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AlgR, a Response Regulator Controlling Mucoidy in *Pseudomonas aeruginosa*, Binds to the FUS Sites of the *algD* Promoter Located Unusually Far Upstream from the mRNA Start Site

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Strong transcriptional activation of *algD*, a key event in the overproduction of alginate and establishment of mucoidy in *Pseudomonas aeruginosa*, depends on the functional *algR* gene. The predicted gene product of *algR* shows homologies to response regulators from bacterial signal transduction systems. The *algR* gene was overexpressed in *Escherichia coli*, its product (AlgR) was purified by utilizing its apparent affinity for heparin, and its sequence was verified by partial amino acid sequence analysis. AlgR was found to interact directly with the *algD* promoter. Deletion mapping analysis, in conjunction with mobility shift DNA-binding assays, indicated the presence of three regions within the *algD* promoter capable of specifically binding AlgR. A relatively weak interaction was observed with the *algD* promoter fragment containing the region immediately upstream of the *algD* mRNA start site (−144 to +11). However, when fragments spanning regions located very far upstream from the *algD* mRNA initiation site (−533 and −332) were used, strong specific binding was observed. These regions were separated by a DNA segment not binding AlgR and spanning positions −332 to −144. DNase I footprinting analysis further established the presence of discrete AlgR binding sites overlapping with FUS, the far-upstream sites required for full induction of *algD* transcription and its environmental modulation. There were two distinct binding sites: RB1, spanning nucleotides −479 to −457, and RB2, spanning nucleotides −400 to −380. Both of these sequences shared a highly conserved core region, ACCGTTCGTC. These results established a direct interaction of AlgR with the *algD* promoter and revealed an arrangement of binding sites highly unusual for response regulators of the AlgR type.

Pseudomonas aeruginosa has a genetic potential to synthesize the exopolysaccharide alginate (24). In most ecological niches, alginate production is either very low or strongly suppressed (3, 40). *P. aeruginosa* strains that synthesize and secrete excessive amounts of alginate are frequently isolated from the lungs of patients with cystic fibrosis (CF) (21, 24). Overproduction of alginate results in development of a mucoid capsulelike coating, a virulence determinant of particular importance for the course of chronic respiratory infections in CF (26). Mucoidy plays a critical role in the unusually high resistance of *P. aeruginosa* to natural defense and clearance mechanisms and therapeutic control in CF (24).

Alginate overproduction is contingent upon strong transcriptional activation of *algD*, the gene encoding GDPmannose dehydrogenase (14). The *algD* gene heads the cluster of alginate-biosynthetic genes (11, 14). Regulation of the *algD* promoter has taken center stage in studies of transcriptional control of mucoidy and may hold the key to the understanding of the unusual adaptation phenomena underlying the emergence of mucoid strains in CF. There are several regulatory elements proposed to converge upon the *algD* promoter: the *algR* (13) and *algB* (49) genes encoding putative proteins of the response regulator type from the superfamily of bacterial signal transduction systems (43); the *algP* and *algQ* genes (17–19, 33) (also termed *algR3* and *algR2*, respectively [31]), with the former encoding an unusual polypeptide whose carboxy-terminal domain resembles the tails of eukaryotic H1 histones (18); and the *muc* (23, 34) and *algST* genes (22), with unknown function. The initial finding of the nature of the putative *algR* gene product as an element capable of transducing environmental signals into transcriptional activation events has incited analysis of the environmental modulation of *algD* (6, 16, 37). Although many factors, such as nitrogen source (16), osmolarity (6, 16), general nutrient limitation (47), and oxygen tension (5), etc., have been proposed as conditions affecting alginate synthesis, there is no consensus of opinion regarding the signals and mechanisms mediating environmental modulation of mucoidy (20). In addition, involvement of numerous regulatory elements poses the question of which, if any, of the known factors directly interact with the *algD* promoter.

To answer these questions and dissect the mechanisms of the environmental modulation of mucoidy via factors controlling the *algD* promoter, we have recently initiated a systematic analysis of the regulation of *algD* transcription, which can be summarized as follows. (i) High-level transcription of *algD* has been found to be absolutely dependent on the functional *algR* gene (36); (ii) modulation in response to two factors, nitrogen source and osmolarity, can be separated by insertional mutations in *algR* (37) and in a region downstream of *algR* (36), indicating that transmission of these signals occurs via independent pathways (which, nevertheless, require *algR* for maximum-level transcription of *algD*); and (iii) deletion analysis of the *algD* promoter has indicated a requirement for the far-upstream sites (FUS) for high-level expression of *algD* (37). To determine whether the gene product of *algR* acts directly on *algD*, AlgR was overproduced and purified in this work. This protein was used in DNA-binding studies, and its direct interaction with the *algD* promoter and FUS was established. The results presented here indicate a peculiar arrangement of the AlgR binding sites which may provide a basis for additional

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regulatory networking of factors governing the complex expression of mucoidy in *P. aeruginosa*.

MATERIALS AND METHODS

Growth conditions, bacterial strains, and DNA constructs. The medium used for all growths was LB, supplemented with 40 μg of ampicillin per ml and 30 μg of chloramphenicol per ml (for plasmids) or 5 g of NaCl per liter (for M13) when required. Bacteria were grown at 37°C.

All protein expression studies were performed with *Escherichia coli* BL21(DE3) harboring pLysS (44). This bacterial strain contains chromosomally encoded T7 RNA polymerase controlled by the inducible *lacUV5* promoter. The plasmid pLysS has the 3.5 T7 gene encoding T7 lysozyme cloned in pACYC184 (p15A Cm^r). This plasmid expresses moderate amounts of T7 lysozyme, which binds T7 RNA polymerase and inhibits its noninduced levels in BL21(DE3). This increases the tolerance for plasmids containing genes to be expressed from the T7 ϕ10 promoter. Use of this strain was a critical factor in stabilizing *algR* expression clone pCMR7.

The construct used for overexpression of *algR* (plasmid pCRM7) was produced by cloning the *algR* gene in M13 bacteriophage mp18 and deleting (10) the sequences upstream of the predicted *algR* coding region to position −52 relative to the initiation codon (13). The conveniently located *Sma*I site 24 bp downstream of the stop codon (unpublished results) was used to excise *algR*. This site was converted to *Bam*HI by using linkers, and the fragment was excised as *Hin*dIII-*Bam*HI (the *Hin*dIII site being introduced at the 5′ end by the deletion cloning procedure [10]). This procedure generated an 826-bp fragment containing the *algR* coding region, which was inserted into *Hin*dIII- and *Bam*HI-digested T7 expression vector pT7-6 (ColE1 Ap^r) (46), thereby placing the *algR* gene under control of the ϕ10T7 promoter.

All deletion clones of the *algD* promoter have been previously described and used in transcriptional-fusion studies (37). For generation of uniformly labeled probes used in mobility shift DNA-binding assays, the original M13 deletion clones were employed (37). For generation of competitor DNA used in binding reactions, the *Hin*dIII-*Eco*RI fragments containing *algD* promoter sequences were subcloned in pUC12 (48).

DNA manipulations and determination of nucleic acid and protein concentrations. All recombinant DNA work was done by the standard protocols (4). DNA concentration was determined spectrophotometrically by measuring optical density at 260 nm and verifying these results on agarose gels stained with ethidium bromide. All plasmids used in competition studies were purified twice through CsCl gradients and displayed identical distributions of physical forms on agarose gels. Protein concentration was determined by the method of Bradford (7).

AlgR overproduction and purification. Freshly transformed BL21(DE3)LysS cells harboring pCMR7 were inoculated in 500 ml of LB supplemented with antibiotics and grown to an A_{600} of 0.25 to 0.35. For induction, isopropyl-β-D-thiogalactopyranoside was added to 1 mM and the cells were grown for another 4 h. The cells were harvested, washed in saline, suspended in the lysis buffer (20 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg of pancreatic DNase I [Sigma Chemical Co.] per ml), and freeze-thawed three times by alternating cycles of short incubations on dry ice and in a water bath at 67°C. All following steps were performed at +4°C. The cells were sonicated four times for 45 s each time and centrifuged at 10,000 rpm in an SM24 rotor for 10 min, and the supernatant was centrifuged in a Ti50 rotor for 1 h at 100,000 × *g*. The supernatant was removed, and 10 mg of the total protein was loaded onto a 6-ml heparin–Sepharose CL-6B bed packed in an XK16/20 column and equilibrated with 20 mM Tris-HCl (pH 7.5)–1 mM phenylmethylsulfonyl fluoride (buffer A). A Pharmacia-LKB fast protein liquid chromatography system was used to run a linear salt gradient from 0 to 1 M NaCl made in buffer A. This was followed by elution with 3 ml of 1 M NaCl in buffer A. The 1 M NaCl fractions contained most of the AlgR protein. The fractions containing AlgR were pooled and concentrated by centrifugation in Centricon 10 microconcentrator tubes (Amicon), the buffer was exchanged for storage buffer (20 mM Tris-HCl [pH 7.5], 100 mM NaCl, 5 mM $MgCl_2$, 20% glycerol), and samples were stored at −20°C. When the proteins were chromatographed by ion exchange on MonoQ HR 5/5, a linear gradient of 0.01 to 0.6 M NaCl in buffer A was used.

Protein gel electrophoresis, microsequencing, and cyanogen bromide cleavage. Proteins were separated on sodium dodecyl sulfate–12% polyacrylamide gels and stained with Coomassie blue or electroblotted onto an Immobilon membrane and stained with Ponceau S. The purified protein was characterized as AlgR by microsequencing of protein electroblotted onto polyvinylidene difluoride membrane (35) (Immobilon-P; Millipore) and Edman degradation chemistry in a 477A/120A Protein/Peptide Sequencer (Applied Biosystems). For cleavage with cyanogen bromide, the immobilized protein was digested by overnight incubation of the filter-bound protein with 5 mg of cyanogen bromide in 25 μl of 70% formic acid in a sealed microcentrifuge tube kept in the dark.

Mobility shift DNA-binding assay. The DNA probes used for mobility shift assay were prepared by uniformly labeling deletion clones (37) of the *algD* promoter in M13mp18 with [α-^{32}P]dCTP (800 Ci/mmol; DuPont NEN) with the 17-bp universal primer and the Klenow DNA polymerase I fragment. The radiolabeled double-stranded fragments were obtained by digestion of the reaction products with appropriate enzymes, separation on a 5% polyacrylamide gel, and electroelution. The standard binding reaction with AlgR contained 80 pg of radiolabeled probe and 90 ng of AlgR in a 10-μl total volume of binding buffer (25 mM Tris-HCl [pH 8.0], 6 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 20 mM KCl, 5% glycerol, 10 μg of native salmon sperm DNA per ml). Incubation was carried out for 10 min at room temperature. Where indicated, an appropriate amount of competitor DNA was added to the reaction mixture. The protein-DNA complexes and free DNA were separated on a 5% native polyacrylamide gel in 6.7 mM Tris-HCl (pH 7.9)–3.3 mM sodium acetate–1 mM EDTA with buffer recirculation. The gels were dried and autoradiographed. The same procedure was used with end-labeled fragments, except that the amount of the competitor DNA was 2.7 μg per standard reaction mixture.

DNase I footprinting analysis. For DNase I footprinting analysis, DNA fragments were digested with the appropriate restriction enzyme and labeled at the 5′ end by phosphorylation with [γ-^{32}P]ATP (6,000 Ci/mmol; DuPont NEN) catalyzed by polynucleotide kinase or at the 3′ end with the Klenow fragment of DNA polymerase I and [α-^{32}P]dCTP (800 Ci/mmol; DuPont NEN). This was followed by digestion with a second restriction endonuclease. The resulting fragments were gel purified and electroeluted. The standard

binding reaction with AlgR was modified by removing $MgCl_2$ from the binding buffer and doubling the reaction volume. This did not reduce the binding activity of AlgR and was not affected by addition of 2.5 mM EDTA to the modified binding reaction (see Fig. 4). After incubation of 3×10^5 cpm of radiolabeled probe with 1.2 μg of AlgR, the concentration of $MgCl_2$ was adjusted to 5 mM and 200 ng of DNase I (Cooper Biomedical, DPFF) was added. After incubation for 1 min at room temperature, the reaction was stopped by adding an equal volume of 1% sodium dodecyl sulfate–100 mM EDTA–200 mMNaCl–2 mg of tRNA per ml, followed by phenol extraction and ethanol precipitation. The extent of the binding reaction and DNase I digestion was monitored by electrophoresing half of each sample on a native polyacrylamide gel. The products of DNase I digestion were separated on sequencing gels, and the boundaries of the binding sites were determined by comparing the footprints with a sequencing ladder generated by using appropriate M13 clones and a custom-made primer matching the beginning of the fragment used for footprint analysis. Because of the presence of an additional run of T residues within the deletion clones, the observed footprint positions determined by comparison with the sequencing ladder had to be corrected by subtracting 9 bp.

Nucleotide sequence accession number. The *algD* promoter nucleotide sequence used for Fig. 5 has been reported previously (37) GenBank accession no. M37205.

RESULTS

Overexpression of *algR*. Initial attempts to overproduce AlgR in *E. coli* were performed with an *algR*-containing DNA fragment spanning coordinates −153 to +775 of the *algR* sequence relative to the presumptive initiation codon (13, 17). This fragment was placed behind the T7 promoter in vector pT7-6. With this construct we could not overexpress *algR* to the extent that its gene product was distinguishable on the background of the total cellular proteins in *E. coli*. To bypass any difficulties because of inclusion of the proximal promoter of *algR* (17), a different deletion at the 5′ end of the insert was created, resulting in a fragment starting at −52 and ending at +775 relative to the *algR* initiation codon. This fragment contained the *algR* open reading frame, the 52 bp 5′ to the initiation codon, and the 28 bp 3′ to the *algR* stop codon. When this insert, cloned in pT7-6 (plasmid pCMR7), was used in conjunction with the expression system, affording high repressibility of the T7 promoter prior to its desired induction (44), high levels of AlgR production were obtained (Fig. 1). Equally important, most of the AlgR was in the soluble state, since the protein was retained in supernatants, even after centrifugation at 100,000 × *g* (Fig. 1). The polypeptide observed in gels upon induction with isopropyl-β-D-thiogalactopyranoside migrated through sodium dodecyl sulfate-polyacrylamide gels with mobility matching that of the previously identified [^{35}S]methionine-labeled *algR* gene product (36).

Purification of AlgR. Extracts containing AlgR were initially chromatographed on a MonoQ column. These preparations of enriched AlgR (25%) were used in DNA-binding studies and displayed the ability to associate specifically with DNA fragments containing *algD* promoter sequences (see below) but not with fragments containing parts of the *argH* structural gene (positions 362 through 603 of the published sequence [36]) and the promoter region of *algQ* (positions −129 through +163 relative to the *algQ* mRNA start site [33]), which displays expression independent of the mucoid or nonmucoid status of the cells (33; data not shown). These studies were performed under conditions in which DNA and other anionic polymers were added to the reaction mixture as competitors of nonspecific and specific associations. While performing these experiments, we observed that one such competitor, heparin, displayed an unusually high effectiveness in reducing the putative AlgR-binding activity. This indicated that AlgR could have a significant affinity for heparin and that perhaps this could be utilized for affinity chromatography.

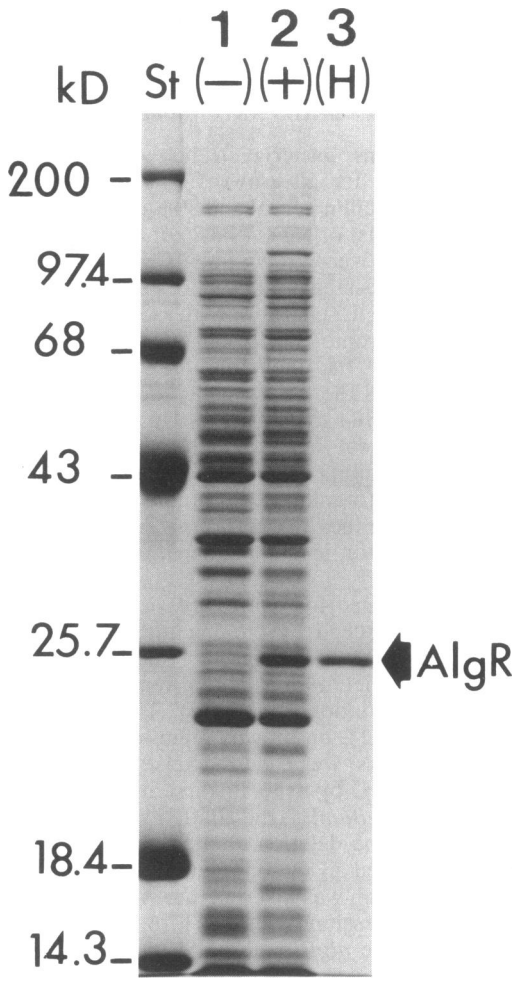

FIG. 1. Overproduction and purification of AlgR. Lanes: 1 and 2, 100,000 × *g* supernatants from extracts of noninduced (−) and induced (+) (with 1 mM isopropyl-β-D-thiogalactopyranoside) *E. coli* BL21(DE3)pLysS harboring *algR* expression clone pCMR7; 3, AlgR purified on heparin-Sepharose (H); St, molecular mass standards. The band visible in lane 2 above the 97.4-kDa standard is T7 RNA polymerase.

To explore this possibility, a heparin-Sepharose column was prepared and the 100,000 × *g* supernatants of extracts with overproduced AlgR were loaded on the column. The proteins were eluted from the column by using a linear gradient of 0 to 1 M NaCl, followed by a half-column volume of 1 M NaCl. This resulted in a high level purification of AlgR (Fig. 1), which eluted when the NaCl reached a concentration of 1 M. Under these conditions, most cellular proteins were eluted to completion from the column prior to elution of AlgR. The purity of AlgR in the pooled fractions

was estimated to be over 90% on the basis of laser densitometry of Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gels (Fig. 1).

The amino acid composition of purified AlgR was determined. The results of this analysis corresponded to the values of amino acid ratios predicted from the AlgR sequence (13) (data not shown). A larger amount of AlgR (approximately 100 pmol) was immobilized on Immobilon-P membrane and digested with cyanogen bromide, and the resulting peptides were sequenced by using eight cycles. The results of this analysis matched the predicted peptide products of cyanogen bromide cleavage based on the translated *algR* sequence (13) at amino acid residues 1 to 9 (Met-Asn-Val-Leu-Ile-Val-Asp-Asp-Glu [a combination of the N-terminal sequence and the first cyanogen bromide fragment]) and 58 to 66 (Pro-Gly-Leu-Asp-Gly-Leu-Gln-Val) and the carboxy-terminal tripeptide 246 to 248 (His-Gln-Leu). These analyses identified the purified protein as AlgR and also confirmed the previously determined *algR* sequence (13).

AlgR binds to the region of the *algD* promoter containing the FUS sites. To determine whether AlgR binds to the *algD* promoter or exerts its action indirectly (e.g., via a cascade [12] or a phosphorelay [9] mechanism), the purified AlgR preparations were used in a mobility shift DNA-binding assay. The *algD* mRNA start site has been mapped (15), and the entire 1,143-bp region upstream from this site has been sequenced (37). Also, a set of overlapping deletions within this region has been produced and their end points have been sequenced (37). The choice of fragment [$F_{(-533/-144)}$ (the numbers in the index denote the sequence coordinates relative to the *algD* mRNA start site)] used in the initial studies (Fig. 2) was meant to incorporate the FUS sites, previously identified as a *cis*-acting region required for full activity of the *algD* promoter (37). As shown in Fig. 2, specific complexes were formed (C1 and C2; Fig. 2) when AlgR was added to the radiolabeled DNA probe. These complexes were resistant to addition of the nonspecific (pUC12) cold competitor DNA to the reaction mixture (Fig. 2, lane 3). When the same amount of the specific competitor DNA (pUC12 with the insert containing the *algD* sequences spanning the region from −533 to the *Eco*RI site at +112 bp relative to the mRNA start site [plasmid pD533]) was added to the binding reaction mixture, the amounts of complex C1 were significantly reduced and the pattern resolved into two components (C1 and C2; Fig. 2, lane 2). This was not the case when the region from −533 to −332 was deleted from the specific competitor DNA (removing the high-affinity binding sites [see below]) (Fig. 2, lane 6). When the amount of the full-size competitor DNA (pD533) was increased another fourfold, complex C1 was almost completely titrated out and most of the probe was in the unbound state (Fig. 2, lane 4). Addition of increased amounts of the nonspecific competitor DNA (Fig. 2, lane 5) also affected the C1 complex but to a much lesser degree than the observed effect of the specific competitor DNA. Even in the presence of four times more pUC12 DNA as the nonspecific competitor (Fig. 2, lane 5), the relative distribution of radiolabel among C1, C2, and the unbound probe was 41, 43.5, and 15.5%, respectively, relative to the sample with 1 μg of the specific competitor DNA, i.e., 25, 45, and 30%, as determined by densitometric analysis (Fig. 2, lane 2). Thus, AlgR appeared to form specific complexes with at least two binding sites within the sequences in the region located from −533 to −332 bp upstream of the *algD* mRNA start site. In addition, no observable retardation of the *algD* fragment (indicative of association with a protein) was detected when identical

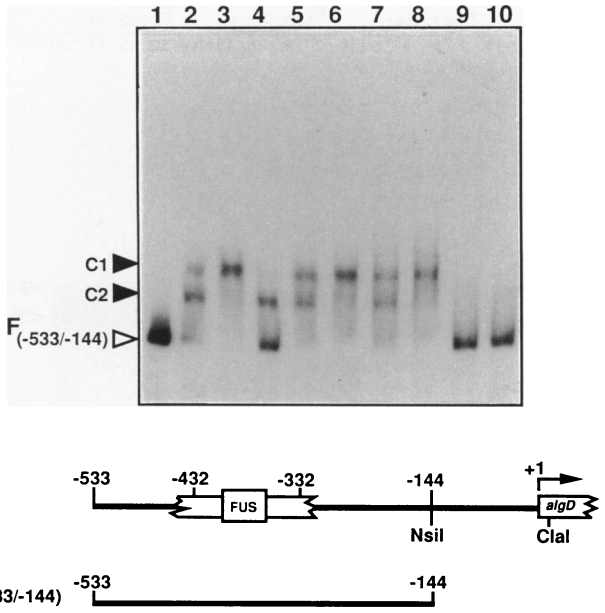

FIG. 2. AlgR binding to the FUS of the *algD* promoter. Shown is a mobility shift DNA-binding assay on a native 5% polyacrylamide gel, using fragment $F_{(-533/-144)}$ extending from positions −533 to −144 relative to the *algD* mRNA start site. The position of FUS, based on previously published transcriptional-fusion studies (37), is indicated by a box with jagged sides to denote the uncertainty of the previously determined limits of FUS (37). Lane 1 contained the fragment alone. Lanes 2 through 6 contained equal amounts of heparin-Sepharose-purified AlgR (90 ng) and different amounts and types of competitor DNA. Lanes 2 and 3 contained 1 μg of specific (sequences spanning the *algD* promoter region from −533 to +112 cloned in pUC12 [plasmid pD533]; see Materials and Methods) and nonspecific [pUC12] competitor DNAs, respectively. Lanes 4 and 5 contained 4 μg of competitor DNAs of the same types as lanes 2 and 3, respectively. Lane 6 contained the same amount of competitor DNA as lane 2, except that sites −533 to −332 were deleted from pD533. Lanes 7 and 8 were the same as lanes 2 and 3, except that a different preparation of AlgR was used. Lanes 9 and 10 were the same as lanes 2 and 3, except that instead of AlgR, the appropriate pooled fractions from the heparin-agarose-chromatographed extracts of *E. coli* B121(DE3)pLysS harboring vector pT7-6 in place of pCMR7 were used. C1 and C2 are the two protein-DNA complexes observed.

fractions of cell extracts containing vector pT7-6 instead of pCMR7 were added to the reaction mixture in place of AlgR (Fig. 2, lanes 9 and 10).

Localization of AlgR binding sites by using deletion subclones of the *algD* promoter. To ascertain that the observed binding was due to association of AlgR to discrete sites within the *algD* promoter, as well as to delimit the potential binding sites further to permit their mapping by DNase I footprinting analysis, we used a set of deletion constructs of *algD* and investigated their abilities to bind AlgR. The results of these studies are shown in Fig. 3, which displays a typical binding pattern obtained with fragments $F_{(-432/-144)}$, $F_{(-332/-144)}$, and $F_{(-332/+11)}$. The $F_{(-432/-144)}$ fragment showed strong binding of AlgR but, unlike $F_{(-533/-144)}$ (Fig. 2), formed only one DNA-protein complex distinguishable on the gel (complex C3; Fig. 3, lanes 1 to 5). Densitometric analyses indicated that 46% of the probe was in the unbound state when in competition with 1 μg of the cold specific competitor DNA, as exemplified in Fig. 3, lane 2. The

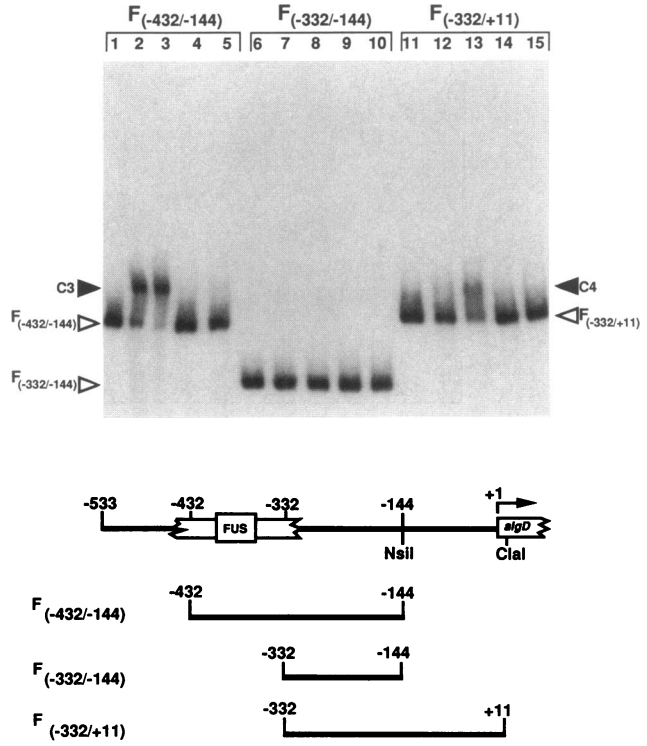

FIG. 3. Analysis of AlgR binding using deletion clones of the *algD* promoter. A mobility shift assay was performed as described for Fig. 2 and in Materials and Methods. Three different fragments are shown spanning sequences from −432 to −144 [$F_{(-432/-144)}$], −332 to −144 [$F_{(-332/-144)}$], and −332 to +11 [$F_{(-332/+11)}$] relative to the *algD* mRNA start site. Lanes 1, 6, and 11 contained radiolabeled fragments alone; lanes 2, 3, 7, 8, 12, and 13 contained AlgR; and lanes 4, 5, 9, 10, 14, and 15 contained the fractions from *E. coli* harboring the vector instead of pCMR7 (Fig. 2, legend). Lanes 2, 4, 7, 9, 12, and 14 each contained 1 μg of pD533 specific competitor DNA (Fig. 2). Lanes 3, 5, 8, 10, 13, and 15 contained 1 μg of pUC12 nonspecific competitor DNA. C3 and C4 indicate protein-DNA complexes. The other symbols are as in Fig. 2.

fragment with a deletion removing an additional 100 bp [$F_{(-332/-144)}$] was no longer capable of binding AlgR (Fig. 3, lanes 6 to 10). However, when this last fragment was additionally extended to include sequences from −144 to +11 [$F_{(-332/+11)}$], weak binding was observed (complex C4; Fig. 3, lanes 11 to 15). When the amount of AlgR was sufficiently increased, most of probe $F_{(-332/+11)}$ could be forced into complex C4 (data not shown). In each experiment shown, the specific and nonspecific competitor DNAs were used (Fig. 3, legend) to establish the specificity of the observed DNA complexes bound. In addition, equivalent fractions processed identically to the AlgR-containing fractions but obtained from cells harboring vector pT7-6 instead of pCMR7 were used as a control (Fig. 3, lanes 4, 5, 9, 10, 14, and 15) and did not show formation of DNA-protein complexes.

These results led to the conclusion that AlgR had at least three binding sites within the *algD* promoter and that the highest-affinity binding sites were located very far upstream from the *algD* mRNA start site. On the basis of deletion mapping, at least one of these sites had to be in the region between the nucleotides −533 and −432.

DNase I footprinting analysis of the AlgR binding sites in the FUS region. To demonstrate the existence of discrete binding

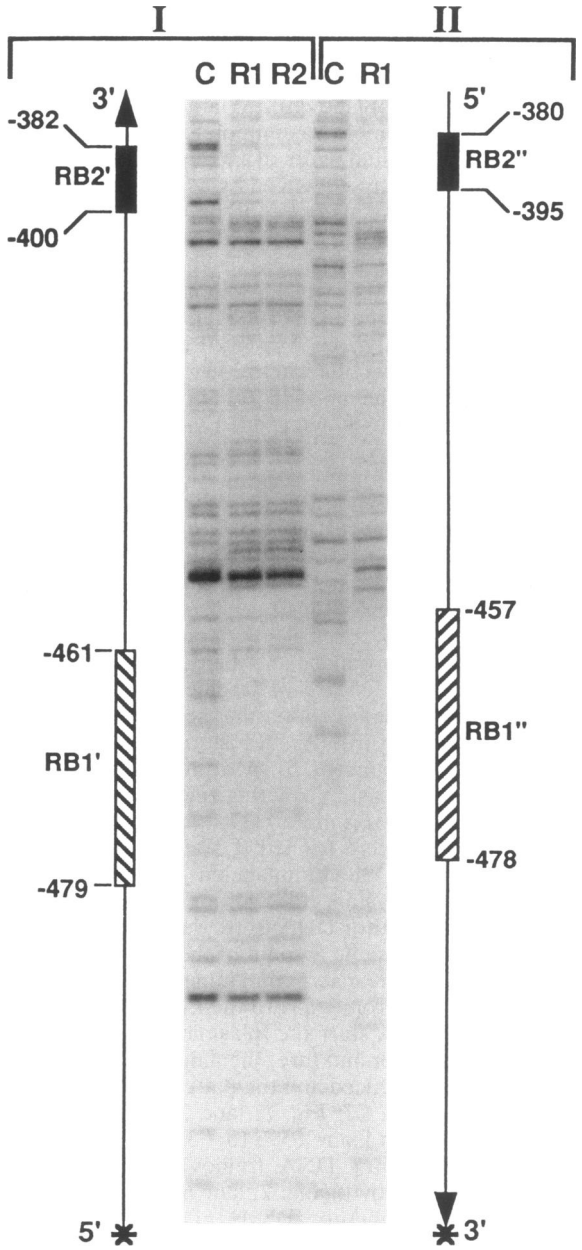

FIG. 4. DNase I footprint analysis of AlgR binding to the FUS. The −533 deletion clone was digested with *Hin*dIII, and two different end-labeled probes were produced. (I) Fragment radiolabeled at the 5′ end with ^{32}P by phosphorylation. (II) Fragment labeled at the 3′ end by filling in the recessed end of the *Hin*dIII-digested DNA, thereby labeling the complementary DNA strand. Probe I, lanes: C, control (probe alone treated with DNase I); R1, 0.6 μg of AlgR; R2, 0.6 μg of AlgR in the presence of 2.5 mM EDTA. Probe II, lanes: C, control; R1, 0.6 μg of AlgR. The arrowheads denote the antiparallel nature of the probes. The labeled end is indicated by an asterisk. Boxes indicate regions of protection due to binding of AlgR: RB1′ and RB1″, footprints of AlgR on strands I and II, respectively, comprising binding site RB1 (hatched boxes); RB2′ and RB2″, footprints comprising binding site RB2 (filled boxes). The nucleotide positions are given relative to the *algD* mRNA start site.

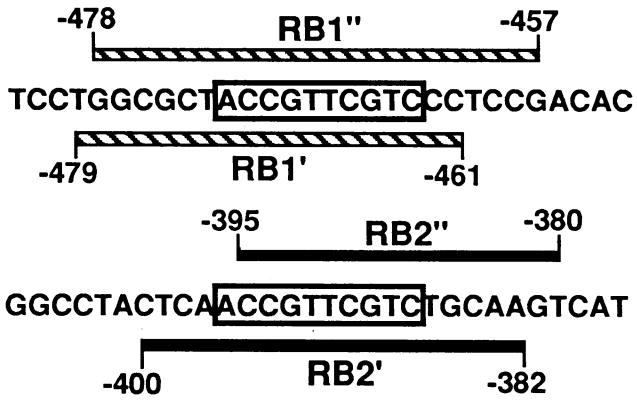

FIG. 5. Sequences of RB1 and RB2. The positions of AlgR footprints on both strands within each region are given by rectangles above and below the sequences. Nucleotide numbers are relative to the mRNA start site, and the sequence source was the previously published *algD* promoter sequence (37). RB1 and RB2 span the same core sequence, ACCGTTCGTC (boxed). This sequence has been previously noted as a pair of perfect direct repeats separated by the endpoint (−432) of the first in a series of consecutive deletions reported to reduce *algD* promoter activity significantly (37).

sites within the FUS region and localize these sequences precisely, we performed a DNase I footprinting analysis. Fragment $F_{(-533/-144)}$ was end labeled at the −533 end by cutting at the *Hin*dIII site (introduced by the deletion process [10]; see Materials and Methods), phosphatase treatment, and phosphorylation with [γ-^{32}P]ATP. When this probe (Fig. 4, probe I) was incubated with AlgR under conditions permitting formation of DNA complexes when most DNA was in the bound state (verified by a mobility shift DNA-binding assay run in parallel on half of the sample; data not shown), two discrete areas of protection from digestion with DNase I could be discerned (Fig. 4, probe I, lanes R1 and R2). The identical protection pattern was also observed when the DNA-protein complexes were treated with DNase I and eluted from the native gels after electrophoretic separation from trace amounts of other forms of the probe (data not shown).

The position of observed footprints was also confirmed by using the complementary DNA strand. The previously used fragment, −533, was also end labeled at the *Hin*dIII site (−533), except that in this case the complementary DNA strand was labeled with ^{32}P. This was done by using the Klenow fragment of DNA polymerase I and filling in the recessed 3′ end of *Hin*dIII with [α-^{32}P]dCTP. When this probe (Fig. 4, probe II) was incubated with AlgR, followed by digestion with DNase I, a footprint pattern nearly identical to the one observed with the antiparallel DNA strand was obtained (Fig. 4, probe II, lane R1). These experiments permitted localization of the AlgR binding sites as follows: RB1, spanning nucleotides −479 to −457, and RB2, spanning nucleotide −400 to −380. The chosen boundaries extend to the outer limits of the detected footprints on either strand.

Once RB1 and RB2 were mapped, it became apparent that they spanned a set of two perfect direct repeats (ACCGT TCGTC) previously observed in this region (37) (Fig. 5). These repeats, termed the core region, appeared to be the only highly conserved sequences between the RB1 and RB2 sites, indicative of their role in recognition by AlgR, a notion supported by a previously reported deletion analysis and functional dissection of the *algD* promoter (37).

DISCUSSION

In this work, the *algR* gene was overexpressed, the AlgR protein was purified, its predicted primary structure was confirmed by partial amino acid sequence analysis, and the site-specific interaction of AlgR and the *algD* promoter was established. These findings suggest that AlgR plays a direct role in the regulation of *algD* transcription.

Purification of AlgR was facilitated by its overexpression; however, the most critical step was based on an apparent high affinity of AlgR for heparin. Chromatography on heparin-Sepharose was highly reproducible and was used to obtain different batches of highly pure AlgR in a relatively small number of steps. These preparations of AlgR showed stability upon storage and very little or no variability in binding to *algD*, although AlgR displayed a tendency to form aggregates under conditions of low ionic strength. A propensity to form aggregates has also been observed with OmpR, a homolog of AlgR (39).

AlgR belongs to a large superfamily of bacterial signal transduction systems (42, 43). Several proteins from this group have been shown to undergo rapid phosphorylation-dephosphorylation modifications dependent on the cognate kinase-phosphatase activities (2, 28, 30, 43). These interconversions can also be executed to a certain degree by heterologous proteins (2, 29, 43). Phosphorylation of NtrC affects its affinity for the binding sites of the *glnA* promoter (38). The state of phosphorylation of AlgR in the preparations reported here is not known, but we did notice that the AlgR protein which appeared homogeneous on heparin-Sepharose showed reproducible separation into two fractions when chromatographed on a MonoQ column. The nature of this separation is not known. Future studies with the in vitro-phosphorylated forms of AlgR, using either heterologous broad-spectrum kinases (43) or the AlgR cognate kinase, once it is identified, will permit analysis of how the post-translational modifications may affect the association pattern of AlgR and its relative affinities for the binding sites identified within the *algD* promoter.

Binding of AlgR to the RB1 and RB2 sites within FUS is consistent with the previously determined role for FUS in the high-level transcription of *algD* (37). Transcriptional fusion analyses have indicated that the absolute levels of *algD* promoter activity are dramatically reduced by deletions affecting the FUS region (37). Removal of sequences from −533 to −432 results in twofold reduction in activity, whereas removal of the −432 to −332 region diminishes *algD* transcription 10-fold, significantly below the levels compatible with the mucoid phenotype (16). These results are in agreement with the binding of AlgR on both sides of position −432. Thus, the stepwise reduction of the *algD* promoter activity reported previously (37) can be attributed to sequential removal of the AlgR binding sites. The residual activity and induction of the *algD* promoter, previously observed with the promoter region starting at −116 (37), can be attributed to the association of AlgR with a low-affinity site(s) closer to the mRNA start site detected in the present work. The precise position of this site(s) remains to be determined once conditions that permit footprinting of these relatively low-affinity interactions are developed. There are several sequences within this region of DNA resembling, to some degree, the highly conserved core of RB1 and RB2 (ACCGTTCGTC; Fig. 5). Somewhat surprisingly, the best

match (8 of 10 residues) was obtained with a sequence running in the direction opposite to that of RB1 and RB2 (positions −36 through −45 relative to the *algD* mRNA initiation site).

The location of RB1 and RB2 exceeds the distance of upstream activator sequences observed in other prokaryotic promoters controlled by signal transduction systems (8, 41, 43). Earlier work (15, 16) and our more recent analyses using S1 nuclease mapping and primer extension revealed no additional transcriptional start sites upstream from the mRNA initiation site already mapped for *algD* (data not shown). It is also important to note that the *algD* mRNA already has a very large nontranslated 5′ leader sequence (369 nucleotides [15]). It appears unlikely that such a long mRNA leader would be additionally extended. In addition, we placed a fragment containing sequences extending from the *Nsi*I site (−144) to the naturally existing *Hin*dIII site (located approximately 1.2 kb upstream of the *algD* mRNA start site) in promoter probe vector pVDX18 (32) and detected no transcription above the background level (unpublished results). Thus, binding of AlgR to RB1 and RB2 must play a role in the control of transcription from the previously determined *algD* mRNA start site (15).

What could be the reason for the development of such a structural arrangement, in which the high-affinity binding sites for an activator are positioned in a relatively remote place? A clue for understanding of this phenomenon may come from recent analyses of the *nifH* and *glnA* promoters (27, 45). In both cases, protein-facilitated bending or apparently spontaneous looping of the intervening sequences has been shown to play a role in promoter control by factors bound to upstream sites (27, 45). Similar mechanisms could play a role in the activation of *algD*. If protein-assisted bending is required to enhance the interactions of AlgR bound to RB1 and RB2 with the RNA polymerase or AlgR bound near the mRNA start site, possible candidates for such a function could be bacterial histonelike elements, as in the case of *nifH* (27). Recently, a unique bacterial protein factor (AlgP) required for full expression of *algD* has been described (18, 19, 33). AlgP has a carboxy-terminal domain closely resembling the tails of eukaryotic H1 histones (18) (overall homology with H1 histones of the product of a gene termed *algR3*, allelic with *algP*, has also been reported by others [31]). One hypothetical model (20) predicts that the tail of AlgP plays a role similar to the function of the histone H1 tails, which are known to participate in the folding of chromatin fibers into higher-order structures (1). Alternatively, other bacterial histonelike elements, e.g., a possible *Pseudomonas* integration host factor analog (the integration host factor enhances NifA-dependent *nifH* expression [27]), the HU protein of *P. aeruginosa* (25), or other factors affecting the nucleoid structure of *P. aeruginosa* (e.g., those controlling superhelicity), could influence the conformation of the *algD* promoter. Studies of the intrinsic or protein-assisted capacity of the *algD* promoter for bending, using the purified integration host factor, HU, and segments of the AlgP tail, are under way.

The peculiar arrangement of AlgR binding sites could permit integration of different environmental cues governing *algD* activation. Binding of additional regulatory factors, such as AlgB (49), may be accommodated by the physical organization of the *algD* promoter. These additional elements and factors affecting the nucleoid structure may participate in the transduction of environmental signals, such as nitrogen limitation (16), medium osmolarity (6, 16), oxygen tension (5), or stress in general, into a concerted response of the *algD* promoter. Moreover, the complex organization of the *algD* promoter may represent a security latch preventing spurious activation of the alginate-biosynthetic apparatus under conditions in which mucoidy (which causes a tremendous loss of carbon) provides no selective advantage. In contrast, when the environmental conditions are adequate, such as in a particular ecological niche, the *algD* promoter may be in the state poised for high levels of transcription. Since mucoid strains of *P. aeruginosa* are isolated with a high frequency from patients with CF and many of them display properties consistent with mutations causing constitutive or upregulated expression of *algD* (16, 22, 23, 34), future analysis of these mutations may provide information necessary for the understanding of complex regulation of the *algD* promoter.

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