Transcriptional Organization of the *trans*-Regulatory Locus Which Controls Exoenzyme S Synthesis in *Pseudomonas aeruginosa*

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The transcriptional organization of the exoenzyme S *trans*-regulatory locus was studied by using promoter fusion and transcriptional start site mapping analyses. The 5' regions flanking open reading frames encoding ExsC, ExsB, ExsA, and ExsD were cloned in both orientations into the promoter vector pQF26, which contains the chloramphenicol acetyltransferase reporter gene (*cat*). CAT activity from each promoter fusion transformed into *Pseudomonas aeruginosa* and *Escherichia coli* was measured. The *trans*-regulatory locus promoters demonstrated low to undetectable CAT activity in *E. coli* regardless of the orientation of the DNA fragment relative to the reporter gene. In *P. aeruginosa* two of the promoter clones containing DNA located 5' of *exsC* (pC) and *exsD* (pD) demonstrated significant CAT activity. Transcriptional initiation from pC and pD was dependent on the orientation of the DNA fragment, the inclusion of a chelator in the growth medium, and the presence of a functional *exsA* gene. Transcriptional start sites were mapped for the pC and pD promoter regions by using total RNA isolated from *P. aeruginosa* strains grown in medium including a chelator. Our data are consistent with an operon model for the transcriptional organization of the exoenzyme S *trans*-regulatory locus. In addition, ExsA appears to be involved in controlling transcriptional initiation from both the *trans*-regulatory locus and a region located immediately downstream of the *exsA* gene.

Exoenzyme S is an extracellular ADP-ribosyltransferase produced by the opportunistic pathogen *Pseudomonas aeruginosa* (2, 14). The native form of exoenzyme S has been purified from the supernatant of *P. aeruginosa* 388 as an aggregate consisting of two proteins of 49 and 53 kDa (6, 15, 20). The 49-kDa form of exoenzyme S possesses ADP-ribosyltransferase activity, while the 53-kDa peptide appears to be enzymatically inactive (6, 15, 20). In vitro enzymatic activity of the 49-kDa protein depends on the presence of an accessory eukaryotic protein termed Fas, for factor activating S (6, 11). The principal eukaryotic targets for exoenzyme S include vimentin (5) and members of the H-Ras and K-Ras family of GTP-binding proteins (4, 6). Mutations introduced in the chromosome of *P. aeruginosa* which render the bacterium deficient in exoenzyme S production correlate with a reduction in the ability of *P. aeruginosa* to disseminate from epithelial colonization sites (19, 21). However, the relationship between a toxic form of exoenzyme S and the 53- and 49-kDa polypeptides has not been established.

Our studies have focused on the regulation of exoenzyme S production in *P. aeruginosa*. The expression of exoenzyme S is induced by growing *P. aeruginosa* in medium containing the chelator nitrilotriacetic acid (NTA) or EDTA (26). A cloned regulatory locus encoding three complete open reading frames, ExsC, ExsB, and ExsA, and one truncated open reading frame, ExsD, is required for exoenzyme S synthesis (9). ExsA is postulated to be a transcriptional activator protein, since it is a member of the AraC family of proteins and is highly homologous to the *Yersinia* transcriptional activators VirF and LcrF (9). To analyze the function of each protein in exoenzyme S production, interposon mutations utilizing the Ω cartridge were introduced into or upstream of the *exsC*, *exsB*, and *exsA* genes (10). Return of the insertionally inactive alleles

to the chromosome resulted in a severe reduction in exoenzyme S synthesis and secretion. These data suggested either that each gene was required for exoenzyme S production or that the locus was organized as an operon and Ω was exerting a polar effect on transcription.

The transcriptional organization of the *trans*-regulatory locus was examined by using promoter fusion analysis and transcriptional start site mapping. The studies reported in this article indicate that the *trans*-regulatory locus for exoenzyme S synthesis in *P. aeruginosa* is organized as an operon consisting of the *exsC*, *exsB*, and *exsA* genes. In addition, a second, coregulated promoter controlling the transcription of *exsD* (downstream locus) was identified. The initiation of transcription of the *trans*-regulatory operon and the downstream locus correlates with the induction of exoenzyme S synthesis and secretion. It appears that the induction of exoenzyme S synthesis in response to an environment with low cation levels is at the level of transcription of the *trans*-regulatory operon.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used are listed in Table 1. *Escherichia coli* HB101 and TB1 were cultivated in Luria-Bertani broth or agar at 37°C with ampicillin (100 μg/ml) as required. M13 phage propagation was performed according to standard protocols with *E. coli* TG1 as a host. *P. aeruginosa* strains were grown in Luria-Bertani broth at 37°C or on Vogel-Bonner minimal agar at 37°C with carbenicillin (400 μg/ml) as required (27). Chloramphenicol acetyltransferase (CAT) and exoenzyme S activities were measured after growth in a deferrated dialysate of Trypticase soy broth with 1% glycerol and 100 mM monosodium glutamate and containing NTA (0 to 10 mM) as the inducing agent for exoenzyme S production (26). The broth cultures were grown at 32°C in a shaking water bath (26).

Construction of promoter fusions. Restriction endonuclease sites used to clone 5' regions for each open reading frame are

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Reference or source
P. aeruginosa strains		
388	Wild type	2
PAK	Wild type	Stephen Lory
PAK *exsA*::Ω	Mutation in the chromosomal copy of *exsA*	10
E. coli strains		
HB101	*supE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	3
TB1	F⁻ *ara* Δ(*lac-proAB*) *rpsL* φ-80d *lacZΔM15 hsdR17*(r_K^- m_K^+)	1
TG1	*supE hsdΔ5 thi* Δ(*lac-proAB*) F'[*traD36 proAB⁺ lacIq lacZΔM15*]	16
Plasmids		
pQF26	CAT reporter vector	7
pC	*Eco*RI-*Nco*I-CAT fusion	This study
pC'	*Nco*I-*Eco*RI-CAT fusion	This study
pB	*Cla*I-*Rsr*II-CAT fusion	This study
pB'	*Rsr*II-*Cla*I-CAT fusion	This study
pA	*Bgl*II-*Xho*I-CAT fusion	This study
pA'	*Xho*I-*Bgl*II-CAT fusion	This study
pD	*Sal*I-*Ava*I-CAT fusion	This study
pD'	*Ava*I-*Sal*I-CAT fusion	This study
pDF120	pUC18 containing the exoenzyme S *trans*-regulatory locus	10

summarized in Fig. 1. Promoter fragments were extracted from agarose gels by using a GeneClean II kit (Bio 101, La Jolla, Calif.). The fragments were made blunt by using the Klenow fragment of DNA polymerase I and ligated into the *Sma*I site of pQF26 (7). To determine the orientation of the insert, transformants (12) were subjected to restriction endonuclease mapping analysis. Both orientations of each insert were cloned into M13 vectors and confirmed by nucleotide sequence analysis (24). Following confirmation of each construct, the plasmids were isolated and transformed into *P. aeruginosa* PAK and PAK *exsA*::Ω made competent by an $MgCl_2$ method (22). *P. aeruginosa* transformants were analyzed for the presence of each construct by restriction endonuclease mapping of purified plasmid DNA.

CAT assays. Strains containing promoter fusion constructs were grown under inducing (in the presence of the chelator) and noninducing (absence of the chelator) conditions for exoenzyme S production and were assayed for CAT activity as previously described (18, 25). Briefly, 1.6×10^9 cells were harvested, washed in 100 mM Tris (pH 7.8), and lysed by sonication. Supernatants were collected by centrifugation ($12,500 \times g$) at 4°C. CAT activity was determined by assaying the supernatant in the presence of 1.0 mM chloramphenicol–10 μl of [*butyryl*-1-¹⁴C]butyryl coenzyme A (4.0 mCi/mmol; New England Nuclear Research Products, Wilmington, Del.)–100 mM Tris-HCl (pH 7.8) overlaid with 5 ml of Econofluor II (New England Nuclear Research Products). Reaction mixtures were incubated at 37°C for 50 min and subjected to scintillation counting (6 s per sample) with a Beckman LS 6000CI counter. A standard curve was generated by using purified CAT (Boehringer Mannheim Corporation, Indianapolis, Ind.). CAT activities were normalized by protein content and calculated as units per microgram of total protein. The values for CAT activity are reported as the averages of at least three independent experiments.

Exoenzyme S assays. Exoenzyme S ADP-ribosyltransferase activity was quantitated from supernatant and lysate material as previously described (10, 15).

RNA isolation and primer extensions. RNA was isolated from *P. aeruginosa* strains grown under inducing and noninducing conditions for exoenzyme S production. At an A_{540} of 1.0, 2×10^{10} cells were collected and extracted from sodium dodecyl sulfate-lysed cells by an acid phenol method as previously described (8, 28). Transcriptional start sites were mapped by using a primer extension kit (Promega Biochemicals, Madison, Wis.) according to the manufacturer's instructions with the following modifications. Annealing reactions were performed by denaturing 25 μg of total RNA with 500 pmol of high pressure liquid chromatography-purified primer (Operon Technologies, Inc., Alameda, Calif.) at 90°C for 1 min, annealing at 58°C for 40 min, and cooling to room temperature for 10 min. End-labeled primers (labeled with [γ-³²P]ATP [6,000 Ci/mmol]; New England Nuclear) were extended at 42°C with reverse transcriptase for 45 min. Reaction mixtures were analyzed with 6.6% denaturing polyacrylamide gels and subjected to autoradiography. DNA sequencing reactions were performed by using a *Taq* DNA polymerase kit (Promega Biochemicals) according to the manufacturer's instructions.
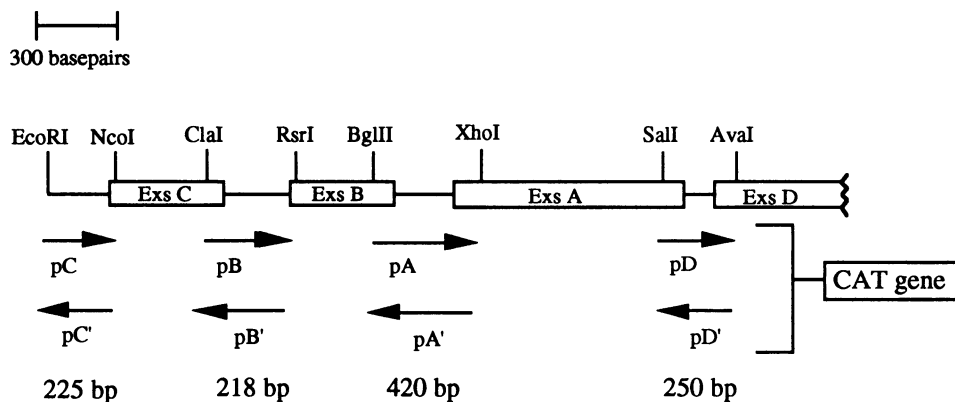

FIG. 1. Regions of the exoenzyme S *trans*-regulatory locus cloned in the promoter probe vector pQF26. The open reading frames encoding ExsC, -B, and -A and a partial open reading frame encoding ExsD are shown as open boxes. Arrows indicate the orientation of each fragment relative to the *cat* reporter gene in pQF26. Specific restriction sites used to isolate each fragment are shown on the map. The size of each fragment is listed.

TABLE 2. CAT activities of promoter fusions in *P. aeruginosa* PAK

Promoter clone	CAT (U) (mean ± SD)	
	With NTA[a]	Without NTA[b]
pC	0.66 ± 0.28	0.29 ± 0.09
pC'	ND[c]	ND
pB	0.06 ± 0.03	0.07 ± 0.03
pB'	ND	ND
pA	0.1 ± 0.04	0.22 ± 0.12
pA'	ND	ND
pD	2.68 ± 0.92	0.25 ± 0.15
pD'	ND	ND

[a] *P. aeruginosa* transformants were grown for induction of exoenzyme S in medium containing 10 mM NTA.

[b] *P. aeruginosa* transformants were grown in the absence of NTA.

[c] ND, not detectable.

Northern (RNA) and dot blot analyses. Total RNA isolated from *P. aeruginosa* cultures grown for maximal exoenzyme S production was denatured with glyoxal and dimethyl sulfoxide and separated by size with 1.2% agarose gels (8, 16). The RNA was transferred to either nitrocellulose or Nytran and probed with ^{32}P-labeled fragments of the *trans*-regulatory locus, using labeling and hybridization conditions as described previously (8, 10). Dot blots were performed as described previously, using total RNA denatured with formaldehyde (8, 16). DNA probes were isolated from agarose gels by using a GeneClean II kit (Bio 101) and labeled with [^{32}P]dCTP (3,000 Ci/mmol; New England Nuclear Research Products) by using a random primer kit (Bethesda Research Laboratories, Gaithersburg, Md.) according to manufacturer's instructions.

RESULTS

Promoter fusion analysis. The transcriptional organization of the exoenzyme S *trans*-regulatory locus was examined by constructing promoter fusions in a shuttle plasmid capable of replicating in both *E. coli* and *P. aeruginosa*. Regions corresponding to the 5' ends of each complete open reading frame (*exsC*, *exsB*, and *exsA*) and a truncated open reading frame (*exsD*) were isolated and cloned into the *Sma*I site of the promoterless reporter shuttle vector pQF26 (7). Clones were screened for orientation of the insert sequence initially by restriction endonuclease mapping. Subsequently, the insert DNA was removed by endonuclease digestion and cloned into M13 vectors for nucleotide sequence analysis (24). Nucleotide sequence analysis confirmed (i) that each promoter region matched the nucleotide sequence previously reported (9), (ii) the orientation of each promoter region relative to the *cat* reporter gene, and (iii) that only single and not concatameric copies of the cloned fragment existed within each reporter construct. Only constructs that adhered to all three criteria were analyzed further.

Each of the eight constructs (four promoter clones, pC, pB, pA, and pD, in both orientations) was transformed into *P. aeruginosa* PAK. CAT activity was measured in cultures induced and not induced for exoenzyme S synthesis (9, 26). Transformants were harvested at an A_{540} of 4.0 to 6.0 (stationary phase) and lysed by sonication. Units of CAT activity were calculated on the basis of protein content to control for variations in lysis efficiency. Constructs in which the promoter was oriented as in the *trans*-regulatory locus (reading 5' to 3' through the predicted open reading frames) demonstrated CAT activity (Table 2). Constructs in which the promoter

regions were oriented in the opposite fashion (pC', pB', pA', and pD') were inactive in CAT assays (Table 2).

Three patterns of CAT expression were observed when cultures were grown under inducing or noninducing conditions for exoenzyme S synthesis. In the absence of induction, two promoter constructs, pC and pD, demonstrated a reduction in the accumulation of CAT activity. Initiation from the pB promoter region remained the same whether transformants were grown in the absence or presence of NTA. Slightly more CAT activity was detected in the pA clone when cells were grown under noninducing conditions for exoenzyme S production (Table 2). Taken together, these data suggested that the direction of transcription corresponded to the predicted open reading frames (sense orientation) and that the pC and pD promoter regions were coordinately regulated with exoenzyme S synthesis.

NTA titration studies. To determine if the concentration of NTA was critical for transcriptional initiation, an NTA titration experiment was performed with constructs in the sense orientation. A *P. aeruginosa* PAK(pQF26) control culture was grown under the same conditions to monitor production of both extracellular and cell-associated exoenzyme S by ADP-ribosyltransferase activity. As shown in Fig. 2A, ADP-ribosyltransferase activity was maximal in lysate fractions when cells were grown in the presence of approximately 2 mM NTA. In supernatant fractions, growth in the presence of 5 mM NTA appeared to result in maximal exoenzyme S yields. CAT activity for pB, pA, and the vector plasmid pQF26 was low regardless of the concentration of NTA in the growth medium (Fig. 2B). The pC and pD promoter constructs demonstrated a rise in CAT activity accumulation as the concentration of NTA in the medium increased from 0 to 0.5 mM. CAT activity remained constant for the pC construct but continued to rise for the pD construct as the concentration of NTA in the growth medium increased above 0.5 mM.

Comparison of reporter activities in *P. aeruginosa* and *E. coli*. *P. aeruginosa* PAK and *E. coli* TB1, transformed with the various promoter fusions, were grown for maximal exoenzyme S expression and tested for CAT activity (Fig. 3). In *E. coli*, the CAT activity for each construct was below that measured for a vector control. Since promoters pC and pD were inducible in *P. aeruginosa* and reporter activity was absent in *E. coli*, we reasoned that a positive activator may be required for initiation of transcription from these promoter regions.

Reporter activity in PAK *exsA*::Ω. To determine the requirement of ExsA for transcriptional initiation of pC and pD, promoter fusions were transformed into a strain of PAK in which *exsA* was insertionally inactivated (PAK *exsA*::Ω) (10). Exoenzyme S production was severely reduced in PAK *exsA*::Ω, indicating that *exsA* is required for exoenzyme S expression (10). Transformants were grown under inducing conditions for exoenzyme S production, and CAT activity was measured. In the absence of a functional *exsA* gene, pC and pD CAT activities (Table 3) were reduced to levels expressed when wild-type transformants were grown without induction (Table 2). A marginal reduction in CAT activity was detected with the pA clone. CAT activity measured from the pB promoter clone remained unchanged, regardless of the host strain. These results suggested that ExsA or a gene controlled by ExsA was required for the initiation of transcription from pC and pD.

Transcriptional start site mapping. Transcriptional start site mapping was performed by using primers for each promoter region. *P. aeruginosa* PAK and 388 were grown under inducing and noninducing conditions for exoenzyme S synthesis. Total RNA was isolated, and annealed primers were extended with
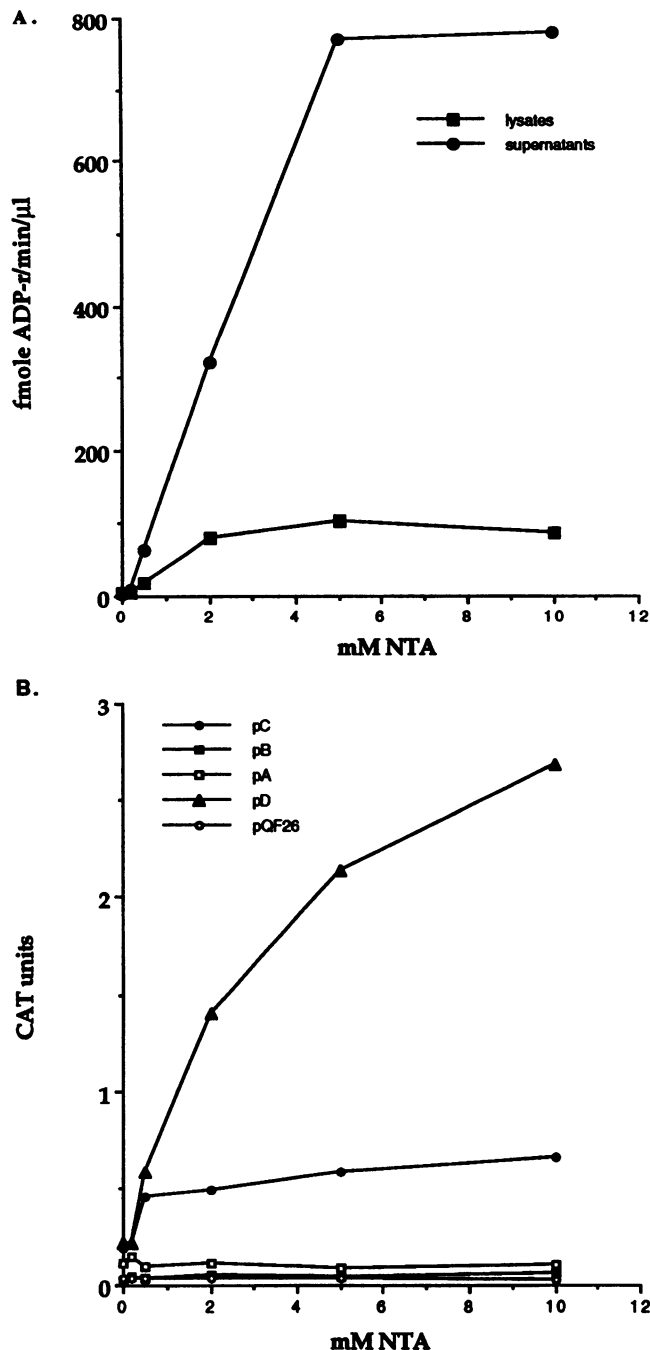
A.

B.

FIG. 2. Expression of exoenzyme S ADP-ribosyltransferase activity and promoter clone CAT activity in response to the concentration of NTA in the growth medium. (A) ADP-ribosyltransferase activity was monitored in supernatant and lysate fractions of *P. aeruginosa* PAK containing the vector plasmid pQF26. ADP-ribosyltransferase activity is reported as femtomoles of ADP-ribose incorporated per minute per microliter with ^{32}P-labeled NAD as a substrate and the specific target protein soybean trypsin inhibitor (10, 15). (B) CAT activity of PAK transformants containing the indicated promoter fusion constructs.

reverse transcriptase. Start sites for the pB and pA promoter regions were undetectable regardless of the growth conditions or the strain of *P. aeruginosa* used as a source for RNA (data not shown).
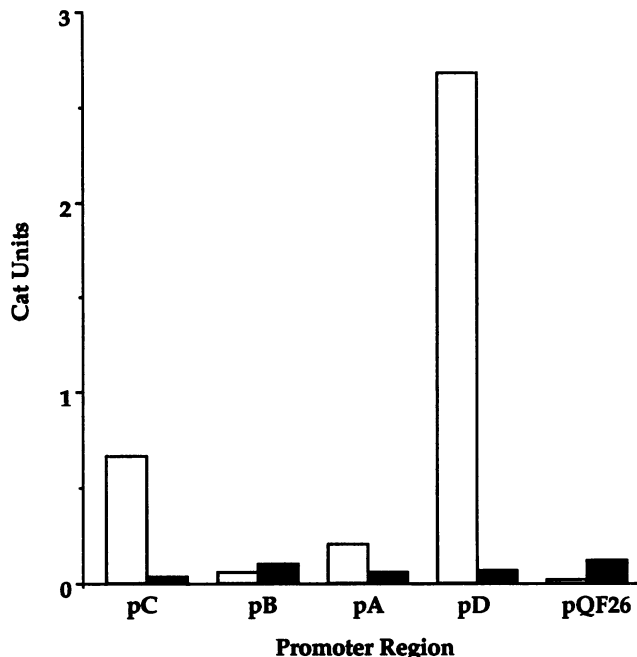

FIG. 3. Comparison of CAT activities of *E. coli* TB1 and *P. aeruginosa* PAK as host strains for promoter constructions. Open bars represent *P. aeruginosa* PAK and filled bars represent *E. coli* TB1 as hosts for promoter fusion clones. The pQF26 set of bars represents a vector control in each host strain. Cultures were grown under inducing conditions for exoenzyme S production in *P. aeruginosa*.

In the two strains of *P. aeruginosa* tested (PAK and 388), transcriptional start sites detected for the pC and pD promoter regions mapped to identical nucleotides when cells were grown for exoenzyme S production (Fig. 4). The start of transcription for pC mapped evenly to two nucleotides located 85 and 86 bp upstream of the ATG start codon for ExsC. These products were undetectable when RNA was isolated from cells grown in the absence of NTA. The start site for pD was located 54 bp 5′ of an ATG start codon for the truncated open reading frame, *exsD*. Under noninducing conditions, initiation of pD transcription was reduced, and in some experiments an additional product was detectable in total RNA from PAK. This start site was located 93 bp upstream of the ATG start codon of ExsD (Fig. 4B). We were, however, unable to confirm the start of this message (i) by using a second primer, (ii) by using *P. aeruginosa* 388 total RNA isolated from cells grown in the absence of NTA, or (iii) in replicates of these gels with different batches of total RNA. For these reasons, the pD start site detected under noninducing conditions was not analyzed further. The signal

TABLE 3. CAT activities of promoter fusions in PAK and PAK *exsA*::Ω[a]

Promoter clone	CAT (U) (mean ± SD) in:	
	PAK	PAK *exsA*::Ω
pC	0.66 ± 0.28	0.041 ± 0.028
pB	0.06 ± 0.03	0.054 ± 0.047
pA	0.1 ± 0.04	0.065 ± 0.042
pD	2.68 ± 0.92	0.073 ± 0.043

[a] PAK is wild type for exoenzyme S production; PAK *exsA*::Ω contains a chromosomal insertional mutation in the *exsA* gene.
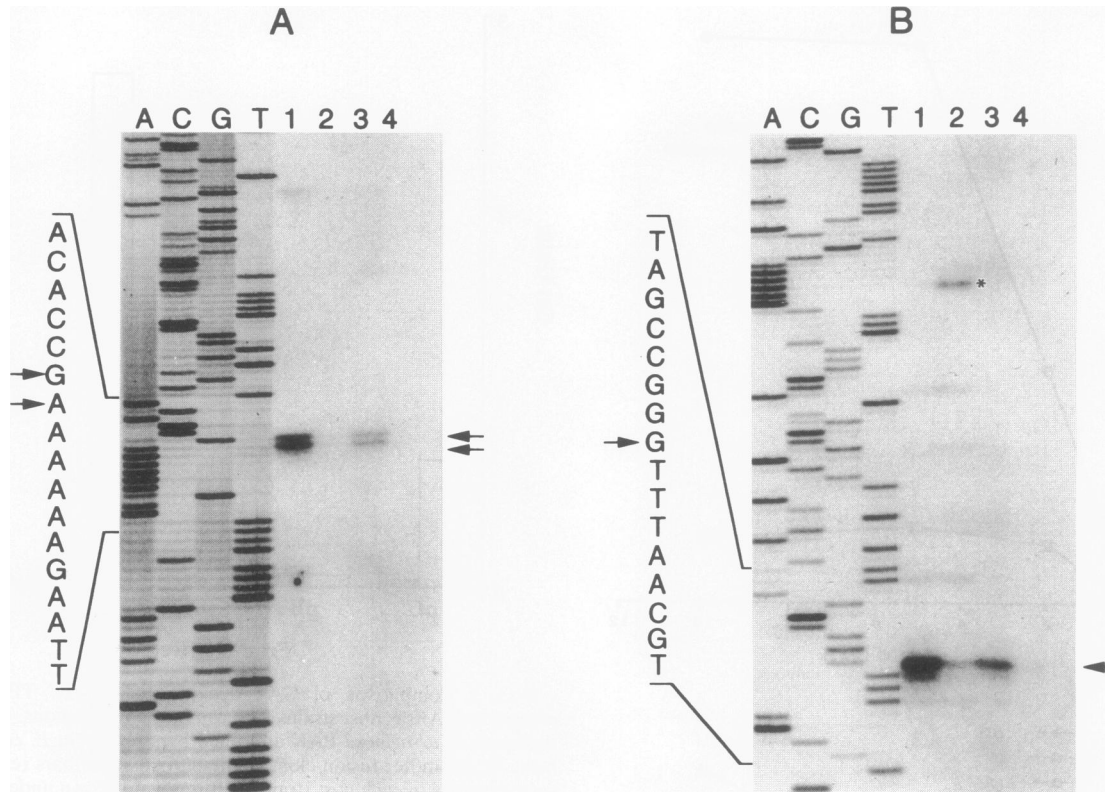
FIG. 4. Transcriptional start site mapping of pC (A) and pD (B). Nucleotide sequence analysis using the same primer for transcriptional start site mapping is shown in the lanes marked A, C, G, and T. Lanes 1, products of primer extension with PAK total RNA isolated from cells grown in the presence of NTA as a template. Lanes 2, products mapped from PAK total RNA isolated from cells grown in the absence of NTA. Lanes 3 (presence of NTA) and 4 (absence of NTA), same set of samples as for lanes 1 and 2 except that *P. aeruginosa* 388 was used as a source of total RNA. The nucleotide sequence for each region is indicated at the left of each panel, with arrows denoting the start site for transcription. A start site detected under noninducing conditions in PAK RNA is indicated by an asterisk.

intensity appeared to be higher when PAK RNA was compared with 388 RNA in transcriptional start site mapping experiments. This result suggested that strain PAK accumulated higher levels of pC and pD transcripts than strain 388 did.

Analysis of promoter regions. We examined the DNA sequence of each promoter clone to identify common *cis*-acting regulatory elements, since our data indicated that pC and pD (i) were coordinately regulated with exoenzyme S synthesis, (ii) initiated transcription by responding to the same environmental stimulus, and (iii) required a functional *exsA* gene product to initiate transcription. Analysis of the pC promoter revealed that the −10 (CACAGC) and −35 (TCTCCG) regions exhibited weak homology to the *E. coli* −10 (TATAAT) and −35 (TTGACA) consensus sequences (17) (Fig. 5). In contrast, the pD promoter utilized under inducing conditions for exoenzyme S production had strong homology to the *E. coli* consensus sequence, with only a single mismatch in both the −10 (TAGAAT) and −35 (TTGAAA) regions.

Two common features of pC and pD that may be involved in transcriptional initiation were identified (Fig. 5). An A+T-rich region immediately following the +1 initiation nucleotide was found in each promoter clone. In addition, the pD promoter had two A+T-rich stretches located upstream of the −35 region and centered at −40 and −54. A common 10-bp sequence, CACCCAGAGC, was centered at the −83 position of the pC promoter and the −125 position of the pD promoter.

Regions of significant homology were not identified near the RNA polymerase-binding sites. Studies are in progress to determine if ExsA directly binds to pC and pD.

Northern and dot blot analyses. To determine the length of the mRNA of the *trans*-regulatory locus, total RNA isolated from *P. aeruginosa* PAK and 388 was subjected to Northern blot analysis. mRNA from this region was undetectable regardless of (i) the probe used, (ii) the stage of growth, or (iii) the batch of RNA used. Specific but weak signals were detectable in dot blot analysis when RNA was isolated from cells grown for exoenzyme S production. Signals were not detectable in dot blot analysis using total RNA isolated from cells grown in the absence of NTA (data not shown).

DISCUSSION

Data from promoter fusion experiments and transcriptional start site mapping analysis are consistent with an operon structure for the exoenzyme S *trans*-regulatory locus. Intergenic regions between the four predicted open reading frames, ExsC, ExsB, ExsA, and ExsD, were cloned in both orientations within a promoter fusion vector containing a *cat* reporter gene. CAT activity was detected in two clones which localized active promoters to the regions 5′ of ExsC (pC) and ExsD (pD). The direction of transcription through the *trans*-regulatory locus was established, since only one orientation of the DNA fragments resulted in the ability to detect CAT activity.
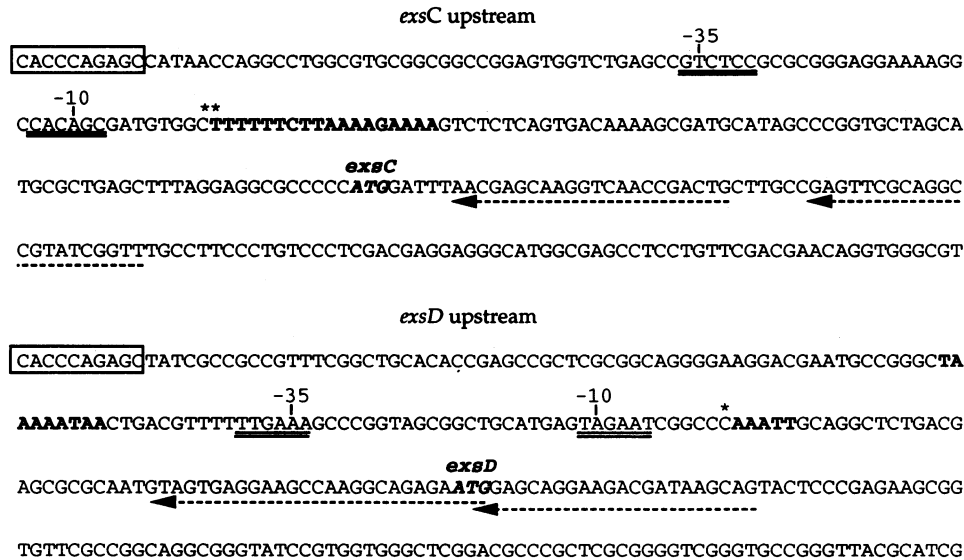
exsC upstream

-35

CACCCAGAGC ATAACCAGGCCTGGCGTGCGGCGGCCGGAGTGGTCTGAGCCGTCTCCGCGCGGGAGGAAAAGG

-10 **
CCACAGCGATGTGGCTTTTTTCTTAAAAGAAAAGTCTCTCAGTGACAAAAGCGATGCATAGCCCGGTGCTAGCA

exsC
TGCGCTGAGCTTTAGGAGGCGCCCCCATGGATTTAACGAGCAAGGTCAACCGACTGCTTGCCGAGTTCGCAGGC
◄----------------------------- ◄---------------

CGTATCGGTTTGCCTTCCCTGTCCCTCGACGAGGAGGGCATGGCGAGCCTCCTGTTCGACGAACAGGTGGGCGT
·----------------

exsD upstream

CACCCAGAGC TATCGCCGCCGTTTCGGCTGCACACCGAGCCGCTCGCGGCAGGGGAAGGACGAATGCCGGGCTA

-35 -10 *
AAAATAACTGACGTTTTTTTGAAAGCCCGGTAGCGGCTGCATGAGTAGAATCGGCCCAAATTGCAGGCTCTGACG

exsD
AGCGCGCAATGTAGTGAGGAAGCCAAGGCAGAGAATGGAGCAGGAAGACGATAAGCAGTACTCCCGAGAAGCGG
◄------------------------- ◄--------------------------

TGTTCGCCGGCAGGCGGGTATCCGTGGTGGGCTCGGACGCCCGCTCGCGGGGTCGGGTGCCGGGTTACGCATCG

FIG. 5. Comparison of nucleotide sequences of the coordinately regulated pC and pD promoter regions of the exoenzyme S *trans*-regulatory locus. −10 and −35 regions, based on the transcriptional start site, are indicated by double underlines. A+T-rich regions are shown in boldface. An identical 10-bp site identified in both pC and pD is boxed. The start codons for the ExsC and ExsD proteins are shown as *ATG*. Dotted arrows shown proximal to the start of translation are the primers used with reverse transcriptase to map the transcriptional start sites, which are marked with asterisks.

Internal promoter regions, pB and pA, were either inactive or marginally active under all conditions tested. Results from transcriptional start site mapping studies corresponded to those from promoter fusion studies. Start sites were detectable with primers for the pC and pD promoter regions but were undetectable when primers for pB or pA were used in reverse transcription experiments. These data, and the finding that chromosomal insertional mutations within *exsC*, *exsB*, or *exsA* result in a similar exoenzyme S-deficient phenotype, support the operon model for transcription of the exoenzyme S *trans*-regulatory locus.

Northern blot analysis was performed to identify the size of the mRNA from the *trans*-regulatory locus. Although several batches of RNA were isolated and different probes corresponding to the whole locus as well as internal probes for *exsC*, *exsB*, and *exsA* were used, mRNA remained undetectable (data not shown). mRNA was also undetectable with cells harvested at early, middle, or late stages of growth. A more sensitive dot blot assay was able to distinguish a specific but weak hybridization signal when RNAs isolated from cells grown in the presence and absence of NTA were compared. Two explanations may account for our inability to detect mRNA in Northern blot hybridization analysis. The initiation of transcription from the pC promoter region appears to be relatively weak compared with the initiation of pD transcription. This observation may indicate that the products encoded by the *trans*-regulatory operon are needed in small amounts and that tight control is exerted at the level of transcriptional initiation. Alternatively, the mRNA for the *trans*-regulatory locus operon may be unstable, like the message encoded by the *lcrF* gene (13).

Transcriptional initiation from pC and that from pD have both similar and distinct properties. Both promoter clones initiate transcription in response to an environment with low cation levels, simulated by the inclusion of chelators in the growth medium. However, the responses to titration of the chelator were dissimilar. The promoter pC reached a maximal level of CAT accumulation and then remained constant over several concentrations of NTA. This pattern closely corresponded to the pattern of accumulation of cell-associated exoenzyme S ADP-ribosyltransferase activity. Transcriptional initiation from pD demonstrated a pattern that correlated with the expression of extracellular ADP-ribosyltransferase activity; as the NTA concentration in the growth environment increased, extracellular ADP-ribosyltransferase and intracellular CAT activities tended to accumulate. These data, combined with the requirement of *exsA* for transcriptional initiation of pC and pD, indicate that synthesis and secretion of exoenzyme S may be mediated by at least two sets of coordinately regulated genes. Synthesis appears to be regulated by the operon containing *exsC*, *exsB*, and *exsA*, while secretory functions may be encoded by a separately transcribed downstream locus containing at least one gene product, ExsD.

Comparison of the nucleotide sequences of pC and pD revealed both similar and unique features. Both promoter regions contained a set of 10 identical nucleotides which may serve as a binding region for ExsA. The pD promoter was highly homologous to the *E. coli* consensus −10 and −35 sequences and contained A+T sequences. In some cases A+T-rich upstream sequences increase promoter activity (23). Although the pC promoter contained the 10-bp sequence and A+T-rich sequences, they were spaced differently from the same features noted for pD. Since the relative strength of transcription differed for each promoter region, transcriptional initiation may be modulated by proteins other than ExsA, the spacing of *cis*-acting elements, or the presence or absence of certain *cis*-acting elements. DNA-binding and footprinting analyses with recombinant ExsA may resolve these questions.

The discovery of pD defined the 3' end of the *trans*-regulatory locus as including the *exsA* gene and identified a second locus which we have termed the downstream locus. Since pD is coordinately regulated with the transcriptional initiation of pC and exoenzyme S production, we speculate that the downstream locus may contain genes involved in either

exoenzyme S synthesis or secretion. In support of this hypothesis, we mapped the point of insertion of Tn*1* in strain 388 *exs1*::Tn*1* to the downstream locus by Southern blot analysis (7a). The transposon insertion reduced exoenzyme S production (20) and was complemented by the *trans*-regulatory locus (9). Although synthesis of exoenzyme S was restored in complementation experiments, the 53- and 49-kDa forms of exoenzyme S were found to be cell associated rather than secreted (9). These data implicate the downstream locus as contributing to exoenzyme S secretion. A search for homologous genes or peptides in current data bases suggests that ExsD is unique. Further cloning, sequencing, and mutagenesis of this region will be needed to identify potential gene products and determine their functions in exoenzyme S expression and localization.

ACKNOWLEDGMENT

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