performed with each substrate nucleotide set in the presence of $ExsA_{His}$. (C) Abortive initiation assays with the P_{exsD} and modified $P_{exsD+GG}$ promoters in the absence or presence of $ExsA_{His}$. Abortive initiation reactions were allowed to proceed for 240 min in the presence of RNAP, unlabeled ATP/UTP, and labeled GTP. Control reactions in the presence of $ExsA_{His}$ were incubated for 40 min.

RNAP-σ^{70} can bind to the P_{exsD} and P_{exsC} promoters independent of ExsA and that ExsA facilitates transcriptional initiation by enhancing recruitment of RNAP-σ^{70} to the promoter and/or promoting isomerization to an open complex.

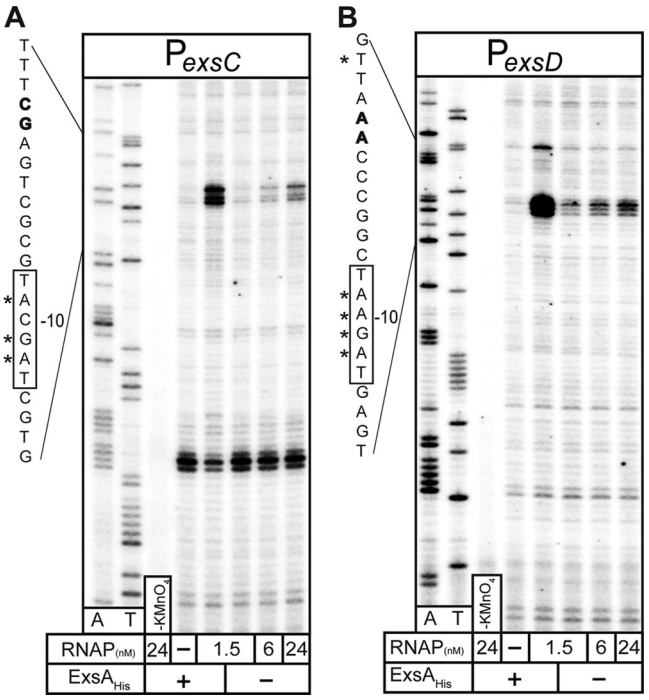

FIG. 6. ExsA stimulates formation of open complexes as measured by potassium permanganate footprinting. (A and B) Supercoiled minicircles carrying the P_{exsC} (A) or P_{exsD} (B) promoter (1.6 nM) were incubated in the absence (−) or presence (+) of $ExsA_{His}$ (30 nM) for 10 min. RNAP was added to the indicated concentrations and incubated for 3 min. Reaction mixtures were then treated with potassium permanganate (except for the control [KMnO4]), and the modified minicircles were used as templates in primer extension reactions with a radiolabeled coding-strand primer. Primer extension products were subjected to denaturing electrophoresis and phosphorimaging. Dideoxy sequencing reactions for A and T are indicated. The diagrams to the left show the transcriptional start sites (bold), the −10 regions (boxed), and the nucleotides modified by potassium permanganate (indicated with asterisks).

ExsA facilitates a rate-limiting step prior to open complex formation at the P_{exsC} and P_{exsD} promoters. Potassium permanganate footprints indicate that ExsA functions at the level of transcription initiation, but this method cannot discern whether ExsA recruits RNAP and/or promotes isomerization to the open complex. The abortive initiation assay, however, can be used to estimate the isomerization rate constant and overall reaction rate for a given promoter by measuring the lag time to steady-state synthesis of abortive transcripts. To analyze the kinetics of transcription initiation at the P_{exsC} and P_{exsD} promoters, abortive transcripts were generated at various concentrations of RNAP-σ^{70} in the absence or presence of saturating concentrations of $ExsA_{His}$, and the lag time (τ_{obs}) to open complex formation was recorded for each RNAP-σ^{70} concentration on a τ plot. The resulting τ plots for both the P_{exsC} and P_{exsD} promoters show an inverse linear relationship between the lag time to open complex formation and the RNAP-σ^{70} concentration (Fig. 7A and B). The overall reaction rates for the P_{exsC} and P_{exsD} promoters increased 13- and 11-fold in the presence of $ExsA_{His}$, respectively (Table 1). In both cases, the stimulatory effect of $ExsA_{His}$ resulted primarily from an increase in the equilibrium binding constant for

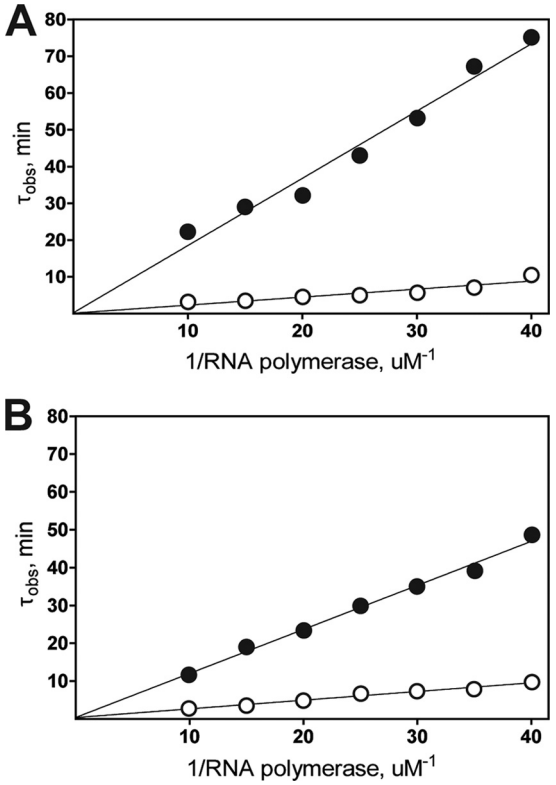

FIG. 7. τ plots for the P_{exsC} (A) and P_{exsD} (B) promoters in the presence (open circles) and absence (closed circles) of $ExsA_{His}$. Values for τ_{obs} were calculated from abortive initiation assays measuring synthesis of the products pppGCUUU (P_{exsC}) and pppAAAUU (P_{exsD}). Calculated values for τ_{obs} were plotted on the ordinate as a function of reciprocal RNAP concentration.

RNAP-σ^{70} (five- to eightfold), although there was also a modest increase in the isomerization rate constant (~2-fold). These data indicate that the primary mechanism by which ExsA stimulates transcription is through recruitment of RNAP-σ^{70} to the P_{exsC} and P_{exsD} promoters prior to open complex formation.

DISCUSSION

In the present study we found that purified $ExsA_{His}$ and RNAP-σ^{70} isolated from either *P. aeruginosa* or *E. coli* is sufficient to activate transcription from T3SS promoters in vitro. This is consistent with previous studies demonstrating that T3SS genes are expressed maximally during exponential growth phase (22, 29, 47) and with the fact that the −10 and −35 regions of T3SS promoters are similar to the σ^{70} consensus sequences from *E. coli* (21, 26) and *P. aeruginosa* (14). In addition, potassium permanganate footprinting assays reveal RNAP-σ^{70}-dependent unwinding of the P_{exsD} and P_{exsC} −10 regions, and this was greatly enhanced in the presence of $ExsA_{His}$. Based on these data, we conclude that ExsA primarily utilizes RNAP-σ^{70} to activate T3SS gene expression. Our findings, however, do not preclude the possibility that alternative sigma factors might also be involved in ExsA-dependent gene expression.

5′ RACE was used to map the general region of the P_{exsC} and P_{exsD} transcription start sites in vivo, and abortive initiation products were used for more precise mapping in vitro. Although the P_{exsD} start site matched previously published primer extension data, two apparent start sites were observed for the P_{exsC} promoter. Whereas the first site is located upstream of the ExsA binding site, the second site is located downstream of the −10 element (Fig. 4). We believe that the latter site is the true P_{exsC} start site, given its proximity to the ExsA binding site and RNAP recognition elements and its similarity to the positions of the P_{exsD}, P_{exoS}, and P_{orf1} start sites. The apparent upstream start site may result from transcriptional read-through from the upstream P_{pcrG} promoter. In this regard it is worth noting the poly(dA) and poly(dT) tracts located just downstream of the start site mapped by primer extension and 5′ RACE (Fig. 4) and that both of those techniques rely upon RT extension from mRNA templates. RT is known to pause at sites of secondary structure and at poly(A) and poly(U) nucleotide runs (25, 34). We believe that the apparent upstream start site represents pausing of RT that results from either the poly(A)/poly(U) sequence or secondary structure associated with a transcriptional terminator. In either case this finding suggests that transcriptional read-through from the upstream P_{pcrG} promoter, which is also ExsA dependent, contributes to expression of the *exsCEBA* operon and could represent another point at which ExsA expression levels are regulated.

AraC activates transcription by enhancing the RNAP equilibrium binding and the open complex isomerization rate constants (57). These activities have been attributed to protein-protein interactions between AraC and RNAP-σ^{70} (37). Given the similarities between ExsA and AraC with respect to the location of the activator binding site, we hypothesized that ExsA might activate transcription through similar mechanisms. Abortive initiation assays, however, indicate that $ExsA_{His}$ only marginally altered the isomerization rate constant but had a significant effect on the equilibrium binding constant (five- to eightfold) at both the P_{exsC} and P_{exsD} promoters. These results indicate that ExsA functions primarily by enhancing recruitment of RNAP-σ^{70} to the promoter prior to open complex formation. The effect of $ExsA_{His}$ on transcriptional initiation in vitro (11- to 13-fold) is much lower than that observed in vivo (100- to 1,000-fold) (38). The most likely explanation for this discrepancy is that the level of ExsA-independent transcription seen in vitro is artificially elevated due to the absence of competing promoters (Fig. 3D), a condition never seen in vivo. Other contributing factors include the inherent limitations of

TABLE 1. Kinetic parameters of the P_{exsC} and P_{exsD} promoters

Promoter	τ(s)[a]	k_2 (s^{-1}, 10^3)[b]	K_B (M^{-1}, 10^{-5})[c]	k_2K_B (M^{-1} s^{-1}, 10^{-3})[d]
P_{exsC}				
Without $ExsA_{His}$	13.8	72	1.3	9.4
With $ExsA_{His}$	8.1	123	10	123
P_{exsD}				
Without $ExsA_{His}$	52.7	18.9	3.4	6.4
With $ExsA_{His}$	22.6	44	16.5	72.6

[a] Lag time to open complex formation.
[b] Isomerization rate constant.
[c] Equilibrium binding constant for RNAP.
[d] Overall reaction rate for open complex formation.

an in vitro assay, the use of minicircle transcription templates as a mimic for chromosomal DNA, and the absence of factors that may be required for maximal ExsA-dependent transcription. For example, ExsA may possess a coactivator, as is seen for the AraC family members (VirF and MxiE) that regulate T3SS gene expression in *Salmonella enterica* serovar Typhimurium and *Shigella flexneri*, respectively. Alternatively, some AraC family members function together with catabolite activator protein to regulate gene expression. It is interesting to note that the *P. aeruginosa* homolog of catabolite activator protein (Vfr) is required for T3SS gene expression through an undetermined pathway (52). Development of an in vitro transcription assay provides a means to test whether factors known to influence T3SS gene expression in *P. aeruginosa* do so by directly modulating ExsA-dependent activation.

ExsA-dependent promoters are unusual in that the putative −10 and −35 promoter elements are separated by 21 or 22 bp, compared to the 17 bp typical of σ^{70}-dependent promoters. Employing a panel of promoter spacing mutants, we demonstrated that P_{exoT} promoter activity shows a strict dependence on spacing of 21 or 22 bp. Increased spacing between the −10 and −35 regions has been described in only a few cases for transcription factors that activate σ^{70}-dependent promoters (28, 56). The mechanism of Spo0A-dependent transcriptional activation in *Bacillus subtilis* closely parallels the ExsA situation, where in both cases the −10 and −35 sites are separated by 21 to 22 bp and the activator binding sites overlap with and extend upstream of the −35 regions. These observations initially raised concerns as to whether the −35 region was an authentic determinant for binding of RNAP-σ^A (the equivalent of RNAP-σ^{70} in *B. subtilis*) to Spo0A-dependent promoters (56). The findings that reducing the spacing between the −10 and −35 regions of the *spoIIG* promoter to 17 bp results in Spo0A-independent transcription and that RNAP-σ^A footprints the −35 site independently of Spo0A (5), however, indicate that the −35 region is recognized by RNAP-σ^A and that Spo0A activates transcription by suppressing the −10 and −35 spacing constraint. The current model proposes that RNAP binds to the *spoIIG* promoter independently of Spo0A through low-specificity interactions between RNAP-σ^A and the −35 region. Spo0A binding then repositions RNAP-σ^A 4 bp downstream of the −35 region such that region 2 of σ^A can interact with the −10 region resulting, in open complex formation (36).

While it is clear from permanganate footprinting experiments that the −10 regions of P_{exoT} and P_{exsD} isomerize to open complexes, the role of the putative −35 regions remains unclear. Permanganate footprints and abortive transcripts demonstrate that RNAP-σ^{70} is capable of binding to the P_{exoT} and P_{exsD} promoters in the absence of ExsA. In this regard, ExsA-dependent promoters are similar to Spo0A-dependent promoters in that RNAP can bind independently of the activator. Binding of RNAP-σ^{70} to the P_{exoT} and P_{exsD} promoters, however, was detected only in the absence of competing promoters and may not reflect the in vivo situation. A notable difference between the ExsA and Spo0A systems is the effect of altered spacing between the −10 and −35 regions. Based upon the Spo0A model, we hypothesized that reducing the spacing between the −10 and −35 regions of ExsA-dependent promoters to 17 bp would result in ExsA-independent activity. Both the P_{exoT} and P_{exsD} promoters, however, lacked ExsA-independent expression at the optimal spacing of 17 bp (Fig. 1B and 2B). This was particularly surprising for the P_{exsD} promoter because the −10 and −35 regions closely match the σ^{70} consensus. Even more striking was the finding that the −10 and −35 regions of P_{exsD} are poor substitutes for the corresponding elements of the P_{tacI} promoter (Fig. 2D). These findings suggest that the −10 and −35 regions of P_{exsD} function as poor recognition elements for RNAP-σ^{70} and support our conclusion that the primary role of ExsA is to facilitate recruitment of RNAP-σ^{70} to the promoter. Future experiments will focus on characterization of the ExsA–RNAP-σ^{70} interaction, the regions of σ^{70} that interact with T3SS promoters in both the presence and absence of ExsA, and whether the −35 region contributes to the binding of RNAP-σ^{70}.

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