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Mucoid-to-Nonmucoid Conversion in Alginate-Producing *Pseudomonas aeruginosa* Often Results from Spontaneous Mutations in *algT*, Encoding a Putative Alternate Sigma Factor, and Shows Evidence for Autoregulation

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The mucoid phenotype is common among strains of *Pseudomonas aeruginosa* that cause chronic pulmonary infections in patients with cystic fibrosis and is due to overproduction of an exopolysaccharide called alginate. However, the mucoid phenotype is unstable in vitro, especially when the cells are incubated under low oxygen tension. Spontaneous conversion to the nonmucoid form is typically due to mutations (previously called *algS*) that are closely linked to the alginate regulatory gene *algT*, located at 68 min on the chromosome. Our sequence analysis of *algT* showed that its 22-kDa gene product shares homology with several alternate sigma factors in bacteria, suggesting that AlgT (also known as AlgU) interacts directly with RNA polymerase core to activate the promoters of alginate genes. AlgT showed striking sequence similarity (79%) to σ^E of *Escherichia coli*, an alternate sigma factor involved in high-temperature gene expression. Our analysis of the molecular basis for spontaneous conversion from mucoid to nonmucoid, in the cystic fibrosis isolate FRD, revealed that nonmucoid conversion was often due to one of two distinct missense mutations in *algT* that occurred at codons 18 and 29. RNase protection assays showed that spontaneous nonmucoid strains with the *algT18* and *algT29* alleles have a four- to fivefold reduction in the accumulation of *algT* transcripts compared with the wild-type mucoid strain. Likewise, a plasmid-borne *algT-cat* transcriptional fusion was about 3-fold less active in the *algT18* and *algT29* backgrounds compared with the mucoid wild-type strain, and it was 20-fold less active in an *algT*::Tn*501* background. These data indicate that *algT* is autoregulated. The spontaneous *algT* missense alleles also caused about fivefold-reduced expression of the adjacent negative regulator, *algN* (also known as *mucB*). Transcripts of *algN* were essentially absent in the *algT*::Tn*501* strain. Thus, *algT* regulates the *algTN* cluster, and the two genes may be cotranscribed. A primer extension analysis showed that *algT* transcription starts 54 bp upstream of the start of translation. Although the *algT* promoter showed little similarity to promoters recognized by the vegetative sigma factor, it was similar to the *algR* promoter. This finding suggests that AlgT may function as a sigma factor to activate its own promoter and those of other alginate genes. The primer extension analysis also showed that *algT* transcripts were readily detectable in the typical nonmucoid strain PAO1, which was in contrast to a weak signal seen in the *algT18* mutant of FRD. A plasmid-borne *algT* gene in PAO1 resulted in both the mucoid phenotype and high levels of *algT* transcripts, further supporting the hypothesis that AlgT controls its own gene expression and expression of genes of the alginate regulon.

Pseudomonas aeruginosa is an opportunistic pathogen that is responsible for a wide variety of infections, including chronic pulmonary tract infections in patients with the autosomal recessive disorder cystic fibrosis (CF). Following colonization of the CF respiratory tract with *P. aeruginosa*, mucoid variants of the original strain emerge and become predominant (30, 45). The mucoid form of *P. aeruginosa* leads to chronic pulmonary infection and a poor prognosis for the patient (30). The mucoid phenotype is the result of an overproduction of the exopolysaccharide alginate. Non-CF-related strains of *P. aeruginosa*, and even other species of *Pseudomonas*, have the potential for alginate biosynthesis, yet their genes for the production of this polymer are usually not highly expressed (26, 28, 29). However, the lung environment of the CF patient apparently selects for variants of *P. aeruginosa* which overproduce this polymer. The mucoid phenotype appears to confer upon the pathogen increased resistance to phagocytosis (3) and a mechanism of adherence that permits the formation of microcolonies in the lung (35).

Alginate produced by *P. aeruginosa* is an O-acetylated linear polymer of D-mannuronate and L-guluronate residues (15). The alginate biosynthetic gene cluster is at 34 min on the chromosome and appears to form an operon (6). Enzymes involved in the early (42) and late (20, 21, 50) steps of the pathway have been described. The alginate biosynthetic gene cluster is transcriptionally regulated by the products of several genes located in the 9- to 13-min region of the chromosome. These include two distinct response regulators encoded by *algR* (9) and *algB* (54) which share sequence homology to environmentally responsive two-component regulatory proteins. Fyfe and Govan (24) showed that mutations called *muc* in the 68-min region of the chromosome can give rise to the mucoid phenotype in a typical nonmucoid strain.

We have reported the cloning of a 7.6-kb fragment of DNA (pJF15) from the 68-min region of the alginate-producing (Alg^+) CF strain *P. aeruginosa* FRD1. This clone contains *algT*, which encodes a positive regulator of alginate biosynthe-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
E. coli		
HB101	*proA2 leuB6 thi-1 lacY1 hsdRM recA13 supE44*	This laboratory
INVαF′	*endA1 recA1 hsdR17 supE44 thi-1 gyrA relA1* φ80 *lacZ*αΔM15 Δ(*lacZYA-argF*) *deoR*$^+$ F′	InVitroGen
P. aeruginosa		
PAO1	Wild type, prototrophic (Alg$^-$)	33
FRD1	Wild type, prototrophic CF isolate (Alg$^+$) (source of FRD isolates below)	46
FRD2	*algT18* (spontaneous Alg$^-$)	46
FRD39	*hisI1* (Alg$^+$)	46
FRD40	*pro-1* (Alg$^+$)	46
FRD60	*hisI1 algT18* (spontaneous Alg$^-$)	46
FRD75	*hisI1 algT29* (spontaneous Alg$^-$)	46
FRD440	*algT*::Tn*501*-33 (Alg$^-$)	18
Plasmids		
pRK2013	ColE1-Tra[RK2]$^+$ (Km^r)	16
R68.45	IncP1 (Cb^r Tc^r Km^r CMA$^+$)	31
pLAFR1	IncP1 *cos oriT* (Tc^r)	23
pCP13	IncP1 *cos oriT* (Tc^r Km^r)	8
pCRII	ColE1*ori* F1*ori lacZ*α (Ap^r Km^r)	InVitroGen
pJF15	pLAFR1 with 7.6-kb *Eco*RI fragment from FRD1, *algT*$^+$ *algN*$^+$	18
pCD4	pCP13 with 4-kb *Xho*I fragment of pJF15, Tn*501-250*, *algT*$^+$	This study
pJF19-18	pJF19 is a 4.5-kb derivative of pJF15 without *algT* followed by insertion of Tn*501-18*	18
pCD12	pCRII with *nadB* fragment	This study
pCD45	pCRII with *algT* fragment	This study
pCD89	pCRII with *algN* fragment	This study
pTn*3*-Spice	pMB8::Tn*3-inaZ tnpA*$^+$ (Ap^r Sm^r Sp^r)	36
pTn*3-cat*	pMB8::Tn*3-cat* (Ap^r/Cb^s Sm^r Sp^r CMA$^+$)	This study

[a] Abbreviations for phenotypes: Tc^r, tetracycline resistance; Hg^r, mercury resistance; Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Cb^r, carbenicillin resistance; Sp^r, spectinomycin resistance; Tra$^+$, transfer by conjugation; Alg$^+$, alginate producing; CMA$^+$, chromosome mobilization ability.

sis (18). An *algT*::Tn*501* mutant was constructed by gene replacement and became Alg$^-$, indicating that *algT* is essential for alginate production (18). The *algT*::Tn*501* mutant also showed reduced transcription of *algB* (25, 55), *algR* (56), and *algD* (56). Downstream of *algT* is *algN* (*mucB*), whose product acts as a negative regulator (26). A plasmid containing only *algT* activates alginate production in a variety of *Pseudomonas* strains and species, but a plasmid containing both *algT* and *algN* from strain FRD is not changed to mucoid (26). Thus, AlgN can prevent AlgT-mediated activation of the mucoid phenotype by a mechanism not yet defined. Between *algT* and *algN* is the negative regulator *mucA*. It was recently shown that the *muc* mutations resulting in mucoid conversion often inactivate *mucA* (41). The *mucA* gene is defective in strain FRD and may be responsible for the mucoid phenotype of this CF isolate (26, 41). Linked to *mucAB* is a gene called *algU* which shows homology to an alternate sigma factor in *Bacillus* sp. (40). Studies described here show that *algT* and *algU* are actually the same gene and also extend our understanding of its role in alginate gene regulation.

The Alg$^+$ phenotype of mucoid *P. aeruginosa* from CF patients is unstable, and spontaneous reversion to the nonmucoid form is common. Such mutations also occur at 68 min on the chromosome (46). Gene replacement studies showed that the locus responsible for nonmucoid conversion is present on the *algTN* clone pJF15 (18). The locus responsible for switching off alginate production (previously referred to as *algS*) also affects the expression of *algT* (19). No observable restriction fragment length polymorphisms were detected in or around *algT* in spontaneous Alg$^-$ variants, indicating that no gross rearrangement of DNA had occurred (19). However, the nature of the mutations responsible for spontaneous mucoid-to-nonmucoid conversion remained unknown. In this study, we determined that the molecular basis for the spontaneous conversion from mucoid to nonmucoid was often due to missense mutations in *algT*. The sequence analysis of *algT* indicated that its gene product may function as an alternate sigma factor which activates the promoters of alginate genes. We also show that AlgT is an autoregulator of *algTN* transcription in the CF-related strain FRD1.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are described in Table 1. *Escherichia coli* and *P. aeruginosa* strains were routinely cultured in L broth (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter). A minimal medium (51) was used to select for *P. aeruginosa* following triparental matings with *E. coli* or in selections for recombinants. L agar or defined medium AP (46) was used to observe the Alg$^+$ mucoid phenotype. Antibiotics when used were at the following concentrations (per milliliter): ampicillin at 100 μg, carbenicillin at 300 μg, $HgCl_2$ at 18 μg, and tetracycline at 20 μg for *E. coli* and 100 μg for *P. aeruginosa*.

Mobilization of DNA. Triparental matings (27) were used to mobilize plasmids from *E. coli* HB101 to *P. aeruginosa*, using the conjugation helper plasmid pRK2013 (16). Gene replacement was performed in *P. aeruginosa* as previously described (48), using the transducing phage F116L to transfer Tn*501*-marked DNA into FRD strains followed by selection for mercury-resistant recombinants. R68.45-mediated uninterrupted plate matings were used to mobilize chromosomal DNA and repair auxotrophic mutations as previously described (46).

Isolation of nonmucoid variants and chromosomal mapping. Spontaneous nonmucoid variants of mucoid strain FRD39 were obtained as previously described (46). Briefly,

each mucoid colony was incubated under low oxygen tension in 2 ml of L broth for 24 h at 37°C without aeration. Cultures were diluted onto L-agar plates followed by selection of a nonmucoid colony among the many which arose following incubation. Tests to show that the nonmucoid phenotype was due to mutations in the 68-min region of the chromosome were performed by plate matings between donor FRD40(R68.45) and the nonmucoid colonies derived from FRD39 (*hisI1* at ~68 min) with selection for His^+ on VB minimal medium (46). Recombinants inheriting the Alg^+ phenotype were scored on the basis of their mucoid growth on AP medium.

Nucleic acid manipulations and DNA sequencing. General DNA manipulations (39) and isolation of genomic DNA (27) were performed as previously described. RNA was isolated by a hot phenol method (5) from logarithmic-phase *P. aeruginosa* cells, cultured in L broth to approximately 5×10^8 cells per ml, and stored at −70°C. Plasmid DNA was isolated from *E. coli*, using columns and procedures described by the manufacturer (Qiagen Corp.). Sequences on plasmid DNA were determined by the chain termination technique, using Sequenase (United States Biochemical Corp.), [5′-α-^{32}P]dCTP (>6,000 Ci/mmol, 10 mCi/ml; Amersham), and 7-deaza-dGTP (U.S. Biochemical) in procedures described by the manufacturers. Oligonucleotides used for sequencing, primer extensions, and PCR were synthesized on an Applied Biosystems model 380B DNA synthesizer. Oligonucleotides were end labeled as described elsewhere (2) with [γ-^{32}P]ATP by polynucleotide kinase. Using genomic DNA samples as templates, PCR with AmpliTaq DNA polymerase (Perkin-Elmer Corp.) was used to generate 750-bp fragments containing *algT* from FRD strains, using the primers CD13 (5′-AAGACGATTCGC TGGGACGCT-3′) and CD14 (5′-AACGCAGCTCGGCATCCTCG-3′). The PCR product was gel purified by using the Qiaex system (Qiagen), and the nucleotide sequences were determined with a double-stranded DNA cycle sequencing system (Bethesda Research Laboratories, Inc.) in a PCR apparatus under the conditions of 1 min at 95°C, 2 min at 55°C, and then 2 min at 70°C, with specific end-labeled primers. Homology searches and alignments were performed with the Basic Local Alignment Search Tool (BLAST) Network Service at the National Center for Biotechnology Information, National Institutes of Health (1).

RNase protection assays. Specific DNA fragments were generated by PCR to be used as templates for the in vitro synthesis of antisense RNA probes to measure transcript levels of *algT*, *algN*, and *nadB* in *P. aeruginosa* strains. Specific primers used were CD4 (5′-AGATCGAGGCCACCGTGCACC-3′) and CD5 (5′-GATCCGCGACCGTACCGTCCC-3′) for *algT*, CD8 (5′-AGCGCAATGGCAGCTTCTCC-3′) and CD9 (5′-CATCGGCCAGTTGGTCGGCAA-3′) for *algN*, and CD1 (5′-AGAACGGGCATAGACGAAGCA-3′) and CD2 (5′-GA TCCCGGTAGTGCCGGCCGC-3′) for the control *nadB* probe. DNA fragments were amplified by PCR using FRD39 genomic DNA as the template. The DNA fragments of 175 bp (*nadB*), 150 bp (*algT*), and 165 bp (*algN*) were gel purified (Qiaex system; Qiagen), ligated into a PCR fragment cloning vector (pCRII; InVitroGen), and transformed into *E. coli* INVαF′ cells as described by the manufacturer of pCRII. Plasmids with DNA in the nontranscribing orientation, relative to the T7 or SP6 promoter on pCRII, were purified by using plasmid isolation columns (Qiagen). The fidelity of the PCR-generated clones was verified by sequence analysis. Plasmid DNA was treated with Proteinase K (Sigma) to eliminate contaminating RNase before being used in an in vitro transcription system (Riboprobe Gemeni System II; Promega), with SP6 polymerase (Promega) for *nadB* or T7 polymerase (Promega) for *algT* and *algN*, in the presence of [α-^{32}P]CTP (50 μCi at 10 mCi/ml) to generate specific, antisense RNA probes from linearized template DNA. The reactions were carried out for 1 h at 37°C and then the mixtures were treated with DNase (4 U of RQ RNase-free; Promega) at 37°C for 20 min to eliminate residual DNA. The incorporated label was separated from the unincorporated label by centrifugation through 1-ml columns of Sephadex G-50 (Bio-Rad). The relative activity of the RNA probes was measured in a scintillation counter with 1 μl of the reaction mixture in 5 ml of Quantifluor (Mallinkrodt). The RNase protection assays were performed with the RPAII system (Ambion Corp.) as described by the manufacturer. Briefly, ^{32}P-labeled antisense RNA probes (~2×10^4 cpm) were each mixed with *P. aeruginosa* RNA samples (30, 20, 10, and 5 μg) or yeast RNA (10 μg) and allowed to hybridize overnight with gentle agitation at 45°C. The mixture was treated with RNase for 30 min at 37°C to destroy all but duplexed RNA, which was then precipitated as described by the manufacturer at −20°C for 30 min, pelleted by centrifugation (14,000 × *g*, 15 min, 4°C), and dissolved in 8 μl of loading buffer (80% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue, 2 mM EDTA). After heating for 4 min at 90°C, the samples were loaded onto a denaturing 6% polyacrylamide gel, which was run at 80 V for approximately 1 h in Tris-borate buffer (89 mM Tris base, 89 mM boric acid, 1 mM Na_2EDTA). The gel was transferred to chromatography paper, covered with plastic wrap, and exposed to X-ray film (Kodak XAR5) at −70°C for 1 to 5 days. To determine the relative abundance of transcripts, densitometric measurements of the autoradiographs were performed with ScannerOne (Apple Computer Corp.) and Scan Analysis software (Biosoft Corp.).

Primer extension analysis. For primer extensions of *algT*, 50 μg of total RNA from *P. aeruginosa* in 8 μl of diethylpyrocarbonate-treated water was mixed with an end-labeled (5×10^4 cpm) oligonucleotide, CD40 (5′-CCCGTTCAACCAGTTGC TGATCCTGTTCCTGGGTTAGCAT3′), which is complementary to a 40-base sequence at the 5′ end of the *algT* transcript. The suspension was heated for 5 min at 85°C, and the primer was allowed to anneal to the RNA for 3 h at 42°C. After adjustment of the buffer (900 mM Tris-HCl [pH 8.3], 100 mM $MgCl_2$, 100 mM dithiothreitol), deoxynucleoside triphosphates (2.0 mM), RNasin (0.5 μl at 40 U/μl), and avian myeloblastosis virus reverse transcriptase (1 μl at 15 U/μl; U.S. Biochemical) were added, the mixture was incubated 30 min at 42°C, and the nucleic acids were recovered by phenol extraction and ethanol precipitation. The DNA pellet was suspended in formamide sequencing stop mix and heated at 85°C for 3 min, and then an aliquot was applied to a 6% sequencing gel. This was electrophoresed in Tris-borate-EDTA next to a DNA sequencing ladder primed with the same oligonucleotide (CD40) used for primer extension.

CAT transcriptional fusion assays. To construct a system for monitoring transcription of *algT* from a low-copy-number plasmid, a derivative of pJF15 with an *algT-cat* transcriptional gene fusion was generated. For this, we constructed a derivative of transposon Tn*3* that allowed *cat* to be used as a reporter of the transcriptional activity of genes into which it is inserted. The *Eco*RI fragment containing the *inaZ* reporter gene in pTn*3*-Spice (36) was replaced with an *Eco*RI-*Sal*I-*Eco*RI DNA linker (5′-GAATTCCCCGGATCCGTCGACGGATCCGGG GAATTC-3′), into which was inserted a correctly oriented 800-bp *Sal*I fragment containing a promoterless *cat* gene cartridge encoding chloramphenicol acetyltransferase (CAT) from pCM1 (7). This formed pTn*3-cat*, a 10.5-kb plasmid composed of pMB8 and Tn*3-cat* (2.7 kb) encoding resistances

to ampicillin, spectinomycin, and streptomycin. This element was randomly transposed as previously described (36) onto pJF15, and a correctly oriented *algT-cat* transcriptional fusion was identified by restriction analysis. The pJF15::Tn*3-cat* was transferred to *P. aeruginosa* strains by triparental matings with selection for tetracycline resistance, which were then grown under alginate-producing conditions (37°C, L broth, with aeration) to approximately 10^9 cells per ml. Cell extracts for CAT assays were obtained by sonication as previously described (55), and CAT levels were determined by an enzyme-linked immunosorbent assay (ELISA), using procedures recommended by the manufacturer (5 Prime→3 Prime, Inc.), and normalized for relative protein concentration (A_{280}).

Nucleotide sequence accession number. The nucleotide sequence data reported here for *algT* have been deposited in the GenBank database under accession number L36379.

RESULTS

Sequence analysis of *algT*. The *algT* gene was previously cloned on pJF15 from the CF-associated mucoid strain *P. aeruginosa* FRD1 and shown to be essential for expression of the Alg^+ phenotype (18, 19, 26, 55). In this study, a DNA sequence analysis was performed on the *algT* locus to better define its gene product (Fig. 1). An open reading frame was identified in the region known to contain *algT* that started at nucleotide 502 and encoded a 193-amino-acid protein with a predicted molecular mass of 22.18 kDa. A putative ribosome binding site was found 7 bases upstream of the initiating ATG codon. Our comparison of this sequence with other known alginate gene sequences showed that *algT* was recently described as *algU*, a gene in close proximity to genes called *mucAB* from nonmucoid strain PAO. The *mucAB* genes were identified by their ability to suppress the mucoid phenotype in *trans* in some mucoid strains (40). The *algU* (*algT*) gene product was reported to show sequence similarity to an alternate sigma factor, Spo0H, which is involved in the initial stages of sporulation in *Bacillus* sp. (13). Although *algU* was reported to encode a 27.5-kDa protein (40), our calculations for the same sequence (*algT*) would predict a protein of 22.18 kDa.

We also performed a search for homologies to the inferred AlgT sequence by using the BLAST algorithm (1). In the regions considered significant by BLAST, AlgT and Spo0H (σ^H) of *Bacillus subtilis* (GenBank accession number P17869) showed 19% similarity (i.e., 37 of 193 residues matched) (Fig. 2). In addition, this analysis revealed that AlgT has sequences in common with a number of other minor sigma factors of RNA polymerase (Fig. 2). Similarities to other bacterial sigma factors included 17% (34 of 193) to SpoIIAC of *Bacillus megaterium* (accession number X63757), 18% (35 of 193) to SigC of *Myxococcus xanthus* (accession number L12992), 14% (27 of 193) to FliA of *E. coli* (accession number P31804), and 11% (22 of 193) to WhiG of *Streptomyces coelicolor* (accession number P31804). Such limited homologies between sigma factors are not unusual (37). The highest match found in this search was to SigE (also known as σ^E or RpoE) of *E. coli* (accession number P34086), a 21.7-kDa sigma factor involved in high-temperature gene expression (14). The high level of homology between AlgT and SigE, 79% similarity (153 of 193) and 65% identity (126 of 193), suggests that AlgT may play a role in *P. aeruginosa* similar to the one it plays in *E. coli*. In the next-highest match, AlgT showed 32% similarity (63 of 193) to HrpL of *Pseudomonas syringae* pv. syringae (accession number U03854), a putative sigma factor which is part of an environmentally regulated cascade that activates expression of genes for plant pathogenesis (57). AlgT showed 21% (41 of 193) similarity to CarQ of *M. xanthus* (accession number X71062), which is involved in light-induced carotenogenesis but has not yet been reported as a sigma factor. The alignment of these sigma factors (Fig. 2) and the sequence conservation relationships recently described by Lonetto et al. (37) were used to identify the potential locations of polymerase core binding (AlgT residues 21 to 38), an RpoD box (i.e., −10 recognition, AlgT residues 68 to 83), and a 20-mer (i.e., −35 recognition, AlgT residues 164 to 184).

Upstream of *algT* was an open reading frame transcribed in the opposite direction that encoded a product showing 73% similarity (63% identity) to NadB, an L-aspartate oxidase involved in de novo NAD biosynthesis in *E. coli*. A genetic analysis of this putative *nadB* gene from *P. aeruginosa* is being reported elsewhere (12).

Spontaneous nonmucoid strains have mutations in *algT*. The mucoid (Alg^+) phenotype of *P. aeruginosa* strains from CF patients is typically unstable, especially when cultures of this strict aerobe are not maintained under highly aerated conditions (46). The genetic alterations responsible for spontaneous Alg^- conversion are closely linked to *algT* (18). A sequence analysis downstream of *algT* (i.e., in the defective *mucA* region) of spontaneous nonmucoid variants did not reveal any changes in the DNA sequence compared with the mucoid parent strain (17). This finding suggests that reversion to $mucA^+$ was not responsible for conversion to the nonmucoid phenotype among the strains examined. Subsequently, we examined the possibility that spontaneous Alg^- variants arise as a result of mutations in the *algT* locus itself. DNA fragments (750 bp) were generated by PCR amplification of genomic DNA containing *algT* alleles from Alg^+ strains (FRD1 and FRD39) and from spontaneous Alg^- variants (FRD2, FRD60, and FRD75). These PCR fragments (beginning at nucleotide 449 and ending at nucleotide 1226 in Fig. 1) were analyzed by direct cycle sequencing to avoid sequencing errors that could occur by cloning and sequencing one representative fragment. As expected, the PCR product from Alg^+ FRD39 (a His^- auxotroph) produced the same sequence as its Alg^+ parent, FRD1 (Fig. 1). In contrast, the spontaneous Alg^- strains had single base pair changes that caused missense mutations in *algT*. One mutation was at codon 18, where a GAC (Asp) in Alg^+ strains was changed to GGC (Gly) in FRD2 and FRD60. Another change was at codon 29, where TAC (Tyr) in FRD39 was changed to TGC (Cys) in FRD75 (Fig. 1).

To determine whether such *algT* mutations were common upon conversion from Alg^+ to Alg^-, we isolated 28 independent, spontaneous-nonmucoid variants of FRD39. FRD39 has a *hisI* marker at approximately 68 min on the chromosome which shows genetic linkage to *algT* (46). Plasmid R68.45 (31) was used to mobilize chromosomal DNA from the Alg^+ donor strain FRD40 to each of the 28 nonmucoid derivatives of FRD39, followed by selection for $hisI^+$ on minimal medium. The His^+ recombinants obtained showed approximately 50% coinheritance of the Alg^+ phenotype, indicating that all spontaneous Alg^- mutations were in the 68-min region of the chromosome and mapped near *algT*. Direct cycle sequencing of *algT* in the spontaneous Alg^- variants, as described above, revealed that the same two distinct single base pair mutations occurred at codons 18 and 29 within the *algT* coding sequence in several of these strains. A total of five spontaneous Alg^- strains had changed to GGC at codon 18, and four were changed at codon 29 to TGC. Strains representing these two alleles, FRD60 (*algT18*) and FRD75 (*algT29*), were chosen for further study. The spontaneous nonmucoid variants without base pair mutations in the 750-bp region of *algT* are currently

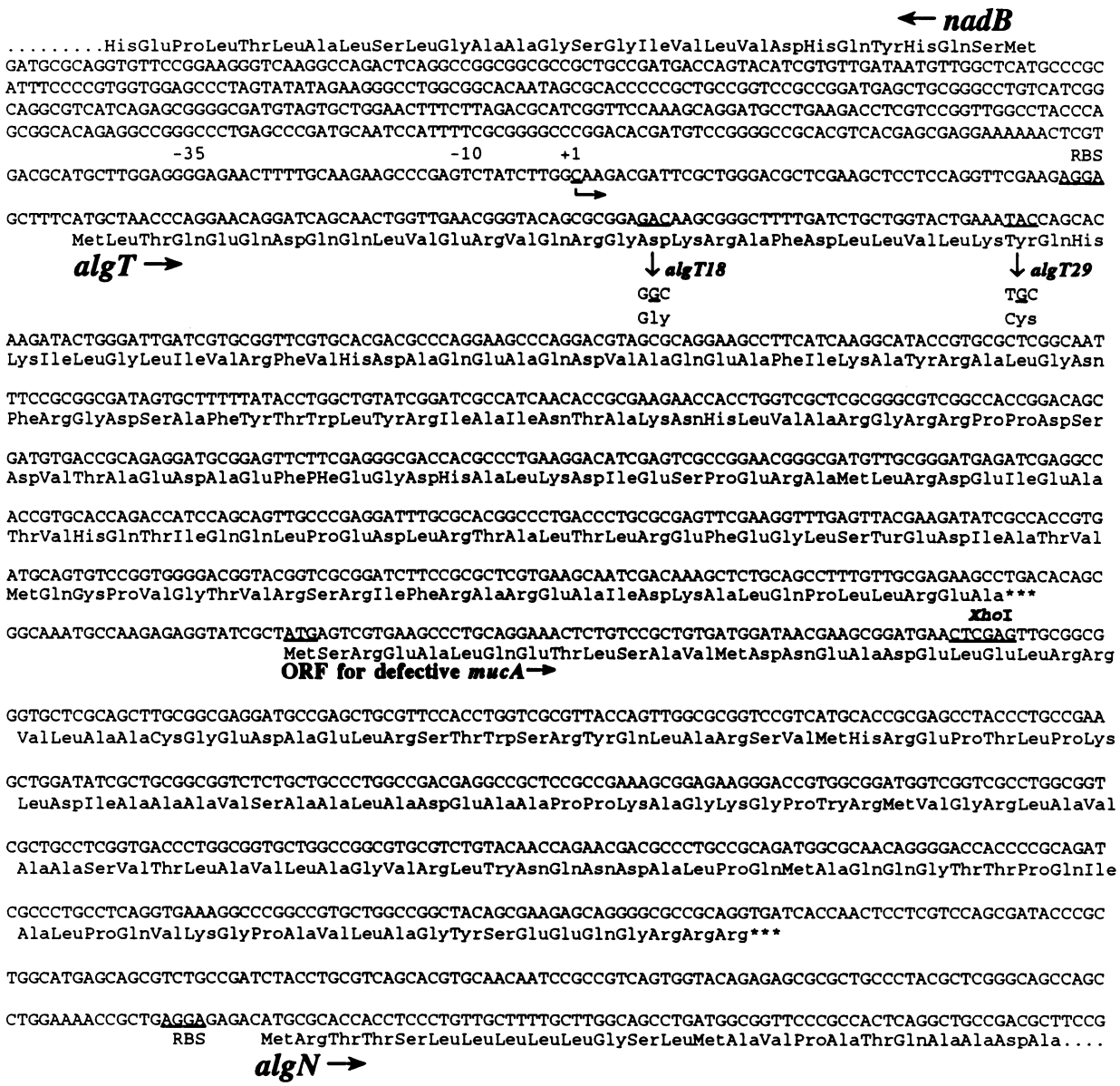

FIG. 1. Nucleotide sequence of the *algT* region and predicted amino acid coding sequence of AlgT in the mucoid CF isolate *P. aeruginosa* FRD. The open reading frame for *algT* starts at nucleotide 502 and encodes a 22.18-kDa protein. The ribosome binding site (RBS) is located 7 bp upstream of the ATG translational start site. The transcriptional start site is indicated by +1 with an arrow. The presumptive *algT* promoter region is indicated by −10 and −35. Two spontaneous mutant *algT* alleles, *algT18* and *algT29*, are denoted at the specific codons (underlined) with the resulting base pair changes and amino acid substitutions. Three asterisks denote stop codons. The open reading frame (ORF) for a defective *mucA* allele in strain FRD is indicated. Downstream, the beginning of the *algN* open reading frame is indicated with its ribosome binding site. Upstream of *algT* is a divergently expressed gene homologous to the *nadB* gene of *E. coli* which is involved in NAD biosynthesis.

undergoing analysis to identify other mutations responsible for their Alg^- phenotype.

Gene replacement with *algT*$^+$ in spontaneous nonmucoid mutants. The foregoing sequence analysis showed a correlation between spontaneous conversion to the Alg^- phenotype and missense mutations in *algT*. Gene replacement with *algT*$^+$ DNA was used to provide evidence that these mutations were indeed responsible for loss of alginate production. A derivative of pJF15 with a Tn*501* insertion adjacent to *algT*$^+$ was used to provide an adjacent selectable marker (i.e., mercury resistance) for selection of recombinants inheriting plasmid-borne *algT* sequences. When the *P. aeruginosa* DNA in pJF15::Tn*501*-215 (Fig. 3) was used to replace homologous DNA in FRD60 and FRD75, both strains produced recombinant colonies with the Alg^+ phenotype. When pCD4, which contains *algT*$^+$ on a 4-kb *Xho*I fragment of pJF15, was used for gene replacement in FRD60, Alg^+ recombinants were obtained. In contrast, when pJF19::Tn*501*-18, a derivative of pJF15 that does not contain *algT*, was used in gene replacement, none of the recombinants were Alg^+ (Fig. 3). These data suggest that the *algT* missense alleles which correlate with the spontaneous Alg^- phenotype were responsible for the Alg^- phenotype.

Mutant *algT* alleles affect *algT* transcription. To better understand the role of AlgT in alginate gene regulation,

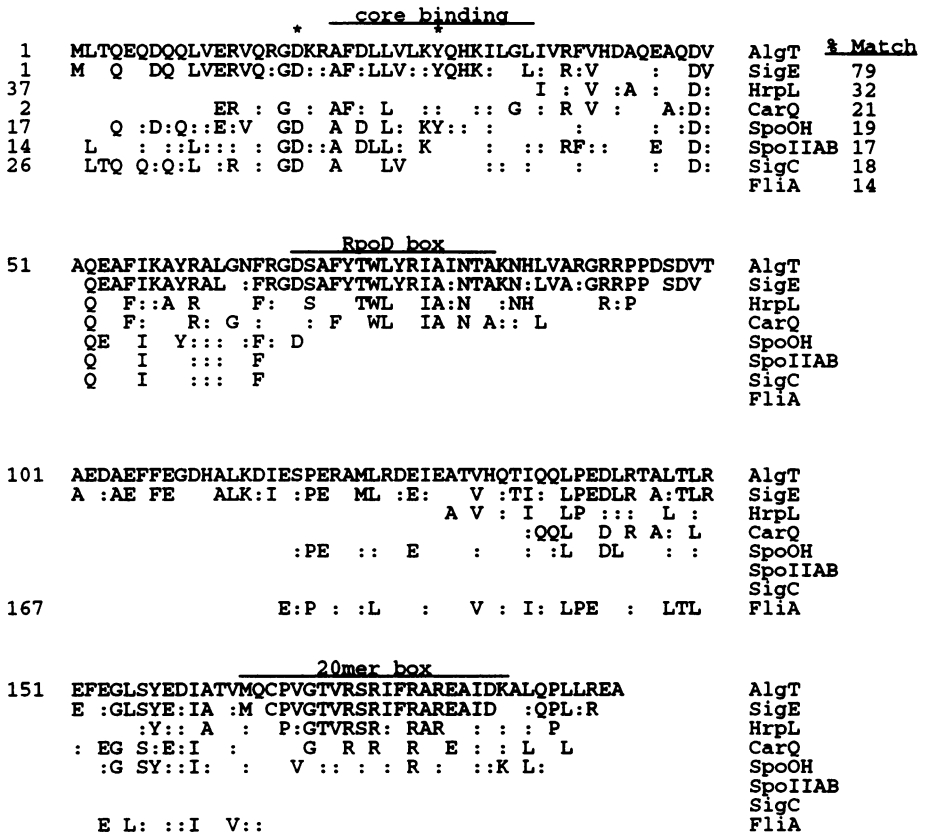

FIG. 2. Similarity of AlgT to known and putative alternative sigma factors as determined by the BLAST algorithm. Partial sequences shown that matched AlgT include those from SigE (σ^E or RpoE) of *E. coli* (GenBank accession number P34086), HrpL of *P. syringae* pv. syringae (accession number U03854), CarQ of *M. xanthus* (accession number X71062), Spo0H (σ^H) of *B. subtilis* (accession number P17869), SpoIIAC of *B. megaterium* (accession number X63757), SigC of *M. xanthus* (accession number L12992), FliA of *E. coli* (accession number P31804), and WhiG of *S. coelicolor* (accession number P31804). The sequence conservation relationships described by Lonetto et al. (37) were used to identify the potential locations of polymerase core binding, an RpoD box (i.e., −10 recognition), and a 20-mer (i.e., −35 recognition). Note that the amino acids at codons 18 and 29 (shown with asterisks) are located within or near the putative RNA polymerase binding site. Amino acids are designated by one-letter symbols. Letters and dots (:) below the AlgT sequence indicate identities and conservative substitutions, respectively, that were considered significant by the BLAST algorithm (1). The numbers to the left indicate either the residue number of AlgT or the first residue in other proteins showing a match with the AlgT sequence. The overall percent match according to BLAST (identical plus conservative amino acid substitutions) relative to the entire sequence of AlgT (193 amino acids) is indicated to the right of the protein name.

RNase protection assays were used to determine whether the mutant *algT* alleles in the spontaneous Alg$^-$ mutants were affected in the chromosomal production or accumulation of transcripts in the *algT* region. As a control for RNA levels, a probe of *nadB* was used (Fig. 4A). The *nadB* antisense RNA probe was used in hybridizations with RNA from strains containing wild-type *algT*$^+$ (FRD39) and mutant alleles *algT18* (FRD60), *algT29* (FRD75), and *algT*::Tn*501*-33 (FRD440). The *nadB* gene is divergently transcribed upstream of *algT* and may be involved in de novo NAD biosynthesis (12). These hybridization reactions were then subjected to RNase, and samples containing the resulting protected, double-stranded fragments were separated on polyacrylamide gels. The autoradiograms (Fig. 4B) and densitometric readings (Table 2) showed that levels of the expected 175-bp protected *nadB* transcript remained relatively constant in the strains examined. Thus, the *nadB* data provided a background control to show that the amounts of total RNA present in the reactions were approximately the same.

An antisense RNA probe of *algT* (Fig. 4A) was used in hybridizations with RNA from strains containing wild-type *algT*$^+$ (FRD39) and mutant alleles *algT18* (FRD60), *algT29* (FRD75), and *algT*::Tn*501*-33 (FRD440). A strong band of the expected 150 bp protected *algT* transcript was observed in the Alg$^+$ strain FRD39. In contrast, strains with mutant *algT18* and *algT29* alleles showed a decrease in the amount of *algT* transcripts (Fig. 4C). Densitometry was used to estimate *algT* transcript levels and indicated a four- to fivefold reduction in the *algT18* and *algT29* strains (Table 2). This result suggests that either *algT* positively regulates its own transcription or the mutations in *algT* affected mRNA stability. As expected, no *algT* transcript was detected in the *algT*::Tn*501*-33 strain (Fig. 4C).

Mutant *algT* alleles also affect *algN* transcription. Since *algT* appeared to promote its own transcription, an RNase protection assay was performed to determine if *algT* also controls transcription of the adjacent gene, *algN*. An *algN* antisense RNA probe (Fig. 4A) was used in hybridizations with RNA from strains containing wild-type (FRD39) and mutant (FRD60, FRD75) *algT* alleles. The results of the RNase protection assays using the *algN* probe showed a pattern similar to that seen with the *algT* probe. There were high levels of the expected 165-bp protected *algN* transcript in the wild-type strain (FRD39), but they were dramatically reduced in

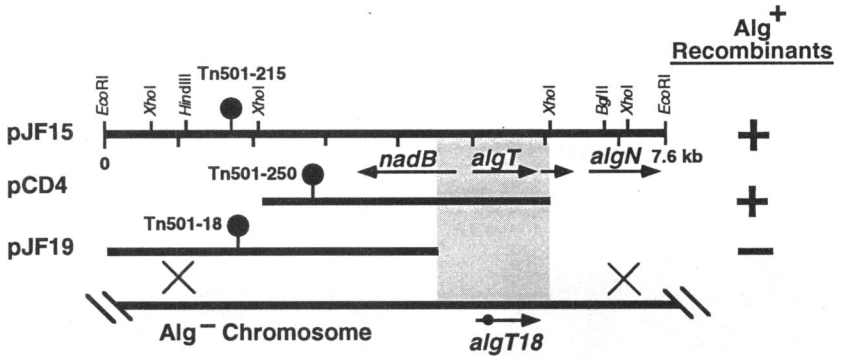

FIG. 3. Depiction of gene replacement experiments used to demonstrate that a spontaneous missense mutation in *algT* was responsible for the nonmucoid phenotype. Derivatives of pJF15, with various Tn*501* insertions to provide the mercury-resistant selectable marker, were used to replace homologous regions of chromosomal DNA of a spontaneous nonmucoid strain with the *algT18* allele (e.g., FRD60). +, mucoid (Alg^+) recombinants were obtained; −, mucoid recombinants were not observed. The grey box shows the $algT^+$ region which was necessary to obtain Alg^+ recombinants.

strains FRD60 and FRD75, which had altered *algT* alleles (Fig. 4D; Table 2). In the *algT*::Tn*501* strain, there was little if any detectable *algN* transcription. These data suggest that *algT* is also a positive regulator of *algN* transcription. Loss of *algN* transcription in FRD440 may be due to the polar *algT*::Tn*501* mutation if *algT* to *algN* forms an operon.

Autoregulation of *algT*. To quantitate *algT* promoter activity, a promoterless *cat* gene was inserted into *algT* for use as a reporter in *P. aeruginosa*. For this analysis, a mini-Tn*3-cat* derivative was transposed into *algT* on the low-copy-number plasmid pJF15 and shown by restriction analysis to have *cat* in the transcribing orientation. The pJF15::mini-Tn*3-cat* construction was then mobilized into *P. aeruginosa* strains containing wild-type and mutant *algT* alleles. An ELISA was used to measure CAT antigen in crude cellular extracts, and the levels obtained relative to protein concentration were assumed to correspond to *algT* promoter activity. The amount of CAT in Alg^+ FRD39 was approximately threefold greater than the levels seen in the Alg^- strains FRD60 and FRD75, which had the mutant *algT18* and *algT29* alleles (Table 3). These relative changes in *algT-cat* transcription due to the spontaneous *algT* alleles were similar to the densitometric readings obtained in the RNase protection assays described above. The *algT-cat* activity in the *algT*::Tn*501* background (FRD440) was about 20-fold lower than that seen in the $algT^+$ (FRD39) background (Table 2). Thus, *algT* was subject to transcriptional activation by its own gene product.

Transcriptional initiation site of *algT*. A primer extension analysis was performed to identify the initiation site of *algT* transcription. A 40-base oligonucleotide, complementary to the start of the *algT* coding strand, was end labeled and hybridized to RNA isolated from FRD39 and FRD60. The primer was extended to the beginning of the transcript, and the product was then examined by polyacrylamide gel electrophoresis. A sequencing ladder, formed with the same 40-base primer using a clone of *algT* (pCD4) as the template, was included to determine the size of the product. A major band was detected (Fig. 5A, lane 1) next to a cytosine located 54 bases upstream of the predicted translation initiation codon of *algT* (shown in Fig. 1). In contrast, the extension product from the *algT* transcript was not readily detected in Alg^- FRD60 (Fig. 5A, lane 2), which was consistent with the results obtained in the RNase protection and *algT-cat* assays above.

Transcriptional analysis of *algT* in nonmucoid strain PAO1. We also did a primer extension analysis of *algT* in *P. aeruginosa* PAO1, a typical nonmucoid strain which has not been associated with CF infection. Our initial hypothesis was that failure to express *algT* may be typical of any Alg^- strain. However, *algT* transcription was readily detectable in PAO1, and it initiated at the same base as in FRD39 (Fig. 5A, lane 3). We then developed a mucoid derivative of PAO1 by introducing pCD4, a plasmid which contains a 4-kb clone of *algT* (shown in Fig. 3). We recently showed that the Alg^+ phenotype can be *trans* activated in PAO1 by such a plasmid because the adjacent negative regulator *algN* is deleted (26). Compared with nonmucoid PAO1, there was a dramatic increase in the amount of *algT* transcripts in mucoid PAO1(pCD4) as a result of the increased copy number of *algT* (Fig. 5A, lane 4). The *algT* gene of PAO1 was sequenced and found to be identical to that of FRD1; thus, AlgT is not defective as it is in the Alg^- revertant FRD60 (data not shown).

Promoter of *algT*. The upstream region preceding the transcriptional initiation site of *algT* showed little similarity to the consensus −10 Pribnow box (TATAATG) or −35 (TTGACA) region in promoters recognized by RNA polymerase containing an *E. coli*-like vegetative σ^{70}. However, striking similarity to the promoter region reported (34) for *algR* was noticed. An alignment of the *algT* and *algR* promoters (Fig. 5B) showed a 7-bp match in the −10 region (CGAGTCT) and a 6-bp match in the −30 region (ACTTTT). Some homology between the *algT* promoter and the reported (10) promoter for *algD* was also observed, and this overlapped the regions common to the *algT* and *algR* promoters. Thus, AlgT may act as an alternate sigma factor to directly activate transcription of promoters at *algT*, *algR*, and possibly *algD*.

DISCUSSION

Adaptation to the changing environment of a new host is frequently important for the success of a microbial pathogen, and this is often manifested through variations in cell surface structures. *P. aeruginosa* strains associated with the chronic infections of CF patients undergo a variety of changes in surface structures, including conversion to the highly mucoid form (45), which is indicative of alginate overproduction. In general, phenotypic variation may be accomplished by a sensory transducer (e.g., two-component system) that responds to specific signals in the changing environment (44) or through the generation of genetic variants that provide the diversity needed to ensure survival (49, 52). *P. aeruginosa* has both of these mechanisms for adaptation to the mucoid form: environmentally responsive two-component regulatory proteins (9, 54)

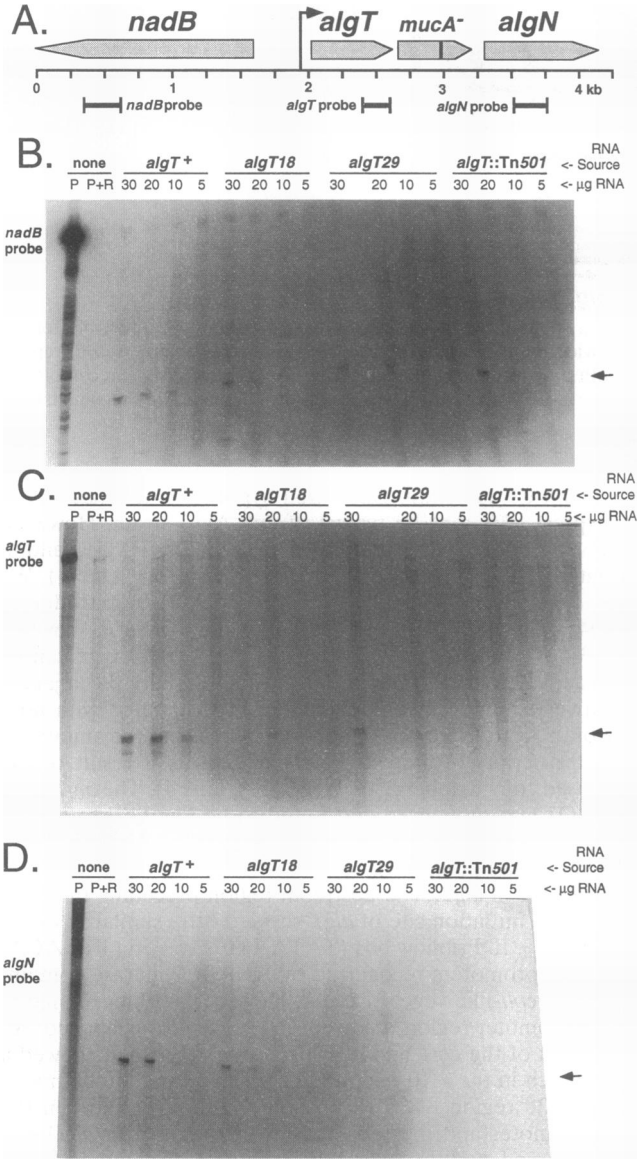

FIG. 4. Results of RNase protection assays used to determine the relative abundance of transcripts for *nadB*, *algT*, and *algN* in Alg^+ strain FRD39 (*algT*$^+$) and in Alg^- strains FRD60 (*algT18*), FRD75 (*alg29*), and FRD440 (*algT*::Tn*501*). (A) Map showing the open reading frames examined and approximate locations of probes containing sequences complementary to the transcripts of *nadB*, *algT*, and *algN*. (B) Assay for *nadB* transcripts, using antisense RNA (309 bases) generated from pCD12 to obtain a 175-bp protected fragment following hybridization and RNase. (C) Assay for *algT* transcripts, using antisense RNA (296 bases) generated from pCD45 to produce a 150-bp protected fragment following hybridization and RNase. (D) Assay for *algN* transcripts, using antisense RNA (311 bases) generated from pCD89 to produce a 165-bp protected fragment following hybridization and RNase. Symbols: P, probe with yeast RNA but no *P. aeruginosa* RNA shows full-length, undigested probe; R, probe with yeast RNA and RNase shows no protected probe in the absence of *P. aeruginosa* RNA. Sources of RNA are shown above each set of lanes. Numbers indicate the amounts of RNA per reaction (30, 20, 10, and 5 µg). Arrows on the right indicate protected probes in the presence of *P. aeruginosa* RNA.

TABLE 2. Densitometry analysis of RNase protection assays to determine relative transcript levels for *nadB*, *algT*, and *algN* in various *P. aeruginosa* FRD strain backgrounds[a]

Strain	Chromosomal genotype	RNA level (% of control)		
		nadB probe	*algT* probe	*algN* probe
FRD39 (control)	*algT*$^+$	9,211 (100)	25,292 (100)	16,614 (100)
FRD60	*algT18*	7,178 (78)	6,142 (24)	3,062 (18)
FRD75	*algT29*	8,201 (89)	5,328 (21)	2,655 (16)
FRD440	*algT*::Tn*501*	8,349 (91)	0 (0)	744 (4)

[a] The map in Fig. 4 shows the open reading frames examined and locations of probes used to detect protected transcripts for *nadB* (175 bp), *algT* (150 bp), and *algN* (165 bp). The values shown (arbitrary units) were determined with a densitometer and indicate the amount of protected RNA in a typical experiment (Fig. 4B to D). Most values represent the data obtained from the reactions containing 20 µg of RNA. Note that *nadB* transcript levels showed little variation among the strains, whereas *algT* and *algN* transcripts were reduced approximately four- to fivefold in the FRD60 and FRD75 backgrounds. The *algN* transcripts were reduced about 20-fold in the *algT*::Tn*501* background.

as well as mechanisms for the generation of genetic variants (reference 40 and this study). The mucoid phenotype also correlates with phenotypes that include reduced production of specific virulence determinants (e.g., proteases, exotoxin A, exoenzyme S, phospholipase C, and pyochelin levels) (38, 47, 53) and conversion to lipopolysaccharide-rough (32, 38). This down-regulation in the production of virulence factors is consistent with a tendency in chronic host-parasite relationships to progress toward a state of commensalism (53).

When strains of mucoid *P. aeruginosa* are no longer associated with the patient and are manipulated in the laboratory, they often spontaneously return to the nonmucoid form (46). The expense in terms of energy and carbon which is devoted to the overproduction of alginate by mucoid *P. aeruginosa* is great (43), and so one might predict the involvement of a genetic switch to turn off its production when this capsule-like polymer is not needed. For example, phase variation of type I pilin expression in *E. coli* is under the control of a promoter within an invertible element that permits random and reversible activation and inactivation of its expression (22). The Alg^- to Alg^+ and back to Alg^- phenotypic variations also suggested that the mechanism may be reversible. In our earlier genetic analyses of the alginate switching locus at 68 min, we have referred to it as *algS*(On) and *algS*(Off) (18). However, the data presented in this study suggest that spontaneous mucoid-to-nonmucoid conversion is usually not the result of a reversible switch mechanism.

A sequence analysis of *algT* in spontaneous nonmucoid variants was undertaken here to better understand the alginate

TABLE 3. CAT levels in cell extracts of *P. aeruginosa* strains containing an *algT-cat* reporter

P. aeruginosa strain[a]	Chromosomal genotype	Amt (ng) of CAT/0.2 µg[b] (% of control)
FRD39 (*algT-cat*) (control)	*algT*$^+$	61 (100)
FRD60 (*algT-cat*)	*algT18*	21 (34)
FRD75 (*algT-cat*)	*algT29*	22 (36)
FRD440 (*algT-cat*)	*algT*::Tn*501*	2 (3)

[a] All strains contained pJF15::Tn*3-cat* in which an *algT-cat* transcriptional fusion was contained on the low-copy-number plasmid.

[b] Cell extracts for CAT assays were obtained by sonication, and specific CAT levels were determined by ELISA. A standardized amount of protein (200 µg per well) was used for each test, and then 10-fold dilutions of the samples were used to obtain values in the linear range of the assay. Data shown represent one of three comparable experiments.

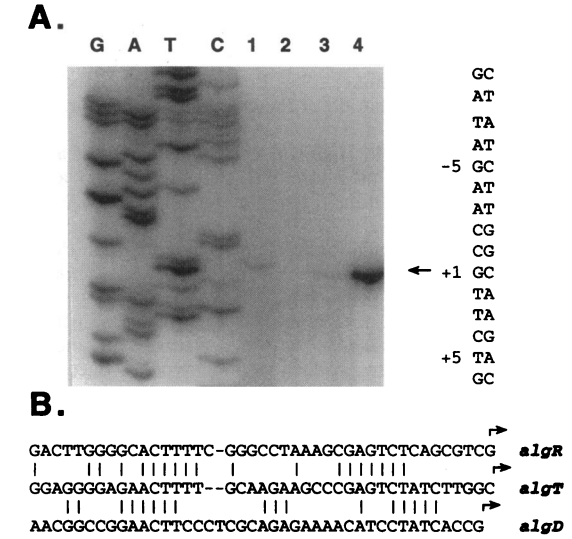

FIG. 5. A. Transcriptional initiation site of *algT* as determined by primer extension analysis. The oligonucleotide used (CD40) was end labeled and used to map the 5′ end of the *algT* transcript. RNA preparations from the following strains were analyzed: FRD39 Alg^+ (lane 1), FRD60 *algT18* (lane 2), PAO1 Alg^- (lane 3), and PAO1 (pCD4) Alg^+ (lane 4). The arrow identifies the start of *algT* transcription at a cytosine, designated +1 on the sequence shown to the right, which is located 54 bases upstream of the predicted translational start site of *algT*. The *algT* sequencing ladder (GATC) was produced with the same oligonucleotide used for synthesis of the probe in the primer extension experiment. Note that the *algT* primer extension product was detected in both Alg^+ FRD39 and Alg^- PAO1, whereas it was overproduced in Alg^+ PAO1(pCD4) but undetectable in the *algT18* mutant FRD60. (B) Comparison of the promoter of *algT* with promoters of *algR* and *algD*. An alignment of the three promoters shows matching bases with the symbol |. The transcriptional initiation site for each gene is depicted with an arrow. The three promoters show little similarity to the −10 and −35 regions typically recognized by vegetative (σ^{70}-like) promoters but do share homology to each other. In that AlgT controls expression of its own promoter, it may also be involved in *algR* and *algD* expression.

conversion process and the role of AlgT in the complex regulation of alginate biosynthesis. This analysis revealed that missense mutations often occurred in the *algT* coding sequence and were responsible for the conversion to Alg^-. It was curious that among the many independently isolated nonmucoid variants, there were two codons in *algT* that often underwent the same mutations. These were designated the *algT18* and *algT29* mutant alleles, and both were the result of A-to-G transitions: GAC (Asp-18)→GGC (Gly) and TAC (Tyr-29)→TGC (Cys), respectively. It is tempting to speculate that there may be a mechanism which controls this specificity in the base pair alterations observed, and this possibility is under investigation.

It is intriguing that spontaneous mutations in *algT* occur most commonly when mucoid *P. aeruginosa* (a strict aerobe) is under the stress of low oxygen tension. A BLAST search revealed that AlgT has striking similarity (65% identity) to SigE (also known as σ^E or RpoE) of *E. coli*, a sigma factor of similar size that is involved in a stress response (14). It was likewise of interest that AlgT shows 32% similarity to HrpL of *P. syringae* pv. syringae, a putative sigma factor which is part of an environmentally regulated cascade that activates expression of genes for plant pathogenesis (57). AlgT is also part of an environmentally regulated cascade of genes (55). AlgT (also referred to as AlgU) shows weaker sequence similarity to σ^H in *Bacillus* sp. (40) as well as other alternate sigma factors in a wide variety of bacteria. This limited homology between AlgT and known alternate sigmas would not be considered unusual (37). Our alignment of several sigma factors related to AlgT revealed that the spontaneous mutations near the N terminus were in codons that are often conserved. Also, the substitutions were observed in or near critical areas identified as potential core binding regions. This finding suggests that the mutations arise to reduce expression of AlgT-dependent promoters in mucoid *P. aeruginosa*.

The missense mutations in *algT* identified here provided tools with which to study AlgT function. RNase protection assays were used to measure and compare the chromosomal production and accumulation of *algT* transcripts in different backgrounds. Compared with the wild-type Alg^+ strain, *algT* transcripts were significantly (four- to fivefold) reduced in the spontaneous Alg^- strains with the *algT18* and *algT29* alleles. Overall, the data suggest that the product of *algT* positively regulates its own transcription. To quantitate *algT* promoter activity, a plasmid-borne *algT-cat* fusion was constructed for use as a reporter in *P. aeruginosa*. Compared with the amount of CAT in the Alg^+ strain, CAT levels were approximately 3-fold lower in the Alg^- strains with the *algT18* and *algT29* alleles and 20-fold lower in the *algT*::Tn*501* background. Thus, the mutations were missense in nature, and the altered AlgT18 and AlgT29 molecules still retained some native activity. On the basis of homology comparisons, AlgT probably acts as an alternate sigma factor required for expression of certain alginate gene promoters, including its own.

We also examined *algT* transcription in *P. aeruginosa* PAO1, a wild-type nonmucoid strain not associated with CF infection. It was interesting that *algT* transcription was readily detectable in PAO1, suggesting that its wild-type Alg^- phenotype was not due to a failure to express *algT*. This was in contrast to the spontaneous $Alg^+ \rightarrow Alg^-$ derivatives of FRD (with *algT* missense alleles) in which levels of *algT* transcripts were reduced. A derivative of PAO1, in which *algT* was supplied in *trans* without its adjacent negative regulator *algN*, demonstrated a mucoid phenotype and showed a dramatic increase in the amount of *algT* transcripts. This finding suggests that there may be a stoichiometric relationship in which an abundance of AlgT can overcome the effects of its negative regulators encoded downstream by *mucA* and *algN* (*mucB*).

The initiation site of *algT* transcription was identified 54 bp upstream of the predicted translational start site of *algT*. The upstream region preceding the transcriptional initiation site of *algT* showed little similarity to the consensus −10 and −35 regions in promoters recognized by RNA polymerase containing an *E. coli*-like vegetative σ^{70}. However, they were remarkably similar to the same region upstream of *algR*. Specifically, the *algT* and *algR* putative promoters showed a 7-of-7-bp match starting at −9, and a 9-of-11-bp match starting at −27, relative to the start of transcription. Weaker homology between the *algT* and *algD* promoters was also observed. A similar comparison that included a more specific analogy to σ^E-dependent promoters was recently made (11). In that AlgT is needed for transcriptional activation of *algR* and *algD* (56), AlgT may act directly at one or both of these promoters. Studies are currently in progress to determine whether AlgT acts as an alternate sigma factor to directly activate transcription of promoters at *algT*, *algR*, and possibly *algD*.

The RNase protection assay also showed that *algT* controls *algN* transcription. There were high levels of *algN* transcripts in wild-type Alg^+ cells, but the levels decreased dramatically in strains with the altered *algT* alleles, especially in the *algT*::Tn*501* strain. Thus, *algT* appears to be a positive regulator of

the *algT-N* cluster. The results of our preliminary genetic experiments suggest that *algT* to *algN* may form an operon (12). Martin et al. (41) have proposed that *mucA*, between *algT* (*algU*) and *algN* (*mucB*), is a negative regulator of AlgT and that *mucA* is often defective in mucoid CF strains, such as the one we used here. It is curious that the gene product of *algT* controls not only its own transcription but apparently that of the two adjacent genes (*mucA*, *algN* [*mucB*]) encoding negative regulators of its own activity. However, a similar arrangement exists in *B. subtilis*, in which the secondary sigma factor σ^B transcribes a subset of three genes, cotranscribed with its own, that negatively regulate σ^B-dependent gene expression (4). This provides an attractive model for alginate regulation via AlgT. However, further study is needed to show how these multiple proteins interact to control AlgT-dependent gene expression.

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