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The *algT* (*algU*) gene of *Pseudomonas aeruginosa*, a key regulator involved in alginate biosynthesis, encodes an alternative σ factor (σ^E)

(cystic fibrosis/virulence/*in vitro* transcription)

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ABSTRACT **Chronic infection by alginate-producing (mucoid) *Pseudomonas aeruginosa* is the leading cause of mortality among cystic fibrosis (CF) patients. During the course of sustained infection, the production of an alginate capsule protects the bacteria and allows them to persist in the CF lung. One of the key regulators of alginate synthesis is the *algT* (*algU*) gene encoding a putative alternative σ factor (σ^E). AlgT was hyperproduced and purified from *Escherichia coli*. The N-terminal sequence of the purified protein matched perfectly with that predicted from the DNA sequence. The purified protein, in the presence of *E. coli* RNA polymerase core enzyme, was able to initiate transcription of an *algT* promoter. Deletion of the −35 region of this promoter abolished this activity *in vitro* as well as *in vivo*. These data indicate that the *algT* gene encodes a σ factor that is autoregulatory.**

Cystic fibrosis (CF) is a common, lethal, genetic disorder. It results from defects in the chloride channels of the epithelial cells, leading to clogging of the CF lung by a thick, dehydrated mucous (1). This environment is fertile ground for infection by many bacteria, including *Haemophilus influenzae, Staphylococcus aureus*, and *Pseudomonas aeruginosa*. Although *H. influenzae* and *S. aureus* can be controlled through antibiotic therapy, *P. aeruginosa* is particularly recalcitrant and eventually becomes the dominant species in the lung. During the course of chronic infection, *P. aeruginosa* undergoes a genetic switch from a normal, nonmucoid phenotype to an alginate-producing mucoid phenotype (2–4). Alginate is a viscous exopolysaccharide, consisting of D-mannuronic and L-guluronic acids. It is a virulence factor that plays a major role in the pathogenicity of *P. aeruginosa* in many ways. Opsonization and phagocytosis by the host immune system are inhibited by the alginate capsule (5, 6). Alginate appears to promote biofilm formation, thus enhancing colonization of the CF lung (7). These alginate-induced biofilms have been shown to confer resistance to antibiotics and to the killing effects of polymorphonuclear leukocytes (8, 9). Mucoidy has also been shown to aid cells in surviving in nutrient-poor environments (10). It is therefore likely that alginate production is selected for and maintained because it confers several advantages, allowing mucoid organisms to persist in the CF lung.

Even though they possess the capability to produce alginate, most *P. aeruginosa* isolates are nonmucoid. Only strains isolated from chronically infected CF patients display the mucoid phenotype, while strains isolated from urinary tract or burn infections are typically nonmucoid. Also, it has been noted that the mucoid phenotype is unstable in cultured isolates of originally mucoid strains (2–4). This clearly suggests that the expression of alginate genes is tightly regulated and the unique environment of the CF lung plays a major role in this regulation. Transcription of the alginate biosynthetic genes is under the control of a complex regulatory cascade and *algT* is one of the key determining factors (Fig. 1) (2–4, 11). When introduced into nonmucoid strains of *Pseudomonas*, the *algT* gene, in multiple copies, is sufficient to induce a switch to mucoidy (12). The activity of AlgT in nonmucoid strains is repressed by MucA and MucB. Many CF isolates are mucoid due to mutations in *mucA* (13). On the other hand, in several spontaneous nonmucoid revertants *algT* itself is mutated, causing the loss of alginate production (4). Thus, *algT* plays a central role in the conversion from nonmucoid to mucoid phenotype.

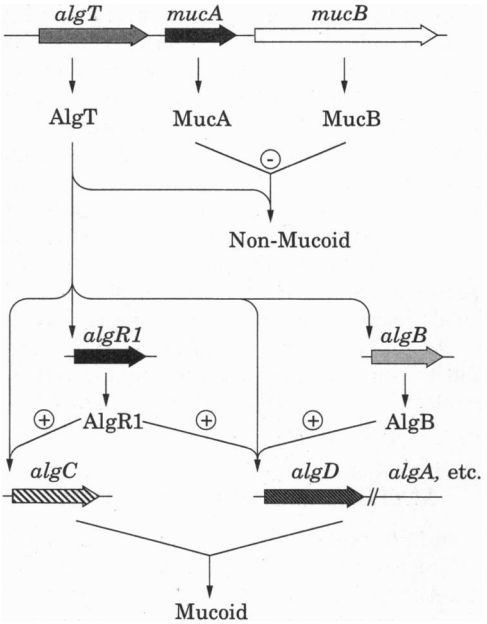

FIG. 1. A model for regulation of alginate production in *P. aeruginosa* (see ref. 11). Shown are most of the genes thought to be involved in regulation of alginate biosynthesis (2–4). Note that when MucA and MucB are present and active, they are able to counteract the activity of AlgT. AlgT acts as a transcriptional activator of many of the alginate biosynthetic genes, including *algR1, algB, algD*, and *algC*. The effect of AlgT on *algC* has been postulated from the observation that the level of *algC* activity (phosphomannomutase) is greatly reduced in AlgT$^-$ mutants (R.W.Y., unpublished data).

The *algT* gene is postulated to encode a σ factor that shows significant homology to σ^E of *Escherichia coli* (2, 4, 14). The σ^E of *E. coli* is a heat shock-related σ factor shown to be necessary for transcription from the P3 promoter of another heat shock σ factor gene, *rpoH* (15, 16). The σ^E analogs from *Streptomyces coelicolor, E. coli*, and *P. aeruginosa* belong to a

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Abbreviation: CF, cystic fibrosis.
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		-35	-10	+1
Ec	*rpoHP3*	CTTGCATTGAACTTGTGGA	TAAAATCACGGTCTGATAAAACAGT	
Sc	*dagAP2*	GCGTTCCGGAACTTTTTGCACGCACGCGAGCTCTCGAATTTTGGC		
Mx	*carQRS*	GAGCGCCGGAAACACTTTCGCAGGTGGCCCGTAGAGGAGTCGGGT		
Pa	*algT*	GGAGGGGAGAACTTTTGCAA	GAAGCCCGAGTCTATCTTGGCAAGACG	
Pa	*algR1*	GACTTGGGGCACTTTTCGGGCCTAAAGCGAGTCTCAGCGTCG		
Pa	*algD*	AACGGCCGGAACTTCCCTCGCAGAGAAAACATCCTATCACCGCGA		
Pa	*algC*	ATCTTCAGGAACTCGGCGGGCAACGG	ACTGCCAAACCCCCTGTGCCT	

FIG. 2. Homology among the promoters recognized by ECF subfamily members (see ref. 11). The −35, −10, and transcriptional start sites are underlined. The primary conserved residues are GAACTT at −35 and TCT at −10. Ec, *E. coli*; Sc, *S. coelicolor*; Mx, *Myxococcus xanthus*; Pa, *P. aeruginosa*.

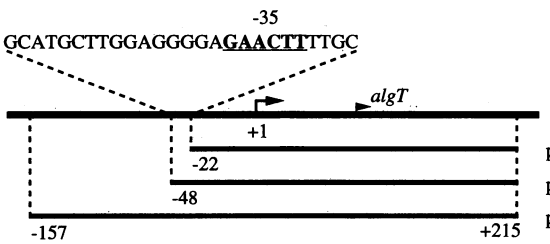
recently defined subclass of the σ^{70}-related class of bacterial σ factors. This subclass is denoted ECF because many of the members are involved in the transcription of genes with extracytoplasmic functions (14). A consensus promoter sequence for the subclass has been proposed (14) (Fig. 2). In addition to mucoidy, AlgT may control the expression of other regulons involved in stress response, which enable the cell to survive exposure to extreme temperature or reactive oxygen intermediates (17). AlgT thus seems to be a global regulator with far-reaching effects on the organism's ability to respond to various stress conditions. Despite the homology of AlgT to σ^E and speculation about its role as a σ factor, no purification or functional characterization of AlgT has been reported. In this paper, we describe the hyperproduction of AlgT and its purification to homogeneity. We also demonstrate its functional homology to *E. coli* σ^E and its role in *in vitro* transcription of the *algT* promoter.

MATERIALS AND METHODS

Strains and Plasmids. *P. aeruginosa* mucoid strain 8821 is a mucoid clinical isolate from the lungs of a CF patient (18). The plasmid pKRZ1 is a β-galactosidase (*lacZ*) promoter probe vector capable of mobilization into and replication in *P. aeruginosa* (19). *E. coli* strain TG1 was obtained from Amersham. *E. coli* strain BL21(DE3)LysS and vector pET11a used for the hyperproduction of AlgT were obtained from Novagen. Plasmid pJET 41 containing the *rpoH* promoters from *E. coli* was kindly provided by Carol Gross (University of California, San Francisco). Protocols for plasmid isolation and plasmid construction were as described (20). To construct pMP7, the supercoiled plasmid used for transcriptional assay, a 240-bp fragment containing the T7 early transcriptional terminator (21, 22) was inserted into the *Eco*RI site of pAN518. RNA polymerase core enzyme and σ^{70} saturated holoenzyme were purchased from Epicentre Technologies (Madison, WI).

Hyperproduction and Purification of AlgT from *P. aeruginosa*. A DNA fragment containing the *algT* gene was generated by PCR (20). PCR was carried out using recombinant *Pfu* polymerase from Stratagene cloning systems. Reaction conditions were as supplied with the polymerase: melting temperature, 95°C (1 min); annealing temperature, 50°C (1 min); elongation temperature, 72°C (1 min). Two primers, TC1 (GAGGAGCTTTCATATGCTAACCCAGGAACAGGATCAGC) and TC2 (TAGTGGATCCTCAGGCTTCTCGCAACAAAGG), containing the restriction sites *Nde* I and *Bam*HI, were used. This PCR product was cloned into the *Nde* I and *Bam*HI site of pET11a, resulting in pT11-1. *E. coli* BL21(DE3)LysS containing pT11-1 was grown in LB medium (Bacto tryptone, 10 g/liter; Bacto yeast extract, 5 g/liter; NaCl, 10 g/liter) in the presence of ampicillin (100 μg/ml) at either 30°C or 37°C. Production of AlgT was induced by adding isopropyl β-D-thiogalactopyranoside to a final concentration of 1 mM when the culture density had reached an A_{600} of 0.6. The cells were disrupted by sonication in TES buffer (50 mM Tris·HCl, pH 7.9/100 mM NaCl/1 mM EDTA) and the hyperproduced AlgT was found to be present in inclusion bodies. These were collected by centrifugation at 15,000 × *g*. The inclusion bodies were resuspended in TES buffer by sonication and were washed three times. The inclusion bodies were solubilized in 3× sample buffer [3% SDS/15% (vol/vol) glycerol/5% 2-mercaptoethanol/50 mM Tris·HCl, pH 6.8] at room temperature. The samples were then electrophoresed on a preparative SDS/12.5% polyacrylamide vertical slab gel. The portion of the gel corresponding to the appropriate molecular mass was excised. Protein was recovered from the gel and renatured as described (23). Protein concentrations were determined by the Bradford protein assay from Bio-Rad. The Coomassie-stained gel and autoradiograms for publication were imaged with the Imagestore 5000 gel documentation system from Ultraviolet Products (San Gabriel, CA).

Cloning of the Promoter Region of *algT*. PCR was used to generate deletions in the promoter region as shown in Fig. 3. The primers used were T2 (CCTTGATGAAGGGATCCTGCGCTACGTCC), T5 (GTCCGGTCGACCTACCCAGCGGCACAGAGGCC),T6(AACTCGTCGACGCATGCTTGGAGGGGAG), and T8 (TTTTGGTCGACAAGAAGCCCGAGTCTATCTTGG). Primers T5, T6, and T8 correspond to the sense strand of DNA in the regions −157, −48, and −23, respectively, relative to the AlgT-dependent transcriptional start site and contain *Sal* I recognition sites near the 5′ ends. Primer T2 corresponds to the antisense strand of DNA in the region +215 relative to the transcriptional start site and contains a *Bam*HI recognition site near the 5′ end. DNA fragments were generated by using the sense primers T5, T6, and T8 and the antisense primer T2. The *Sal* I and *Bam*HI fragments generated were cloned into the *Sal* I and *Bam*HI



	ß-Galactosidase Specific Activity (Miller Units)	
	E. coli	*P. aeruginosa*
pT8PZ	1.4±0.1	5.6±0.2
pT6PZ	20.5±0.5	444±20
pT5PZ	35.7±0.2	600±19

FIG. 3. Diagram of deletions of the *algT* promoter region used in these studies and their *in vivo* activity. Arrow indicates the transcriptional start site (+1) of *algT*. Note that the segment labeled pT5PZ contains the −35 and −10 recognition sites of the *algT* promoter as well as some regions upstream of −35. The plasmid pT6PZ lacks some of the upstream regions but still includes the −35 and −10 recognition sites. pT8PZ lacks the −35 region of the AlgT-dependent promoter. For construction of pT5PZ, pT6PZ, and pT8PZ, fragments from the 5′ end to +215 of the *algT* gene were cloned upstream of the *lacZ* gene in plasmid pKRZ1. For construction of the supercoiled *in vitro* transcription templates pT5P, pT6P, and pT8P, fragments from the 5′ end to +215 were cloned 150 bp upstream of the bacteriophage T7 early terminator in the plasmid pMP7. The predicted size of transcripts from the *algT* promoter in these constructs is 365 bases. The β-galactosidase specific activities of each of the *lacZ* fusions in either *E. coli* (TG1) or *P. aeruginosa* (strain 8821) are indicated on the right.

sites of the plasmid pKRZ1 for *in vivo* assay of *algT* transcriptional activity and the *Sal* I and *Bam*HI sites of the supercoiled plasmid pMP7 to provide the templates for the *in vitro* transcription assay. The clones were designated, to denote the sense primers used in their construction, pT5PZ, pT6PZ, and pT8PZ for the *algT/lacZ* transcriptional fusions and pT5P, pT6P, and pT8P for the supercoiled *in vitro* transcription templates. The promoter activities of the *algT/lacZ* transcriptional fusion constructs were measured by assaying the β-galactosidase specific activity (24).

***In Vitro* Transcription Assay.** The *in vitro* transcription assays were performed with 0.625 pmol of RNA polymerase core or σ^{70} saturated enzyme and 0.6 pmol of AlgT (where indicated). The AlgT/RNA polymerase apoenzyme molar ratio was ≈1:1. The reaction buffer, regardless of reaction volume, was 100 mM potassium glutamate/50 mM KCl/10 mM Tris·HCl, pH 8.0/0.1 mM EDTA/10 μg of bovine serum albumin per ml/1 mM $CaCl_2$/5% glycerol/1 mM dithiothreitol/5 mM $MgCl_2$. Incubations were at 37°C unless otherwise indicated. Association of RNA polymerase and AlgT was carried out in 5 μl for 10 min. Supercoiled template (0.06 pmol) and Promega rRNasin (2 units) were added, and open complex formation was allowed in 10 μl for 10 min. Transcription was initiated in a 12.5-μl reaction mixture by addition of 250 μM each ATP, GTP, CTP; 25 μM UTP; 0.2 μM [α-^{32}P]UTP (2 μCi; 1 Ci = 37 GBq); 50 μg of heparin per ml. Transcription was terminated after 10 min by addition of 7.5 μl of 3.5 M urea/100 mM EDTA/5% glycerol/0.05% bromophenol blue/0.05% xylene cyanol to a final volume of 20 μl. Samples were incubated at 70°C for 5 min, quenched on ice, and electrophoresed on an 8% polyacrylamide gel containing 8 M urea. RNA used for primer extension mapping (see below) was prepared with the same buffer and temperature but with 10-fold the amount of template, RNA polymerase, and AlgT. RNA polymerase preincubation and open complex formation were as described above in 50 and 100 μl, respectively. ATP, GTP, CTP, and UTP (250 μM each) were added to a final volume of 125 μl. Heparin (as above) was added to a 150-μl final volume after 30 min. The transcription reaction was continued for 15 min and then terminated by addition of 5 units (5 μl) of RNase-free DNase (Epicentre Technologies) and incubating for 20 min.

Primer-Extension Confirmation of the *in Vitro* Transcriptional Start Site. An antisense primer, complementary to the region of DNA 100 bp downstream of the predicted transcriptional start site, +100T (TTTTTCTAGAGCTGTACCCGTTCAACCAGT) was end-labeled with T4 kinase using the KinaseMax 5′-end-labeling kit from Ambion (Austin, TX). RNA transcript from the *in vitro* transcription reaction (described above) was isolated using the RNeasy total RNA isolation kit from Qiagen (Chatsworth, CA). The RNA isolation procedure was performed twice to ensure the removal of DNase. The primer-extension reaction was carried out at 37°C for 30 min with 200 units of L-MLV reverse transcriptase and first-strand synthesis buffer from the RiboClone cDNA synthesis kit of Promega.

RESULTS

Hyperproduction and Purification of AlgT from *P. aeruginosa*. It has been observed that it is difficult to hyperexpress the *algT* gene because of the toxicity of its gene product. When *algT* was cloned in high-copy-number plasmids, the cells harboring such plasmids were not viable in liquid medium, making it difficult to clone the *algT* gene under the control of a strong promoter such as *tac* (25). To overcome this problem, the open reading frame of the *algT* gene was directly cloned into pET11a under stringent control of the T7 promoter, and expression was carried out in *E. coli* strain BL21(DE3)LysS. This strain contains the T7 RNA polymerase gene inducible by isopropyl β-D-thiogalactopyranoside. Low levels of uninduced transcription of the RNA polymerase gene are counteracted by the presence of LysS, which inhibits the RNA polymerase. Only when the T7 RNA polymerase is overexpressed by induction will sufficient quantities be present to express AlgT. Hyperproduced AlgT, with an apparent molecular mass of 28 kDa, was found mostly in inclusion bodies as shown in Fig. 4. The σ^E from *E. coli* has a reported molecular mass of 24 kDa. After extensive washing, the AlgT protein in the inclusion bodies was dissolved in 1% SDS solution and purified by preparative SDS/PAGE. The first 10 amino acid residues in the N terminus of the purified protein matched the predicted amino acid sequence of AlgT exactly.

Transcription from a σ^E-Specific Promoter of *E. coli* Using AlgT. The sequence homology of AlgT and *E. coli* σ^E has been described (2, 4, 14). To test the ability of AlgT to act as a σ factor *in vitro*, we performed *in vitro* transcription with the supercoiled template pJET41 (16). The plasmid pJET41 contains the promoters P1, P3, and P4 from the *E. coli rpoH* gene. In the *in vitro* transcription from this template, P1 and P4 are σ^{70} dependent, producing transcripts 290 and 428 nucleotides long. P3 is σ^E dependent, producing a transcript of 295 nucleotides. The results of *in vitro* transcription with the *rpoH* promoters in the presence of AlgT are shown in Fig. 5. Neither core RNA polymerase nor AlgT alone was able to produce any specific transcripts from this template. RNA polymerase saturated with σ^{70} produced two transcripts corresponding to the P1 and P4 promoters. When core RNA polymerase and AlgT were used, a single RNA species of 295 bases, corresponding to the P3 promoter, was produced. This demonstrated that AlgT is capable of substituting for σ^E from *E. coli* in the *in vitro* transcription from σ^E-dependent promoter of *E. coli*. AlgT is thus functionally analogous to its *E. coli* counterpart.

AlgT-Dependent Transcription of the *algT* Promoter. The *algT* promoter shows homology to the newly recognized class of eubacterial promoters of genes involved in extracytoplasmic function (14). An alignment of some of the members of this class of promoters is shown in Fig. 2. Note that the promoters for *algC, algD*, and *algR1* match the consensus sequence well. This suggests that the promoters of the alginate genes may all share common regulation with *algT*. We chose to study the *algT* promoter as a model to gain insight into the activity of other alginate gene promoters. To test this, *in vitro* transcription was performed with the AlgT protein and the *algT* promoter as a template. The *algT* promoter has been shown to be dependent on AlgT for its transcription (4, 17). Plasmids pT5P, pT6P, and pT8P containing progressive deletions of the *algT* promoter

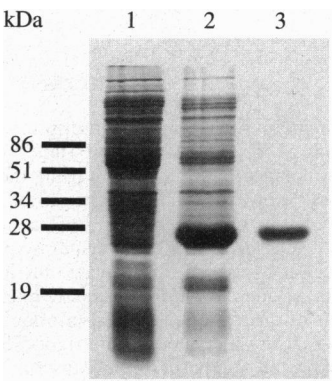


FIG. 4. Purification of the *P. aeruginosa* AlgT protein from *E. coli*. The *algT* gene was overexpressed in *E. coli* strain BL21(DE3)LysS. The inclusion body fraction containing AlgT was obtained by centrifugation. The AlgT protein was subsequently purified by preparative SDS/PAGE as described. Shown is a Coomassie-stained SDS/polyacrylamide gel. Lanes: 1, 37-μg supernatant of the crude extract from cells hyperproducing AlgT; 2, 12-μg inclusion body fraction; 3, 1.2-μg purified protein.

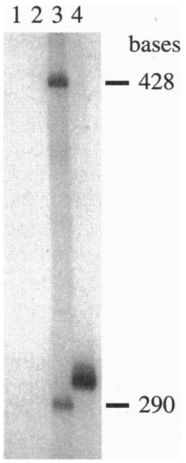


FIG. 5. *P. aeruginosa* AlgT confers *rpoH* P3 promoter recognition on *E. coli* RNA polymerase. Plasmid pJET41 [containing σ^{70} responsive promoters P1 and P4 (generating transcripts of 290 and 428 bases, respectively) and σ^{E} responsive promoter P3 (generating a transcript of 295 bases) from *E. coli* was used as the template for transcription using the following RNA polymerase species. Shown is an autoradiogram of a TBE/urea gel. Lanes: 1, core RNA polymerase alone; 2, AlgT protein alone; 3, σ^{70} saturated holoenzyme producing transcripts from the P1 and P4 promoters; 4, core RNA polymerase preincubated with AlgT protein producing a single transcript from the P3 promoter. Reported sizes (16) of the transcripts produced from P1 and P4 promoters are indicated on the right.

region were cloned so that a transcript of 365 bases would be produced; 215 bases of the transcript are from the *algT* gene and 150 bases are from the vector sequence upstream of the T7 terminator. A diagram of these deletions is shown in Fig. 3. It should be noted that pT5P contains the −10 and −35 sequences of the *algT* promoter and regions further upstream. Plasmid pT6P contains the *algT*-dependent promoter's −10 and −35 sequences but not these upstream regions. Plasmid pT8P lacks the −35 sequence of the *algT* promoter and the regions further upstream. The results of this experiment are shown in Fig. 6*A*. Neither core RNA polymerase, σ^{70} saturated RNA polymerase, nor AlgT alone was able to produce specific transcripts from any template. Core RNA polymerase with AlgT produced a single transcript of the appropriate size. Template pT5P showed the highest level of transcription and pT8P showed essentially no transcription at all. Primer-extension mapping (Fig. 6*B*) of the product of the pT5P reaction confirmed that the transcript corresponded to the known start site for *algT* transcription *in vivo*, previously reported as the adenosine residue 55 nucleotides upstream of the *algT* initiation codon (17). This confirms the ability of AlgT to direct transcription from its own promoter *in vitro*, corresponding with observations *in vivo* (4, 17).

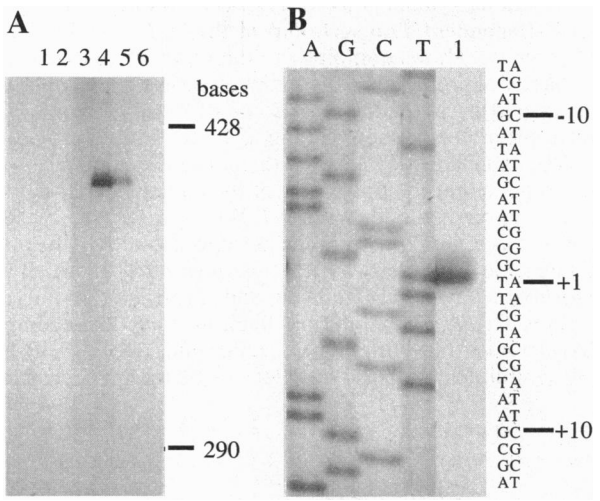


FIG. 6. *P. aeruginosa* AlgT directs transcription of the *algT* promoter. (*A*) Plasmids pT5P (lanes 1–4), pT6P (lane 5), and pT8P (lane 6) were used as templates for transcription using the following RNA polymerase species. Shown is an autoradiogram of a TBE/urea gel. Lanes: 1, core RNA polymerase alone; 2, AlgT protein alone; 3, σ^{70} saturated holoenzyme; 4–6, core RNA polymerase preincubated with AlgT protein. A single transcript is produced only in the presence of core RNA polymerase and AlgT. Molecular size markers corresponding to the products of the σ^{70}-directed transcription of the P1 and P4 promoters of pJET 41 are indicated on the right. The *algT* transcript is ≈365 bases as expected; 215 bases of the transcript are from the *algT* gene and 150 bases are from the vector. Note that pT6P produces transcripts of the same size but with reduced efficiency when compared with pT5P. (*B*) Confirmation of the start site of *in vitro* transcription from the *algT* promoter. Shown is an autoradiogram of a TBE/urea gel. Lane 1, product of the primer-extension reaction. The sequence of the region is indicated on the right. Residue corresponding to the start of transcription is labeled +1. The *algT* sequencing ladder was produced with the same oligonucleotide used to produce the end-labeled primer for primer extension.

Activity of the *algT* Promoter Region *in Vivo*. To determine the relevance of these *in vitro* results, the activities of the same series of deletions of the *algT* promoter region were studied *in vivo*. Plasmids pT5PZ, pT6PZ, and pT8PZ each contain transcriptional fusions of the deletions of the *algT* promoter described above fused to the promoterless *lacZ* gene. These were used as *in vivo* probes of *algT* transcription (Fig. 3). In *E. coli* and in mucoid *P. aeruginosa* strain 8821, the activity from pT6PZ was lower than pT5PZ. In pT8PZ, transcriptional activity was greatly reduced. This corresponded with the transcriptional activities of these regions observed *in vitro*.

DISCUSSION

It has been shown that the *algT* gene is required for expression of many genes involved in alginate biosynthesis (2, 4, 17). The AlgT protein shares 66% identity and 91% overall similarity with σ^{E} from *E. coli* (17). It also has similarities with other sigma factors, especially those belonging to the newly classified ECF subfamily of σ^{70}-related σ factors (14). In this study, we hyperproduced and purified AlgT protein and demonstrated that it initiates transcription from the σ^{E}-dependent *rpoH* promoter (P3) of *E. coli*. This indicates that AlgT can functionally replace σ^{E} from *E. coli*. We have also shown that AlgT can direct transcription of the *algT* gene *in vitro*. These results provide biochemical evidence that the *algT* gene from *P. aeruginosa* encodes an alternative sigma factor, σ^{E}. The −35 and −10 regions of the σ^{E}-dependent promoters from many species and the alginate promoters *algT, algR1, algD, algB*, and to some extent *algC* show strong homology with one another (Fig. 2). Genetic evidence has shown that expression of these genes requires AlgT. Thus, it is likely that in addition to transcription of the *algT* promoter, *P. aeruginosa* AlgT (σ^{E}) is directly involved in transcription of other alginate genes.

Comparing the levels of transcription from the templates pT5P and pT6P in Fig. 6*A*, it is apparent that the region upstream of the −35 recognition site of the *algT* promoter is necessary for full activation of transcription from this promoter. The template pT8P does not contain the −35 recognition sequence of the *algT* promoter and no detectable transcript is produced from this template (Fig. 6*A*). The transcriptional activities of these deletions *in vivo* are similar to the *in vitro* findings (Fig. 3). Thus, the deletion experiments in this study show that the region of DNA, located upstream of the −35 region of the *algT* promoter, may be required for full activation of AlgT-dependent transcription, both *in vivo* and *in vitro*. It is interesting to note the existence of another transcriptional start site, upstream from the σ^{E}-dependent start site, which has been observed in the *algT* and *algR1* promoters (17). In our assays, we did not observe any transcript corresponding to this distal start site. This indicates that any promoter for this site that may exist is most likely not recognized by the σ^{E} protein *in vitro*. Since we used σ^{70} saturated holoenzyme in this assay as well, it is also possible that this promoter is not recognized by σ^{70} either. Indeed, the regions predicted to be −10 and −35 in relation to this start site show very little homology with the required σ^{70} promoter sequences. The contribution of this distal promoter to total *algT* transcription is also of interest. Deletions of the region predicted to be −10 and −35 in relation to this distal transcription site

cause a reduction in the σ^E-dependent transcription of *algT* promoter (Figs. 3 and 6*A*). It is therefore difficult to determine the contribution that transcription from this distal site makes toward total *algT* transcription, because deletions that remove this promoter have an effect on transcription from the σ^E-dependent promoter as well. Further studies with site-directed mutants in both promoters are needed.

It has been reported that the AlgT protein is very difficult to isolate (25). Like many regulatory proteins, it is normally present at very low levels. Previous attempts to hyperproduce the protein have failed. AlgT is thought to be highly toxic when expressed in the absence of MucA (25). It was therefore our goal to hyperproduce AlgT in a manner that would not be toxic to the *E. coli* host cells. The protein sequestered in inclusion bodies has the advantage of being both nontoxic to the cells and easy to purify. The stability of other sigma factors and their ability to be solubilized in SDS and subsequently renatured have been noted (14, 16, 23), raising the possibility that this method may be useful in the study of other systems utilizing alternative sigma factors. Indeed, this method has already been applied to purify other σ factors with similar success (26).

Alternative σ factors have been recognized as important components in the regulation of many bacterial species. Alternative σ factors have been shown to be involved in response to heat and environmental stress (σ^E, σ^{32}) (15, 27), nutrient-limiting stress (σ^{38}, σ^{54}) (27, 28), and flagellin synthesis (σ^{28}) (29). Evidence has been presented that AlgT, in addition to controlling the expression of the alginate biosynthetic cluster, may also control responses to heat shock and environmental stress (17). Furthermore, AlgT has been identified as a member of a newly discovered subfamily (ECF) of sigma factors involved in regulation of extracytoplasmic function. Many of the members of this family play a global role in the cell's response to environmental conditions. Members of this subfamily share some unique characteristics when compared to members of the σ^{70} family. Many of the members of the ECF subfamily lack region 1, which prevents DNA binding in the absence of RNA polymerase. Region 2.3 attributed with promoter melting characteristics is significantly altered, and region 2.4 (−10 recognition) is completely absent. The basic cluster involved in −35 recognition is also poorly conserved. Furthermore, these are the smallest σ factors heretofore identified (14). Further studies of σ^E promise to yield findings of interest to alginate biosynthesis. Of particular interest will be the mechanism of putative interactions between MucA/MucB and σ^E. Other areas of interest such as characterization of the ECF subfamily and of σ factors in general will doubtless benefit as well.

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