JOURNAL OF BACTERIOLOGY, Aug. 1995, p. 4427–4436 Vol. 177, No. 15
0021-9193/95/$04.00+0

Analyses of the DNA-Binding and Transcriptional Activation Properties of ExsA, the Transcriptional Activator of the *Pseudomonas aeruginosa* Exoenzyme S Regulon

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Received 6 March 1995/Accepted 27 May 1995

ExsA has been implicated as a central regulator of exoenzyme S production by *Pseudomonas aeruginosa*. In this study, the DNA-binding and transcriptional activation properties of ExsA were investigated. ExsA was produced and purified as a fusion protein, MALA3A2, which was shown to bind specifically to promoter regions that regulated transcription of the exoenzyme S *trans*-regulatory locus (pC) and a locus located directly downstream of *exsA* (pD). Previously, MALA3A2 was shown to bind the *exoS* 5 *Pst*I-*Nsi*I region, which contained two independent but coordinately regulated (ExsA-mediated) promoters, pS (now termed pORF1) and pS. DNase I footprint analysis of the promoter regions bound by ExsA revealed a common protected consensus sequence of TXAAAAXA. The consensus sequence was located −51 to −52 bp upstream of the transcriptional start sites for pD, pS, and pORF1. Promoter fusion, DNA-binding, and mutagenesis analyses indicated that the consensus sequence was important for transcriptional activation. Each ExsA-controlled promoter region contained at least two consensus sites in close proximity, similar to the arrangement of half-sites seen in AraC-controlled (*Escherichia coli*) or VirF-controlled (*Yersinia enterocolitica*) promoters. However, the results of this study suggested that only one consensus site was required in the exoenzyme S (pS) or *ORF1* promoter (pORF1) to initiate transcription. These data suggest that members of the exoenzyme S regulon can be defined as possessing an ExsA consensus element which maps at bp −51 or −52 relative to the transcriptional start site.

The opportunistic pathogen *Pseudomonas aeruginosa* is the causative agent of acute and chronic infections in cystic fibrosis patients, immunocompromised individuals, and burn victims (3). Several of the extracellular proteins produced by this organism play important roles in the ability of *P. aeruginosa* to colonize, persist, and replicate in host tissues (3, 36). Our studies have focused on exoenzyme S and the regulatory pathway that controls both the synthesis and secretion of exoenzyme S. Exoenzyme S is an ADP-ribosyltransferase (22) whose expression has been correlated with the ability of *P. aeruginosa* to spread from epithelial colonization sites to the bloodstream of individuals with acute infections (2, 6, 33–35). *P. aeruginosa* secretes two immunologically related forms of exoenzyme S of 53 and 49 kDa, which can be differentiated by their levels of ADP-ribosyltransferase activity (24, 34). The gene encoding the enzymatically active 49-kDa form has been cloned (26). Recent studies suggest that a separate gene encodes the 53-kDa form of exoenzyme S (25). Enzymatic activity in vitro requires the participation of a eukaryotic cofactor protein, FAS (9). FAS is a member of the 14-3-3 family of proteins that regulate the activity of several eukaryotic enzymes (15). In the presence of FAS, exoenzyme S has been shown to preferentially ADP ribosylate vimentin and members of the H-Ras and K-Ras family of low-molecular-weight GTP-binding proteins (7, 8).

The synthesis of exoenzyme S by *P. aeruginosa* is regulated. Early studies demonstrated that maximal exoenzyme S production was achieved by growth of *P. aeruginosa* in medium containing low concentrations of cations. Low levels of cations during growth were maintained by the inclusion of chelators such as EDTA or nitrilotriacetic acid in the cultivation medium (49). Genetic studies and complementation analysis of a mutant defective in exoenzyme S production resulted in the cloning of a proposed set of regulatory genes (13). This region, termed the exoenzyme S *trans*-regulatory locus, was shown to contain three complete open reading frames, *exsC*, *exsB*, and *exsA*, and a truncated open reading frame, *exsD* (13). The only gene product in this locus with significant homology to proteins in the database was ExsA.

Homology studies indicated that ExsA was a member of the AraC family of transcriptional activator proteins which contain carboxy-terminal helix-turn-helix DNA binding motifs (13, 16, 19). Members of the AraC family which demonstrated the highest level of amino acid identity (56%) to ExsA included the *Yersinia* spp. transcriptional activator proteins LcrF and VirF (13, 21, 27). Both VirF and LcrF play a major role in the control of the expression of a set of extracellular virulence determinants termed Yops. The Yops include a protein tyrosine phosphatase, YopH (17), and a cytotoxin, YopE, that causes microfilament disruption in eukaryotic cells (41, 46). These virulence determinants protect extracellular bacteria by exerting antiphagocytic effects (39, 40). Like exoenzyme S, the *Yersinia* Yop proteins are optimally expressed in medium containing low levels of cations, particularly calcium (4, 10, 44, 45). These data suggest that the regulatory pathway resulting in Yop synthesis and secretion in *Yersinia* spp. and ExoS synthesis and secretion in *P. aeruginosa* not only share homologous regulatory proteins (LcrF, VirF, and ExsA) but may also respond to similar environmental signals.

In addition to homology information, several pieces of evidence indicate that ExsA is a key regulator in the exoenzyme S pathway. Chromosomal mutations that disrupt *exsA* prevent exoenzyme S synthesis and alter the expression of several ex-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype	Reference
P. aeruginosa		
PAK	Wild type	S. Lory
PAK*exsA*::Ω	Mutation in the chromosomal copy of *exsA*	14
E. coli		
TG1	*supE hsdΔ5 thi* Δ(*lac-proAB*) F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZΔM15*)	42
Plasmids		
pGp1-2	Plasmid expressing the T7 RNA polymerase	47
pT7-5A1A2	*exsA* under transcriptional control of T7 promoter	13
pMAL-c2	IPTG-inducible MalE fusion expression vector	New England BioLabs
pMALA3A2	*exsA* gene fused to *malE* of pMAL-c2	This study
pQF26	CAT reporter vector	12
pS	CAT fusion containing *Pst*I-*Nsi*I (pS) in *exoS* orientation	54
pS′	*Nsi*I-*Pst*I (pORF1)-CAT fusion in *ORF1* orientation	54
pS-101	CAT fusion of pS starting at position −101 of *exoS*	This study
pS-82	CAT fusion of pS starting at position −82 of *exoS*	This study
pS-71	CAT fusion of pS starting at position −71 of *exoS*	This study
pS-66	CAT fusion of pS starting at position −66 of *exoS*	This study
pS-55	CAT fusion of pS starting at position −55 of *exoS*	This study
pORF1-70	CAT fusion of pORF1 starting at nt −70 of *ORF1*	This study
pS-71*GGGG	The pS-71 clone containing deoxyguanosines at positions −54 to −57 of ExsA consensus element, pS_1	This study

tracellular proteins in a strain-dependent manner (14). Transcriptional initiation of the pC promoter which controls the operon containing *exsC*, *exsB*, and *exsA* (the *trans*-regulatory locus) and the pD promoter which drives transcription of *exsD* and perhaps additional downstream genes required a functional chromosomal copy of *exsA* (53). In recent studies, the transcriptional initiation of the gene encoding the 49-kDa form of exoenzyme S, *exoS*, also demonstrated a requirement for a functional copy of *exsA* (54). ExsA was shown to interact specifically with the *exoS* promoter, pS, by gel mobility shift and DNase I footprint experiments utilizing a purified MalE-ExsA fusion derivative, MALA3A2.

In this article, we describe the construction and purification of the ExsA fusion derivative MALA3A2 and demonstrate that this protein specifically binds to the pC and pD promoter regions. These data are consistent with previous genetic data indicating that ExsA controls transcriptional initiation of several operons coordinately regulated with the expression of exoenzyme S in *P. aeruginosa*. Promoters which bound MALA3A2 were subjected to DNase I footprinting assays which identified common protected core sequences of TXAAAAXA. Truncation and mutational analyses of the pS promoter were used to define a minimal ExsA-inducible promoter required for DNA binding and transcriptional initiation by ExsA. Our data are consistent with the model that promoter regions containing an ExsA consensus element (TXAAAAXA) at bp −51 or −52 relative to the transcriptional start site are coordinately regulated with exoenzyme S synthesis and are defined as members of the exoenzyme S regulon.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used are summarized in Table 1. *Escherichia coli* cultures were grown in Luria-Bertani broth or on agar at 37°C with ampicillin (100 μg/ml) as needed. *P. aeruginosa* cultures were cultivated in Luria-Bertani broth with carbenicillin (400 μg/ml). Prior to chloramphenicol acetyltransferase (CAT) assays, *P. aeruginosa* strains were grown on Vogel-Bonner minimal agar with carbenicillin (400 μg/ml) at 37°C for 24 h (50).

Production of cellular extracts as a source of crude ExsA. ExsA was produced as a recombinant protein under the control of the T7 promoter from *E. coli* K38(pGP1-2/pT7-5A1A2) (13). The culture was cultivated under inducing conditions for expression of the T7 RNA polymerase as described previously (13, 47). Cellular extracts were prepared by harvesting a 10-ml induced culture by centrifugation (8,000 × *g*, 10 min, 4°C), suspending the cell pellet in 1 ml of wash buffer (50 mM Tris-HCl [pH 7.9], 1 mM dithiothreitol, 2 mM EDTA, 10 mM benzamidine, and 1 μg of leupeptin per ml), and lysing the cells by passage through a French pressure cell (3/8-in. [ca. 0.9-cm] diameter piston, 1,000 lb/in^2) two times. Insoluble matter and unlysed cells were removed by centrifugation at 13,000 × *g* for 5 min at 4°C. Total protein levels were measured in cellular extracts by the method of Lowry (29). Extracts were stored at −70°C for use in gel mobility shift assays.

Construction and purification of the ExsA fusion derivative MALA3A2. As preliminary gel mobility shift experiments suggested that recombinant ExsA was able to bind to promoter regions from the exoenzyme S *trans*-regulatory locus, a fusion derivative of ExsA was designed to facilitate both expression and purification of the protein. To construct a *malE-exsA* fusion plasmid, *exsA* was excised as an *Eco*RI-*Bam*HI restriction fragment from pKK223-3A3A2 (13) and cloned into the same sites in pMAL-c2 (New England BioLabs, Beverly, Mass.), generating pMALA3A2. Nucleotide sequence analysis was performed to ensure that *exsA* was in frame with *malE*. Expression studies using pMALA3A2 indicated that a fusion protein of an apparent molecular mass of 66 kDa was produced under isopropyl-β-D-thiogalactopyranoside (IPTG) induction. A two-stage purification procedure was used to isolate MALA3A2. In the first stage, MALA3A2 was expressed and a lysate was prepared according to the manufacturer's instructions (New England BioLabs). The lysate was passed through an amylose affinity column, the column was washed, and bound protein was eluted with a buffer containing 10 mM maltose. In the second stage of purification, the amylose column eluate (5 ml) was loaded onto a DEAE-Sephacel column (1 by 6 cm) equilibrated with 10 mM Tris-HCl (pH. 8.0)–25 mM NaCl. The column was washed with 15 ml of equilibration buffer, and bound material was eluted with a 60-ml gradient consisting of 25 mM Tris-HCl (pH 8.0) and 25 to 500 mM NaCl. Fractions were collected and monitored for the fusion protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fractions containing the fusion protein were pooled and concentrated with a Centriprep filtration unit (Amicon, Beverly, Mass.) and a buffer containing 20 mM Tris-HCl (pH 7.5)–50 mM NaCl. Aliquots of purified MALA3A2 were stored at −70°C.

Isolation of DNA probes for gel mobility shift assays. PCR was used to isolate DNA probes corresponding to the exoenzyme S *trans*-regulatory locus promoter clones of previous studies (53). Primer (Operon Technologies Inc., Alameda, Calif.) location was designed to yield fragments of 243 to 320 bp. *trans*-regulatory locus probes, amplified by PCR, were designated pC (nucleotides [nt] 1 to 254), pB (nt 618 to 938), pA (nt 1348 to 1591), and pD (nt 2339 to 2593) (Fig. 1A) (13, 53). For DNA-binding studies, the *exoS* promoter (pS) was isolated as a 285-bp *Pst*I-*Nsi*I fragment and subcloned into the *Pst*I site of pUC18 (Fig. 1B) (54). pS was isolated from pUC18 as an *Eco*RI-*Hin*dIII fragment and purified by phenol extraction from agarose gels (1).

In other studies, pS had been shown to contain divergent promoters controlling the transcription of *exoS* and *ORF1* (54). The divergent promoters were separated by PCR, each fragment contained −10 and −35 hexamers and additional upstream sequences (Fig. 1B). The fragment termed pS-71 was amplified with one primer whose 5′ end started at nt −71 and an opposing primer whose

5′ end was located at nt +28 relative to the *exoS* transcriptional start site (54). The fragment termed pORF1-70 was amplified with a primer whose 5′ end was located at nt −70 and an opposing primer whose 5′ end was located at nt +106 relative to the start of transcription for *ORF1* (54). Other regions of the pS promoter were amplified and used in gel mobility shift studies. The position of the 5′ end of the sense-strand primer relative to the start site of transcription of *exoS* is reported (pS-101, pS-82, pS-66, and pS-55) (Fig. 1B). The 5′ end of the opposing primer (+28 of *exoS* transcriptional start site) for each amplification of pS was constant (Fig. 1B). PCR fragments were isolated from agarose gels (1) and labeled as described in a subsequent section.

End labeling of DNA promoter fragments for gel mobility shift assays. End-labeling reactions (42) were carried out with 100 ng of promoter fragments, T4 polynucleotide kinase (Promega Corporation, Madison, Wis.), and 150 μCi of [γ-^{32}P]ATP (6,000 Ci/mmol; DuPont NEN, Wilmington, Del.). Fragments isolated by restriction endonuclease digestion were pretreated with calf intestinal alkaline phosphatase (Boehringer Mannheim Corporation, Indianapolis, Ind.) before being subjected to the end-labeling procedure.

Gel mobility shift assay. Gel mobility shift assays were performed essentially as described by Hoe et al. (21) with the exception that the binding buffer contained 100 mM KCl and poly(dI-dC) instead of 50 mM KCl and plasmid DNA. Briefly, 20-μl reaction mixtures containing 5 fmol of labeled DNA, 500 ng of poly(dI-dC), 10 μl of 2× DNA binding buffer (20 mM Tris-HCl [pH 7.5], 200 mM KCl, 2 mM EDTA, 2 mM dithiothreitol, 10% glycerol), and MALA3A2 or an *E. coli* T7 expression lysate as a source for ExsA, were incubated for 15 min at 37°C. After the addition of sample buffer, reaction mixtures were electrophoresed on 5% 1× TBE polyacrylamide gels (42) (1:30 bisacrylamide-acrylamide). The gels were fixed, dried, and subjected to autoradiography. Quantitation of the DNA fragments with altered mobility was analyzed by radioanalytic imaging on an Ambis 4000 (Ambis, San Diego, Calif.).

DNase I protection assays. To map the DNA region bound by ExsA, labeled sense (5*) and antisense (3*) strands were used in DNase I protection assays. For the pC and pD promoter regions, PCR was used to synthesize single-end-labeled products in which one primer was end labeled with [γ-^{32}P]ATP (DuPont NEN) using T4 polynucleotide kinase (Promega). PCRs consisted of a single-end-labeled primer and an opposing unlabeled primer. PCR reaction mixes were electrophoresed on 5% polyacrylamide gels and labeled DNA bands were electroeluted (42). DNA was precipitated with ethanol and suspended to a 5 nM concentration in water.

DNase I protection assays were performed essentially as described by Lambert de Rouvroit et al. (27). The binding reaction consisted of 500 ng of poly(dI-dC), labeled probes (15,000 to 30,000 dpm), MALA3A2 (2.5 to 25 pmol) in 50 μl of modified FP buffer (10 mM Tris-HCl [pH 7.9], 100 mM KCl, 6.25 mM $MgCl_2$, 0.05 mM EDTA, 8.5% glycerol). Binding reactions were incubated at room temperature for 30 min. After incubation, 50 μl of a 5 mM $CaCl_2$–10 mM $MgCl_2$ solution was added and then 0.01 μg of DNase I (Worthington Biochemical Corporation, Freehold, N.J.) was added. Reaction mixtures were incubated for 1 min at room temperature and stopped with the addition of a 100-μl solution consisting of 200 mM NaCl, 20 mM EDTA, and 1% SDS. Following a phenol-chloroform extraction, the reaction products were precipitated in the presence of ethanol and 10 μg of carrier tRNA. Precipitated DNA collected by centrifugation (13,000 × *g*) for 15 min at 4°C was suspended in a formamide dye mix (Promega) and heated to 85°C for 5 min prior to electrophoresis on 8 M urea–6.6% polyacrylamide sequencing gels. Chemical cleavage reactions were analyzed on the same gels to determine the nucleotide sequences of regions protected by MALA3A2 (30). The gels were fixed, dried, and subjected to autoradiography.

Construction of promoter fusion clones. All amplified DNA fragments from pS and pORF1 derivatives were isolated from agarose gels and cloned into the *Sma*I site of pUC18. Dideoxy sequencing (43) with the TaqTrack Sequencing Systems (Promega) confirmed the sequence and orientation of each clone. Each promoter fragment was removed from pUC18 by an *Sst*I-*Hin*dIII restriction enzyme cleavage and cloned in an oriented fashion in the same sites of the CAT reporter vector pQF26 (12). The pQF26 subclones were transformed (18) into *E. coli*, TG1, and confirmed by restriction endonuclease analysis. Each clone was transformed (37) into *P. aeruginosa* strains (PAK and PAK*exsA*::Ω) (14).

CAT assays. Lysates of *P. aeruginosa* transformants containing CAT promoter fusions were prepared and assayed for CAT activity as previously described (32, 53). Transformants were grown at 32°C in a deferrated Trypticase soy broth containing 10 mM nitrilotriacetic acid, 1% glycerol, 100 mM monosodium glutamate, and 400 μg of carbenicillin per ml (49).

Site-specific mutagenesis of the ExsA consensus sequence. The pS-71 PCR fragment was cloned into the *Sma*I site of pUC18. Sequence and orientation of the insert was confirmed by nucleotide sequence analysis (43) using the TaqTrack Sequencing Systems (Promega). The pS-71 insert was excised as an *Eco*RI-*Hin*dIII fragment and cloned into the same sites in M13mp18. After confirmation of the M13mp18pS-71 clone by restriction analysis, single-stranded DNA was isolated for synthesis of a 4-bp mutation with the Sculptor in vitro mutagenesis system (Amersham, Arlington Heights, Ill.) based on the phosphorothioate technique of Taylor et al. (48).

RESULTS

Binding of native ExsA to pC and pD. Using promoter fusions, Yahr et al. showed that the transcriptional initiation of several loci required a functional copy of *exsA* (53, 54). Loci coordinately controlled by ExsA included the *trans*-regulatory operon (*exsC*, *exsB*, and *exsA*) (53), an operon that was postulated to encode the genes for exoenzyme S export (*exsD* and the downstream locus) (53), a postulated chaperone for exoenzyme S (*ORF1*) (54), and the structural gene for exoenzyme S (*exoS*) (Fig. 1). The promoter regions for each locus have been designated pC, pD, pORF1, and pS. Direct binding of a MalE-ExsA fusion protein (MALA3A2) has been demonstrated for a DNA fragment which contained both the pORF1 and pS promoters (the *Pst*I-*Nsi*I fragment [Fig. 1B]) (54). To determine if native ExsA bound directly to promoter fragments of the *trans*-regulatory locus (Fig. 1A), gel mobility shift assays were performed. Recombinant ExsA was produced in *E. coli* [K38(pGP1-2/pT7-5A1A2)] under the control of the T7 RNA polymerase. Cellular extracts were prepared for the strain expressing ExsA as well as a vector control strain [K38(pGP1-2/pT7-5)] and used at a total protein concentration of 35 μg per DNA-binding assay. Fragments corresponding to pC, pB, pA, and pD (Fig. 1A) (53) were amplified by PCR, end labeled with [γ-^{32}P]ATP, and used as probes in gel mobility shift assays.

Gel mobility shift analysis with the *trans*-regulatory locus promoter probes indicated that the pC and pD promoters were bound by ExsA. A shift of the pC fragment was detectable with a cellular extract from a strain expressing ExsA (data not shown). In some experiments, a faint but detectable shift with the pD probe was demonstrated (data not shown). Using this crude source of ExsA, mobility shifts were not detected when probes pB and pA were used (data not shown). The mobilities of labeled fragments appeared unaltered with a vector control cellular extract (data not shown). We concluded that native ExsA was a DNA-binding protein that interacted directly with the pC and potentially the pD promoter regions of the exoenzyme S *trans*-regulatory locus.

Expression, purification, and DNA binding activity of MALA3A2. To purify ExsA for DNA-binding experiments, *exsA* was fused in frame to *malE* of the expression vector, pMAL-c2, resulting in a fusion of ExsA to the carboxy terminus of the maltose-binding protein. As shown in Fig. 2 (compare lanes 1 and 2), a fusion protein, termed MALA3A2, was expressed under inducing conditions. Amylose affinity chromatography enriched for the fusion derivative (lane 3) and DEAE chromatography resulted in a relatively pure product (lane 4). The MALA3A2 protein encoded a single factor Xa cleavage site to separate MalE from ExsA. Cleavage of MALA3A2 with factor Xa was approximately 50% efficient as estimated by densitometry with Coomassie blue-stained SDS-PAGs (lane 5). Digestion with factor Xa produced a clear MalE product, but several bands appeared in the molecular mass range for native ExsA (34 kDa). These results suggested that ExsA may be degraded upon factor Xa cleavage.

The MALA3A2 fusion derivative and factor Xa-treated materials were tested in gel mobility shift assays with probes derived from the promoter regions of the exoenzyme S *trans*-regulatory locus. In assays using the full-length MALA3A2 protein, the labeled pC and pD promoter regions demonstrated a mobility shift (Fig. 3, lanes 1 and 4). No binding was detected with the pB promoter region probe or when purified MalE was used as a control (lanes 5 to 8). With high concentrations of MALA3A2 (2,500×), gel mobility shifts with pA were detectable in some experiments (data not shown). Thus, MALA3A2 appeared to have the same DNA-binding specific-

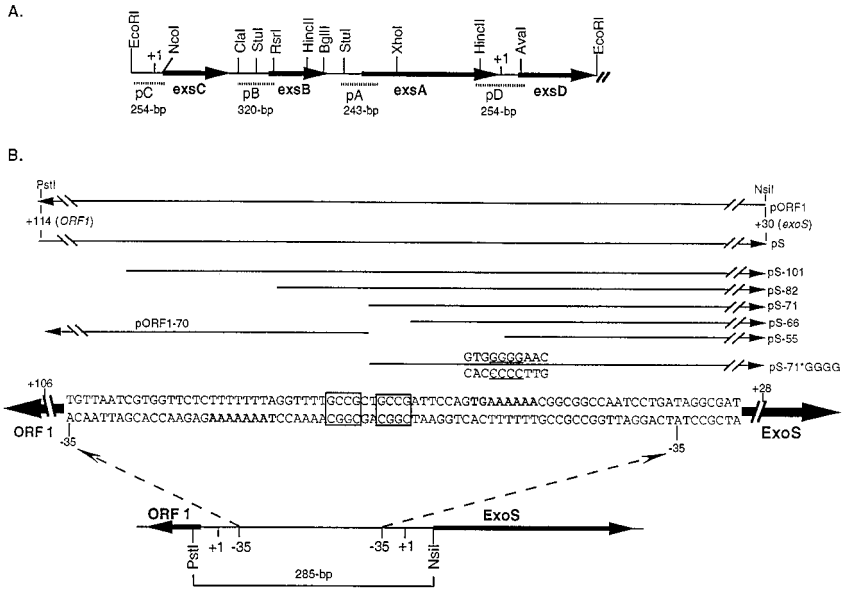

FIG. 1. DNA fragments used in DNA-binding and promoter fusion assays. (A) *trans*-regulatory locus for exoenzyme S. Vertically striped bars below the map show the PCR fragments used for analysis and their sizes. (B) The exoenzyme S locus and promoter regions. All constructs, both restriction fragment and PCR products, are shown as arrows above the detailed nucleotide map of the intergenic region. Below the detailed map is an overview of the *exoS* locus. Bold type indicates the ExsA consensus elements, GCCG boxes are enclosed in a box, the altered sites of the pS-71*GGGG are underlined, and numbers above or below each map are nucleotide positions with respect to the transcriptional start sites of *ORF1* and *exoS*.

ity as that of recombinant ExsA produced in *E. coli* cellular extracts. After treatment with factor Xa, MALA3A2 failed to bind the *trans*-regulatory locus probes in gel mobility shift assays (data not shown). Since factor Xa treatment of MALA3A2 resulted in the apparent degradation of ExsA, we speculate that factor Xa may inactivate the DNA-binding activity of the cleaved ExsA product, as well as MALA3A2, by deleting carboxy-terminal amino acids required for DNA binding.

DNase I footprinting analyses. MALA3A2 was shown to interact directly with the pC and pD promoter regions in this study and the pS promoter fragment (containing pORF1 and pS promoters) in other studies (54). Taken together, these results indicated that a common ExsA binding site should reside within all four promoter regions. To determine the extent of the ExsA protected region, both sense (5*) and antisense (3*) strands of the pC and pD PCR products were labeled at one end. MALA3A2 was incubated with labeled fragments, and reaction mixtures were subjected to DNase I digestion. The products were analyzed by denaturing PAGE which included a chemical cleavage reaction to identify protected DNA sequences.

Patterns of strand-specific protection and/or enhanced susceptibility to DNase I digestion were identified for each tested promoter region (Fig. 4). There was a significant overlap between the protected regions on each strand, indicating that MALA3A2 bound each region at a specific sequence. These protected regions are summarized together with the data from the pS footprinted regions (54) in Fig. 5.

Analysis of MALA3A2 protected sequences. Comparison of the DNase I-protected regions of pC, pD, and pS indicated that at least two core consensus sequences were present in each promoter (Fig. 5A). One sequence, TXAAAAXA, was found at bp −51 or −52 relative to transcriptional start sites mapped for pD (Fig. 5; pD_1) and the divergent promoters of the pS region (Fig. 5; pS_1 and $pORF1_1$). Additionally, most regions contained a 4-bp GCCG box located 3 to 7 bp upstream of the ExsA consensus sequence. A different configuration of consensus sequences was found in the pC promoter. Three ExsA consensus sequences (pC_1, pC_2, and pC_3; Fig. 5) were located downstream of the published transcriptional start site (53). Reexamination of the pC nucleotide sequences located a second set of predicted −35 and −10 RNA polymerase binding sites. These secondary sites would correspond to the appropriate spacing of the upstream ExsA consensus element (pC_1) relative to the −35 position from the transcriptional start site (15- or 16-nt separation) observed in pS, pORF1, and pD. This analysis suggests that pC may contain more than one promoter.

DNA binding and promoter fusion analyses of separate con-

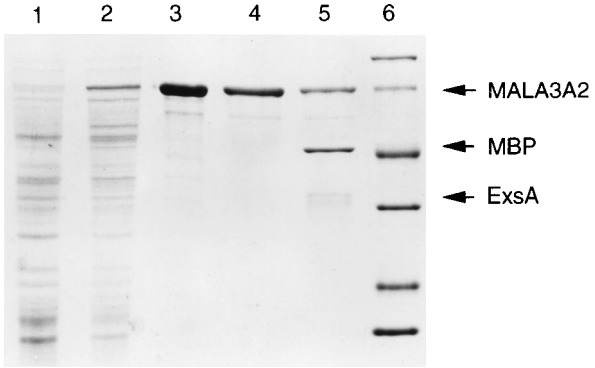

FIG. 2. Induction and purification of the ExsA fusion protein, MALA3A2. SDS-PAGE (11% polyacrylamide) was used to analyze purification and cleavage of MALA3A2. Lane 1, uninduced pMALA3A2 culture (2×10^6 cells); lane 2, induced pMALA3A2 culture (2×10^6 cells); lanes 3 and 4, approximately 1 μg of MALA3A2 from amylose column eluate and DEAE-Sephacel eluate, respectively; lane 5, 1 μg of factor Xa-cleaved MALA3A2 (reaction conditions as follows: 1% factor Xa per microgram of MALA3A2, 22°C, 2 h); lane 6, marker molecular mass proteins: 14.4, 21.5, 31.0, 42.7, 66.2, and 97.4 kDa. MBP, maltose-binding protein.

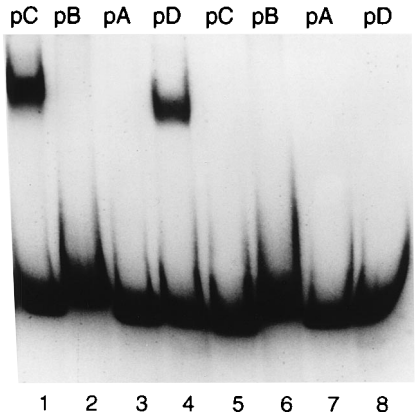

FIG. 3. Gel mobility shift analysis of the *trans*-regulatory locus promoters using the MALA3A2 fusion protein. DNA-binding reactions were performed using 5 fmol of end-labeled promoter fragment with a 500-fold excess of purified MALA3A2. DNA binding was analyzed by PAGE and autoradiography. Lanes 1 to 4 contain promoter probes, pC, pB, pA, and pD, respectively, which had been incubated with MALA3A2; lanes 5 to 8 contain the same sequence of promoter fragments which were incubated with a 500-fold excess of MalE protein purified from the vector, pMAL-c2.

sensus elements. By DNase I footprint analysis and nucleotide sequence comparison, a common consensus ExsA-binding sequence (TXAAAAXA) was identified in four coordinately regulated promoter regions. However, the configurations of the sequences appeared to differ when promoters were compared (Fig. 5B). The pC promoter region had three consensus sequences that were located downstream of the mapped transcriptional start site (53). Two sites were in a direct repeat pattern (pC_1 and pC_2) and two shared some of the same nucleotides (pC_1 and pC_3) (Fig. 5B). The pD promoter possessed two consensus sequences that were arranged in an inverted fashion. The pS promoter region possessed two divergent consensus sequences. To determine the minimal number of consensus sequences required for DNA binding, the divergent consensus sequences present in the pS promoter region were subcloned. The pORF1-70 promoter begins at position −70 relative to the transcriptional start site of pORF1 and the pS-71 promoter begins at position −71 relative to the transcriptional start site of pS (Fig. 1B). Both of these promoters contain one ExsA consensus element along with a conserved GCCG box (Fig. 1B). Analysis of DNA binding by gel mobility shift assays indicated that the pS region (containing both consensus sequences), pS-71, and pORF1-70 were bound by MALA3A2 (Fig. 6A).

To determine whether one consensus site was sufficient for transcriptional initiation, pS-71 and pORF1-70 were subcloned into the CAT reporter vector pQF26 (12). Plasmid constructs were transformed into wild-type (PAK) and into an *exsA* mutant strain (PAK*exsA*::Ω), and CAT activity was compared between constructs containing one or two consensus sequences (Fig. 7). pORF1, pORF1-70, and pS possessed similar levels of CAT activity. pS-71, however, demonstrated approximately half the activity of the full-length segment, pS. These data indicate that a single consensus sequence is sufficient for transcriptional initiation. The same clones in a wild-type host strain, PAK, grown under noninducing conditions (minus nitrilotriacetic acid) or in host strain PAK*exsA*::Ω grown under inducing conditions produced no CAT activity (data not shown). A single consensus sequence appeared to be the only requirement for maximal transcriptional initiation of pORF1-70. Additional upstream sequences may be required for maximal transcriptional initiation of pS-71.

Fine structural analysis of pS. To determine the minimal promoter region required for *exoS* transcriptional initiation, a series of pQF26 reporter subclones (Fig. 1B) were designed to include the following: (i) both consensus sequences and flanking DNA (pS-101), (ii) one consensus sequence and two GCCG boxes (pS-82), (iii) one consensus sequence and one GCCG box (pS-71), (iv) one consensus sequence (pS-66), and (v) part of the consensus sequence (pS-55) (Fig. 1B). CAT activity was measured in PAK transformants. pS-101, pS-82, and pS-71 demonstrated similar CAT activities, indicating that the addition of a second consensus site or GCCG box did not improve transcriptional initiation (Fig. 8). pS-66, which contained only a single consensus site and no GCCG boxes, appeared to have a slightly enhanced ability to initiate transcription (Fig. 8). pS-55 demonstrated CAT activity levels that were indistinguishable from negative-control values (pQF26) (Fig. 8). All *P. aeruginosa* PAK*exsA*::Ω transformants demonstrated CAT activity levels indistinguishable from those of pQF26 controls (data not shown). These data indicated that the core DNA sequence was important for transcriptional activation in vivo by native ExsA.

MALA3A2 was used in DNA-binding analyses to determine if DNA binding in vitro correlated with transcriptional activation in vivo. Gel mobility shift experiments, shown in Fig. 6B, indicated that MALA3A2 bound the pS-101, pS-82, and pS-71 promoters with the same relative affinities (Fig. 6B; Table 2). MALA3A2 bound pS-66 with a lower relative affinity than

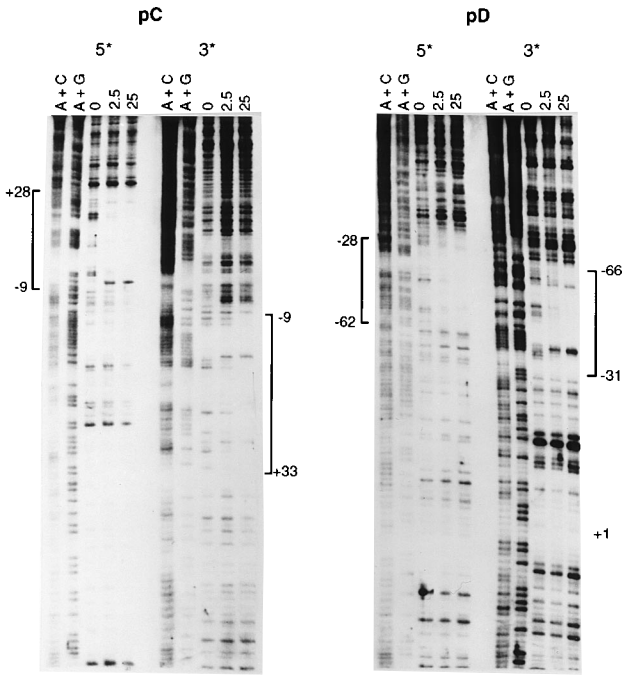

FIG. 4. Footprint analysis of the pC and pD promoter regions. pC and pD were single-end-labeled promoter fragments synthesized by PCR with one radiolabeled primer and one unlabeled primer. The probes were incubated with MALA3A2 (picomoles indicated at the top of each lane), subjected to DNase I cleavage, and electrophoresed on a denaturing 8 M urea–6.6% polyacrylamide sequencing gel together with chemical sequencing reactions (30). The gel was fixed, dried, and exposed to autoradiographic film for analysis. 5* and 3* refer to the ^{32}P end-labeled portion of the promoter fragment. Brackets indicate nucleotide positions relative to the published transcriptional start sites, which are sites of DNase I protection by MALA3A2. A+C and A+G refer to the chemical sequence to give cleavage at those particular residues for the fragments listed.

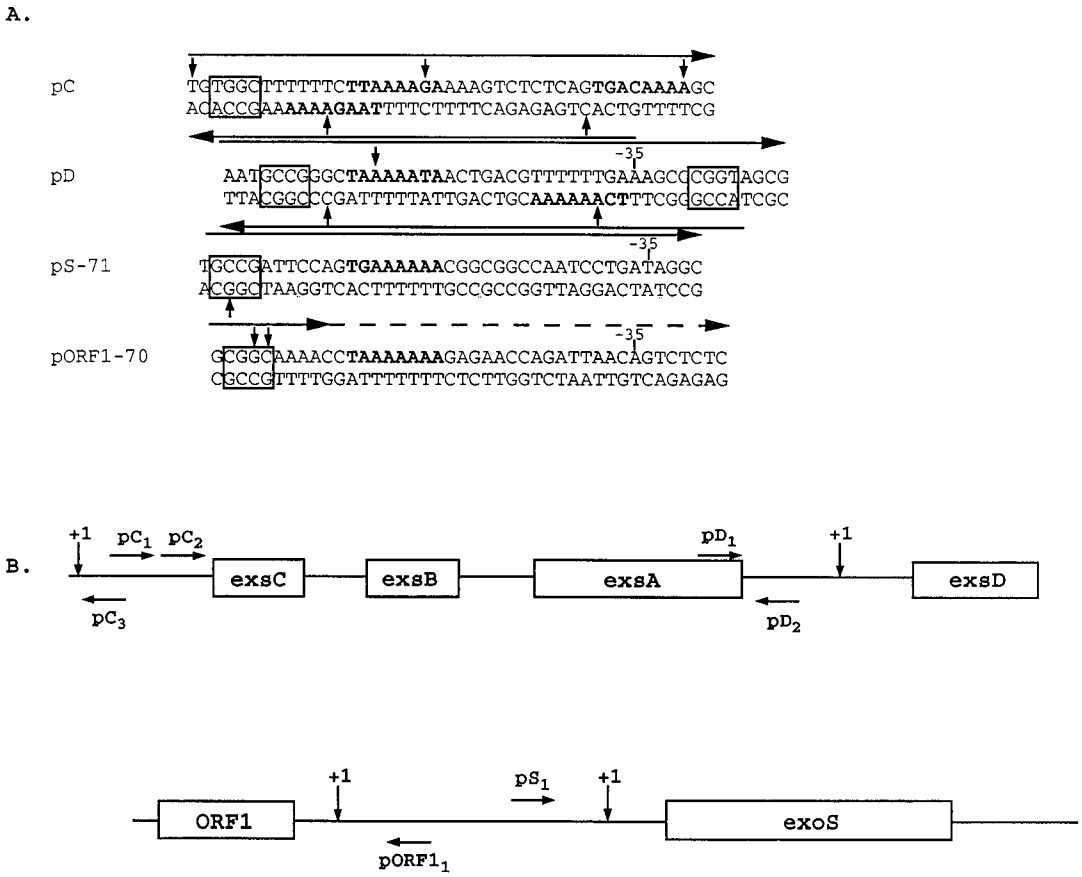

FIG. 5. Summary of the MALA3A2 footprinted regions and the ExsA consensus element. (A) DNase I footprint summary for pC, pD, and pS (54). The pS promoter region was footprinted as the entire *Pst*I-*Nsi*I fragment but is represented as separate promoters in this article. The −35 sites as determined from transcriptional start site mapping (53, 54) are shown. Strong protection is indicated by solid horizontal arrows, and weak protection is indicated by the broken arrow. The ExsA consensus element is in bold print. Nucleotides with enhanced DNase I cleavage (vertical arrows) and GCCG boxes (boxed areas) are shown. (B) Schematic of the *trans*-regulatory and *exoS* loci showing the position and orientation of the ExsA consensus elements. Mapped transcriptional start sites are shown as vertical arrows; the positions and orientations of the ExsA consensus element (TXAAAAXA) are shown as horizontal arrows.

those of the larger promoter regions (Fig. 6B; Table 2). pS-55 bound with the weakest affinity of all the promoter fragments (Fig. 6B; Table 2). The weak binding by pS-55 was unexpected but may indicate that sequences on the 3′ end of the consensus sequence contribute to protein-DNA interactions. All of the pS fragments which showed significant shift activity displayed a multiple band pattern indicative of either multiple binding sites on each fragment or the binding of various oligomers to single sites or both. Because the multiple band pattern varied from batch to batch of purified MALA3A2, we favor the hypothesis that MALA3A2 may exist as various oligomers. This may account for the requirement of large amounts of MALA3A2 relative to DNA for the detection of gel mobility shifts, since only a fraction of the protein preparation may be active.

Site-specific mutagenesis of the core consensus sequence. Our data indicated that the full core consensus sequence (pS-82, pS-71, and pS-66) was required for transcriptional initiation but that the partial core (pS-55) sequence still bound MALA3A2 with reduced affinity. To determine if the four internal deoxyadenosine residues were important for either DNA binding or transcriptional initiation, pS-71 was subjected to site-specific mutagenesis. In this experiment, the internal deoxyadenosine nucleotides were changed to deoxyguanosine residues. DNA gel mobility shift experiments indicated that MALA3A2 failed to bind pS-71*GGGG (Fig. 9). CAT activity was not significantly different than negative-control values or pS-55 (Fig. 8). These data indicated that the internal deoxyadenosine residues of the ExsA consensus element were critical for both DNA binding and transcriptional initiation mediated by ExsA.

DISCUSSION

Several pieces of evidence are consistent with the notion that the ExoS regulon of *P. aeruginosa* is controlled by a key transcriptional activator protein, ExsA, whose activity is mediated by DNA binding. In promoter fusion analysis, a functional copy of *exsA* was required for the transcriptional initiation of several transcriptional units. The individual units include a regulatory operon consisting of *exsC*, *exsB*, and *exsA*, a postulated secretory operon beginning with *exsD*, a divergent promoter controlling the structural gene, *exoS*, and a postulated upstream operon containing several predicted open reading frames, including a homolog (*ORF1*) to the YopE chaperone, SycE (51, 54). In this study, gel mobility shift assays demonstrated that ExsA, as either an expressed recombinant protein or as a purified fusion derivative, MALA3A2, bound to ExsA-dependent promoter regions. DNase I footprinting analysis demonstrated that a common 8-bp core sequence was associ-

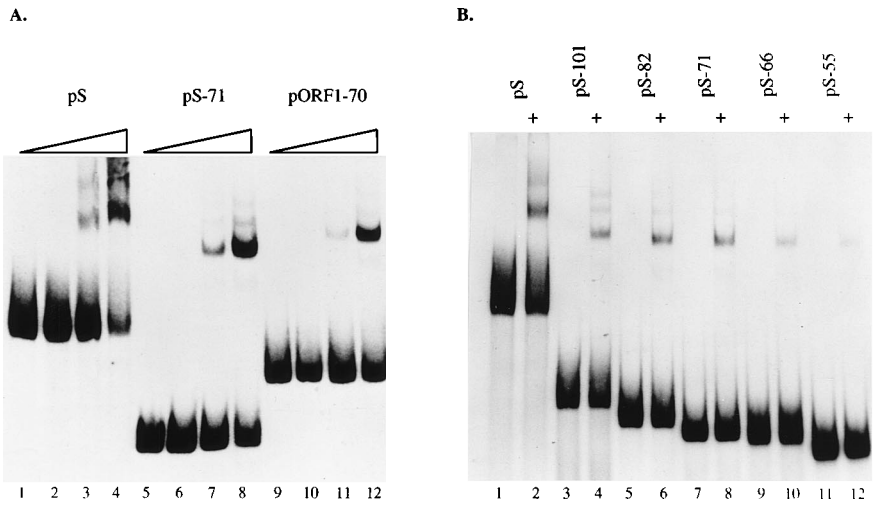

FIG. 6. Gel mobility shift analysis of MALA3A2 to pS derivatives. DNA-binding reactions were carried out as described in Materials and Methods, with 5 fmol of end-labeled DNA and various concentrations of MALA3A2. After incubation at 37°C for 15 min, DNA loading buffer was added, samples were electrophoresed on 5% PAG (1× TBE) and visualized by autoradiography. (A) Each end-labeled DNA fragment was incubated with MALA3A2 at 0×, 250×, 1,000×, and 2,500× (molar fold over DNA) (indicated over the lanes by the triangles). (B) Labeled promoter fragments were incubated in gel mobility shift assays with either MalE (1,000-fold excess over DNA amount) in odd-numbered lanes or MALA3A2 (1,000-fold) in even-numbered lanes (+).

ated with DNA fragments bound by MALA3A2. Truncation and mutation of a portion of this ExsA consensus element in the *exoS* promoter indicated that this region was critical for DNA binding and transcriptional initiation. Together these data suggest that the coordinate expression of regulatory, proposed secretory, and structural genes governing exoenzyme S production was mediated by the DNA-binding and transcriptional activation properties of ExsA upon a specific promoter regulatory element.

Although the data are consistent with a model in which ExsA synthesis correlates with the activation of several sets of genes, other regulatory mechanisms may be controlling the output of each operon. Examination of promoter fusion constructs indicated that reporter activity was approximately equal when pC and the divergent promoters of the structural gene locus (pS and pORF1) were compared (53, 54). Yet mRNA was undetectable from the regulatory operon while messages for *exoS* (controlled by pS) and *ORF1* (controlled by pORF1) appear to be stable enough for detection by Northern (RNA) blot analysis (53, 54). pD is postulated to promote the transcription of *exsD* and other downstream genes that may be important for exoenzyme S export (13, 14). Reporter activity of a pD promoter fusion was four to fivefold higher than for pC, pS, or pORF1, indicating a higher rate of transcriptional initiation (53, 54). In preliminary studies, mRNA of the downstream *exsD* locus appeared unstable (53a). Stability of mRNAs may be one mechanism utilized by *P. aeruginosa* to control the amount of products produced from the coordinately regulated loci involved in exoenzyme S production. The message for one of the ExsA homologs, LcrF, also appears to be undetectable and has been postulated to be labile to degradation by RNase E (20).

Mapping the DNA regions protected by MALA3A2 binding allowed a finer examination and comparison of promoter structure which showed similarities to the structure seen for the *E. coli* AraC inducer binding sites (28). In the presence of its inducer, arabinose, AraC binds to two closely spaced half-sites located between bp −35 and −72 relative to the transcriptional start site of the P_{BAD} promoter (28). At least two ExsA consensus elements are present in each ExsA-controlled promoter site analyzed. This arrangement of multiple binding sites is a common feature in a variety of prokaryotic transcriptional modulating proteins (23) including AraC (28). The arrangement of each set of ExsA consensus elements differs, however. The pC promoter has both a direct repeat of elements 12 bp apart (pC_1 and pC_2), as well as a divergently oriented arrangement of consensus sites separated by 17 bp (pC_2 and pC_3), with the pC_2 and pC_3 elements being imperfect matches to the consensus (1-nt mismatch; Fig. 5). The pD promoter contains an inverted repeat of sites, 7 bp apart. The pS and pORF1 promoters each contain the same set of divergently oriented consensus sequences located 23 bp apart. Despite these differences, one identically oriented ExsA consensus element is found at bp −51 or −52 with respect to the transcriptional start sites of pD, pS, and pORF1. This common location is similar to the specific orientation and distance requirements for the AraC consensus binding sites (38).

The pC promoter showed a significant difference from the other promoter regions. In pC, all consensus elements were mapped to regions that followed the transcriptional start site (pC_1 at position +12 relative to transcriptional start, pC_2 at +32, and pC_3 at +7; Fig. 5). These data suggest the potential for a complex transcriptional regulation system. This complexity may be exhibited both in the variation and the manner in which ExsA can bind this region. Reexamination of the nucleotide sequence of pC indicated that secondary predicted −35 and −10 hexamers (31) could be aligned to the pC_1 ExsA consensus to fit the proposed spacing requirements for transcriptional activation. Alternatively, the ExsA consensus elements may serve to repress transcription of the *trans*-regulatory locus under certain circumstances. This mechanism may limit the amount of ExsA mRNA when ExsA cellular concentrations increase. Further analysis of the pC promoter will be required to determine which hypothesis is correct and to what degree each consensus site contributes to the activity of this promoter or promoters.

The most active promoter, pD (53), has an arrangement of consensus sites that resemble an inverted repeat orientation of half-sites (pD_1 and pD_2). The inverted repeat half-site is observed in other prokaryotic DNA-binding protein binding sites

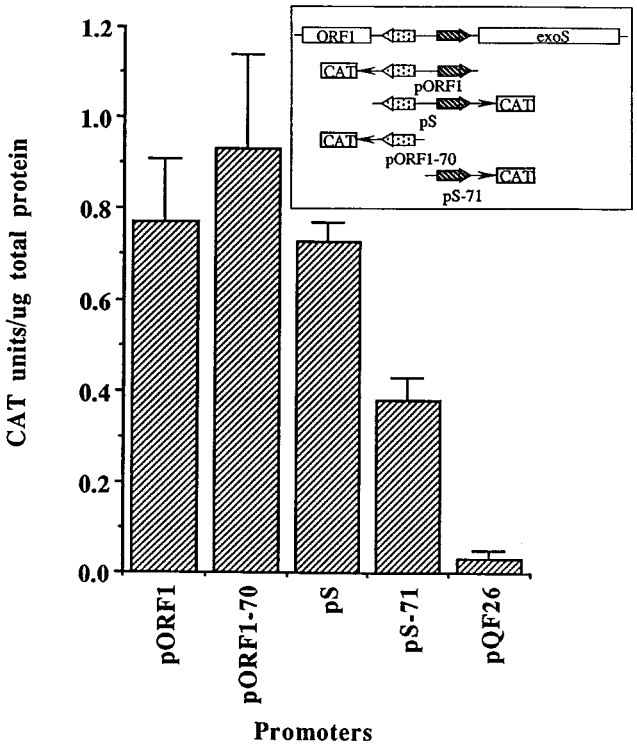

FIG. 7. CAT activity of pS promoter region containing a single ExsA consensus element. CAT activity was measured in pQF26 clones containing both consensus elements (pORF1 and pS) or single consensus elements (pORF1-70 or pS-71) as shown in the inset schematic. Different arrows (dotted or stippled) within the inset figure represent the location and orientation of ExsA consensus sequences. CAT refers to the location of the CAT gene relative to each consensus sequence. Error bars represent the standard deviation. Data from four separate experiments are shown.

(19). These sites are located 7 bp apart in an inverted arrangement with an additional 6-bp sequence, CCGGGC, located near the 5′ end of each consensus site. Of the ExsA-regulated promoters examined to date, pD most closely corresponds to the close arrangement of half-sites found within AraC as direct repeat elements and proposed for VirF-controlled promoters as direct and inverted repeats (5, 52).

A more detailed analysis of the pS promoter region was performed to determine whether both ExsA consensus elements were required for DNA-binding and transcriptional activity and to establish the minimal promoter size required for DNA binding and transcriptional induction by ExsA. CAT fusions of the pS, pORF1, pORF1-70, demonstrated equal

TABLE 2. DNA binding of MALA3A2 to *exoS* promoter fragments

Promoter	% DNA shifted[a]
pS	21.5 ± 5.4
pS-101	11.4 ± 3.6
pS-82	8.7 ± 2.1
pS-71	12.3 ± 2.4
pS-66	2.8 ± 0.8
pS-55	0.4 ± 0.2
pS-71*GGGG	ND[b]

[a] DNA demonstrating a mobility shift was quantitated by radioanalytic imaging. The percentage of DNA altered in mobility was based on the total cpm per lane. Each value is the mean of four trials ± standard deviation.

[b] ND, not detectable.

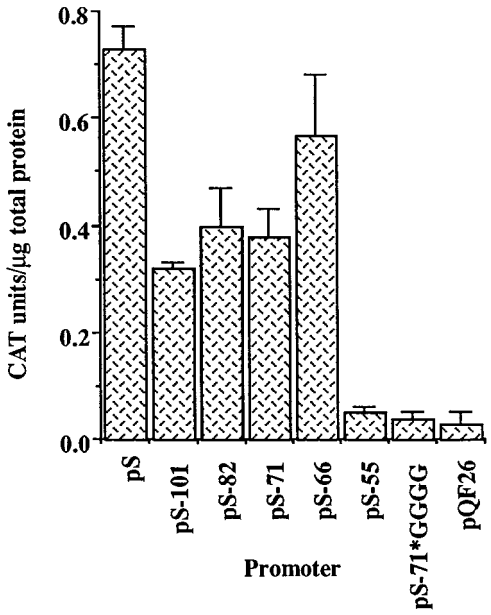

FIG. 8. CAT activity of pS promoter regions containing various combinations of sequence elements (see text). Data from Fig. 7 (pS, pS-71, and pQF26) are reproduced for comparative purposes. PS-71*GGGG refers to the CAT activity of a site-specific mutation replacing the internal four deoxyadenosine residues with deoxyguanosine nucleotides.

enzyme activity, indicating the requirement of only one ExsA consensus element for full activity. A difference was noted in the *exoS* promoter, however. The activity of *exoS* promoters initiating from −101 to −71 relative to the *exoS* transcriptional start site were equal to one another but had approximately half the promoter and DNA-binding activity of the full-length pS region. This result could be linked to the influence of an

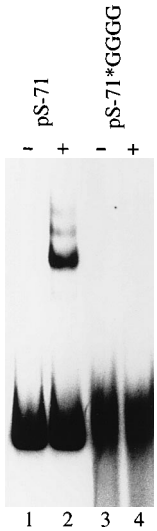

FIG. 9. Gel mobility shift analysis of site-directed mutation of ExsA consensus element of pS. Both the wild-type pS-71 and mutant pS-71*GGGG were isolated as *Sst*I-*Hin*dIII restriction fragments from the pQF26 fusion clones. End-labeling and gel mobility shift assays were performed as described in Materials and Methods. Lanes 1 and 2, pS-71 (restriction fragment) incubated with no (−) or added (1,000× molar concentration greater than the DNA fragment concentration [+]) MALA3A2; lanes 3 and 4, pS-71*GGGG (restriction fragment) incubated with no (−) or 1,000× (+) MALA3A2.

FIG. 10. Comparison of the ExsA consensus element to VirF-protected *Yersinia* promoter regions. Segments of the sense strands of VirF-protected *Yersinia* promoter regions are shown. The −35 sites and the −52 and −53 sites are determined from published transcriptional start information (52). ExsA consensus-like elements (in bold print) were located within each promoter. The 8-bp ExsA consensus of *P. aeruginosa* is shown below the *Yersinia* promoters. *yopH* contains a perfect consensus sequence, and *virC* and *lcrG* sequences differ by one nucleotide. The region with the weakest homology to the ExsA consensus element (2-bp mismatch) is found in the *yopE* promoter which was also a weakly footprinted region (compared with strong footprint regions for the other three).

upstream element, such as found with the upstream cyclic AMP receptor protein binding site of the P_{BAD} operon under the control of AraC (11). The apparent increase of promoter activity under the pS-66 promoter with the concomitant decrease of DNA binding appeared to be linked directly to the GCCG box located between −67 and −70 of pS. The elimination of this GCCG box and the proximity of the consensus site to the end of the DNA in DNA-binding assays and to the vector sequence in promoter fusion assays may influence these activities. The exact role that this region plays, however, is not clear.

The loss of promoter and DNA-binding activities of pS-55, which eliminated the 5′ 4 bp of the 8-bp consensus region (pS_1), and by pS-71*GGGG, which contained a mutation replacing the internal four-deoxyadenosine region with four deoxyguanosines, indicated that the ExsA consensus element was the critical region for ExsA activity. In the divergent pS promoter region, only the proximal (to transcribed gene) consensus element was required for transcriptional activation by ExsA. This result indicates that it does not need to bind both sites ($pORF1_1$ and pS_1) for activity of either promoter.

Analysis of sequences from VirF-controlled *Yersinia* promoters indicate that an ExsA-like consensus element may be present. VirF and ExsA share almost identical carboxy-terminal amino acid sequences containing postulated DNA-binding helix-turn-helix motifs (92.5% identity in an 80-amino-acid carboxy terminal region containing multiple helix-turn-helix motifs) (13, 16). We examined the alignment of VirF-protected regions for the presence of an ExsA consensus element (Fig. 10) (52). Although Wattiau and Cornelis (52) indicate the presence of degenerate half sites in these promoters, an identical ExsA consensus sequence was identified in the *yopH* promoter region with similar sequences appearing in the *virC*, *lcrG*, and *yopE* promoters at bp −52 or −53 relative to their transcriptional start sites. The weakest of these footprinted regions was found in the *yopE* promoter (52) which also contained the weakest homology to the ExsA consensus element. If our analysis is correct, we would predict that ExsA may be able to complement VirF mutations or vice versa.

In conclusion, the binding of ExsA to the promoter regions of genes of the exoenzyme S regulon suggested that this protein directly interacted with DNA containing consensus core sequences of TXAAAAXA. Analysis of the pS promoter region indicated that ExsA may not require binding to half-sites as defined for the ExsA homologs, AraC, and proposed for VirF. ExsA appeared to bind to a defined consensus sequence that was located at position −51 or −52 relative to the transcriptional start sites of promoters of the exoenzyme S regulon.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health-National Institute of Allergy and Infectious Diseases grants R29-AI31665 and K04-AI01289.

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