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# Integration Host Factor and Sequences Downstream of the *Pseudomonas aeruginosa algD* Transcription Start Site Are Required for Expression

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***Pseudomonas aeruginosa* is an extremely important opportunistic pathogen in immunocompromised individuals. Strains of *P. aeruginosa* isolated from chronic lung infections in patients with the genetic disease cystic fibrosis have a mucoid colony morphology. This phenotype is due to overproduction of the exopolysaccharide alginate, which is believed to confer a selective advantage on *P. aeruginosa* in cystic fibrosis lungs. Alginate biosynthesis is controlled by a complex regulatory mechanism. Genes located in the 34-min region of the *P. aeruginosa* chromosome form an operon which encodes most of the biosynthetic enzymes necessary for alginate production. *algD*, the first gene in the operon and a critical point for the transcriptional regulation of alginate biosynthesis, is controlled by several *trans*, *cis*, and environmental factors. In this study, the involvement of the histone-like protein integration host factor (IHF) in *algD* expression was examined. Sequences with similarity to consensus IHF-binding sites of *Escherichia coli* were identified 75 bp upstream (site 1) and 90 bp downstream (site 2) of the start of *algD* transcription. In gel band mobility shift assays, DNA fragments containing either site bind IHF but site 2 has an approximately 90-fold higher affinity for IHF. Mutations in each of the elements were generated, and they resulted in the reduction or loss of in vitro IHF binding and a three- to fourfold decrease in *algD-cat* expression. This indicates that IHF binding is necessary for high-level *algD* transcription. The presence of a high-affinity IHF-binding site located 3′ of the *algD* transcription start site suggested that sequences further downstream of this element are involved in *algD* expression. When a fragment located downstream of site 2 and upstream of the promoterless *cat* gene (+110 to +835) was deleted, *algD-cat* expression was reduced 10-fold supporting the notion that 3′ enhancer elements are required for *algD* transcription. This is the first direct evidence of a 3′ element involved in the control of a *P. aeruginosa* gene. It is postulated that IHF mediates the formation of a higher-order looped structure which is necessary for efficient *algD* transcription.**

The *Pseudomonas aeruginosa algD* gene encodes GDP-mannose dehydrogenase, an enzyme which converts GDP-mannose to GDP-mannuronic acid, a precursor of the exopolysaccharide alginate. Overproduction of alginate contributes to the morbidity and mortality associated with chronic *P. aeruginosa* infections in individuals with the genetic disease cystic fibrosis (20). A committed step in the overproduction of alginate is transcriptional activation of *algD* (8) and subsequent downstream alginate-biosynthetic genes within the *algD* operon. Significantly, *algD* is transcriptionally activated in mucoid (alginate-producing) *P. aeruginosa* but no expression is apparent in nonmucoid strains (8, 9, 51). Thus, several recent studies have focused on the *cis* and *trans* factors which control *algD* activation.

Two of the *trans* factors known to be involved in *algD* activation, AlgR (7) and AlgB (18, 49), are members of the superfamily of response regulators. These regulators control global cellular functions via a signal transduction mechanism (39). These systems also control the production of virulence factors in many important pathogenic bacteria. AlgR has been purified and shown to bind to *algD* sequences at two high-affinity sites (RB1 and RB2) centered at −468 and −391, respectively, from the transcriptional start site (25, 35). In addition, a low-affinity AlgR-binding site, RB3, centered at −40, has been reported to participate in *algD* activation (36). AlgB has also been purified but does not appear to bind to a sequence upstream of the *algD* transcription start site (47).

Some studies have suggested that DNA topology-nucleoid structure is also important for *algD* expression. The peculiar arrangement of the AlgR-binding sites far upstream of the *algD* promoter suggests that DNA looping is required to facilitate interactions between AlgR and RNA polymerase. The histone-like protein AlgP (AlgR3) is required for *algD* transcription (12, 26). However, more recent work indicates a more general role for this protein as a regulator or structural element for other cellular processes in *P. aeruginosa* other than alginate production and AlgP was renamed $H_p1$ (11). Thus, a direct role for $H_p1$ in *algD* activation has not been elucidated. In addition to $H_p1$, the histone-like integration host factor (IHF) protein has been suggested to be involved in *algD* expression (34, 46, 48). Although the genes encoding IHF have not been cloned from *P. aeruginosa*, this organism likely contains an IHF homolog since IHF binding to high-affinity sites can be detected in extracts of *P. aeruginosa* (50) (see below), and antibodies to *Escherichia coli* IHF cross-react with a protein with a similar molecular mass in *P. aeruginosa* extracts (34, 47). Perhaps the best direct evidence of an IHF in *P. aeruginosa* is the recent purification of an αβ heterodimer IHF-like protein from *P. aeruginosa*. The αβ subunits of this protein have N-terminal sequences highly similar to αβ IHF subunits from *E. coli* and *Rhodobacter capsulatus* (46). This

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protein and IHF from *E. coli* binds to *algD* sequences located at −50 to −80 relative to the transcriptional start site (34, 46) (see below). Binding of IHF induces a DNA bend which was proposed to facilitate interactions of proteins bound at distant sites on *algD* such as AlgR bound at RB1 and RB2 with RNA polymerase (34). However, no mutagenesis of this *cis* element or determination of the role of IHF binding in *algD* activation has been reported.

In experiments described here, two *algD* IHF-binding sites were shown to be required for maximal *algD* expression. A low-affinity IHF-binding site (site 1) spanning positions −80 to −68 relative to the *algD* transcription start site was characterized by gel band mobility shift assays and site-directed mutagenesis. In addition, a sequence located downstream of the *algD* mRNA start site spanning nucleotides +79 to +105 (site 2) was shown to bind IHF with approximately 90-fold higher affinity than site 1. Mutagenesis of these elements revealed that IHF binding is required for high-level *algD* transcription. Moreover, deletion of the sequences from +110 to +835 reduced expression of *algD* 10-fold, indicating an essential *cis* element located within the *algD* gene.

(A preliminary report of a portion of this work has appeared elsewhere [48].)

## MATERIALS AND METHODS

**Growth conditions, bacterial strains and plasmids, media, chemicals, and transcriptional fusion assays.** All of the *P. aeruginosa* and *E. coli* strains used were cultured as previously described, in LB medium with appropriate antibiotics (49, 50). Triparental mating experiments were used to mobilize recombinant plasmids from *E. coli* to *P. aeruginosa*. The *E. coli* strains used were TB1 [*ara* Δ(*lac-proAB*) *rspL* F80 *lacZ* ΔM15 *hsdR*] (Bethesda Research Laboratories), JM109 [*endA1 recA1 gyrA96 thi hsdR17* ($r_{K^-}$ $m_{K^+}$) *relA1 supE44* Δ(*lac-proAB* F′ *traD36 proAB lacI*$^q$ZΔM15)] (Promega), BMH71-18 [*mutS thi supE lac-proAB mutS*::Tn*10* (F′ *proAB lacI*$^q$ZΔM15)] (Promega), MH10900 (RBB63) [F$^-$ λ$^-$ *galK2 rpsL200* IN(*rrnD-rrnE*)*1*] (4), and MH10906 (RBB184) [F$^-$ λ$^-$ *galK2 rpsL200* IN(*rrnD-rrnE*)*1 himA* Δ*Sma*I *himD*Δ*3*::*cat*] (4). The *P. aeruginosa* strains used in this study were FRD1 (Alg$^+$ cystic fibrosis isolate) and its spontaneous nonmucoid derivative FRD2 (38). The wild-type *algD-cat* reporter construct pDJW220 was subcloned from pKK61 (49) into pALTER-1 (Promega) on a 3.1-kb *Sph*I fragment. An identical plasmid (pDJW221) was constructed with the 3.1-kb *Sph*I *algD-cat* fragment in the orientation opposite to that of pDJW220. This facilitated isolation of either DNA strand during infection with a helper phage for site-directed mutagenesis (see below). For analysis of *algD-cat* expression in *P. aeruginosa*, the 3.1-kb *Hin*dIII-*Bam*HI fragment of pDJW220, pDJW221, or their derivatives was subcloned into pLAFR3, a low-copy-number, broad-host-range vector which can replicate in *P. aeruginosa* (44). The *algD-cat* alleles generated in this study were each subcloned into pLAFR3 and were as follows: pDJW222, wild type; pDJW232, *algD14*; pDJW233, *algD15*; pDJW240, *algD16* (Δ*Eco*RI fragment from +110 to +835); pDJW267, *algD17*; pDJW241, *algD22*; pDJW242, *algD23*. Extracts for chloramphenicol acetyltransferase (CAT) assays from *P. aeruginosa* strains cultured to the logarithmic phase ($A_{595}$ = 0.7) were prepared by sonic disruption as described previously (50). CAT levels were determined by an enzyme-linked immunosorbent assay technique as indicated by the manufacturer (5 Prime → 3 Prime, Inc.) and normalized for protein concentration, as determined by the Bradford assay (5) with bovine serum albumin (Sigma) as the standard.

**Nucleic acid manipulations.** Routine nucleic acid manipulations were performed as described elsewhere (1, 49). All plasmids were purified from *E. coli* with Qiagen columns as recommended by Qiagen Corp. DNA sequencing was performed by the dideoxy-chain termination technique with modifications as described elsewhere (49). Oligonucleotides used for PCR and DNA sequencing were synthesized on an Applied Biosystems 380B automated DNA synthesizer. Fragments for gel band mobility shift assays were generated by PCR amplification of *algD* plasmids containing wild-type or mutated IHF-binding sites. The oligonucleotides used for PCR amplification of *algD* fragments containing site 1 were 5′-AAG GCGGAAATGCCATCTCC-3′ and 5′-AGGGAAGTTCCG GCCGTTTG-3′, and they generated a 300-bp fragment located from −23 to −323 relative to the start site of *algD* transcription. The primers used to amplify the 200-bp downstream IHF-binding site 2 were 5′-CGCAGAGAAAACATC CTATC-3′ (−23) and 5′-GTGGGTTTCAATAACGGTGC-3′ (+277). PCR amplifications were performed by the GeneAmp process with *Taq* polymerase (Perkin Elmer). The 50-μl reaction mixture included 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 2 mM $MgCl_2$; 200 μM (each) dATP, dGTP, and dTTP; 100 μM dCTP (unlabeled); 100 μM dCTP (labeled with α-$^{32}$P, 3,000 Ci/mmol [10 mCi/ml]; ICN Biochemicals); 1 μM each primer; and 2.5 U of *Taq* polymerase. This was subjected to 30 amplification cycles, each consisting of a 94°C denaturation step (45 s), a 55°C annealing step (30 s), and a 72°C extension step (1 min), followed by a final 15-min 72°C extension step. Following PCR amplification, unincorporated deoxynucleoside triphosphates and primers were removed by chromatography through G-25 Sephadex columns, an aliquot of the suspension was counted by scintillation, and the yield of DNA produced (in picomoles) was calculated by factoring in the G+C content of the DNA fragment being amplified. Site-specific mutagenesis of the *algD* IHF-binding sites was performed by the Altered Sites technique as previously outlined (50), by following the recommendations of the supplier of the mutagenesis kit (Promega, Inc.). Nucleotides within the *algD* IHF-binding sites were mutated on the basis of mutations which affect binding of *E. coli* IHF to specific sequences and IHF binding to *algB* DNA (30, 31, 50). To accomplish this, single-stranded DNA isolated from *E. coli* JM109 cells containing pDJW220 or pDJW221 (wild-type *algD-cat*) infected with helper phage MK404 was mutagenized with the following mutagenic oligomers and a β-lactamase gene repair oligonucleotide: *algD14*, 5′-AGGGACAAACTAACCCTGTT GAAA-3′; *algD15*, 5′-GCCTTTAATTTAACCAGGTTGA GT-3′; *algD22*, 5′-TTGATACCAACGTTATGGCCTTTCC G-3′; *algD23*, 5′-GAAATATCCGTGTTATATCACTTGA T-3′ (altered sites are underlined). To generate the *algD17* allele, a 3.1-kb *Sph*I fragment from pDJW231 (*algD-cat* with the *algD15* allele) was subcloned into pALTER-1 to generate pDJW262. Single-stranded DNA from cells containing pDJW262 was mutagenized with oligomer *algD14* and a β-lactamase gene repair oligonucleotide. In all of the mutagenesis procedures described above, plasmids with mutations were obtained by selection for ampicillin resistance. All *algD* IHF-binding site alleles were verified by DNA sequence analysis.

**Gel mobility shift assays and affinity measurements.** DNA-binding assays were performed essentially as described previously (50). IHF (generously provided by H. Nash) was used at final concentrations described elsewhere in the text and in the figure legends for each experiment. All *algD* fragments used for gel band mobility shift assays were prepared as described above. These fragments were labeled to high specific activities so low concentrations of DNA (1 fmol to 1 pmol) were needed

in the assays. Following electrophoresis, the gels were dried under vacuum and subjected to autoradiography or direct image analysis on an AMBIS radioanalytical imager with version 2.0 software. Total counts from both free and IHF-bound DNA fragments were determined and used to calculate the fraction of DNA in the complex. These values, expressed as the fraction of free DNA, were plotted against the log IHF concentration to generate Bjerrum plots (see Fig. 4A and B) and to estimate the IHF concentration in which 50% saturation ($K_d$) of sites 1 and 2 occurred. In addition, relative binding constants ($K_b$) for IHF binding to sites 1 and 2 were determined as described elsewhere (40) from the fraction of DNA bound ($f_{bound}$) at a given protein concentration ($P$) in the linear range of these graphs by calculating $K_b = f_{bound}/[P(1 - f_{bound})]$. For all calculations, it was assumed that the IHF used in these assays was 100% active.

**Footprinting of site 2.** Footprinting of *algD* IHF site 2 was accomplished in situ by the 1,10-phenanthroline–copper method (42). Briefly, primers identical to those used to generate the PCR fragments for gel band mobility shift studies of site 2 were individually end labeled and purified as described elsewhere (1, 23, 50). These pairs of *algD* oligonucleotides (sense and antisense) were used to generate PCR fragments containing site 2. These fragments were synthesized with only one strand end labeled by using the end-labeled sense oligonucleotide and a cold (unlabeled) antisense oligonucleotide in a PCR amplification of a plasmid containing *algD*. The opposite strand was labeled by reversing the labeled oligonucleotide. The labeled DNA fragments were purified by spun chromatography through Sephadex G25 columns. Approximately 1 pmol of each PCR product was incubated with 500 nM IHF or left untreated, and the samples were separated on 4% polyacrylamide gel electrophoresis gels as described above for the gel band mobility shift assay. The gel was immersed in 200 ml of Tris-HCl (pH 8.0), and 1,10-phenanthroline, $CuSO_4$, and 3-mercaptopropionic acid were added as previously described (42). The reaction mixture was mixed gently for 10 min at ambient temperature, and the cleavage reaction was stopped by addition of 2,9-dimethyl-1,10-phenanthroline (42) for 2 min. The gels were washed several times in distilled water and exposed to X-ray film for 30 min. The bands representing free DNA and IHF-binding site 2-complexed DNA were excised from the gel, and the DNA was eluted from the gel slice for 12 h at 37°C in 0.5 M ammonium acetate–1 mM EDTA. The DNA was precipitated with ethanol, the pellet was dried, resuspended in formamide sequencing stop solution, heated to 85°C for 2 min, and electrophoresed on a 6% polyacrylamide DNA-sequencing gel. This sample was loaded adjacent to a sequencing ladder generated from pDJW220 (*algD*) primed with the same oligonucleotide used to generate the labeled PCR fragment used for footprinting.

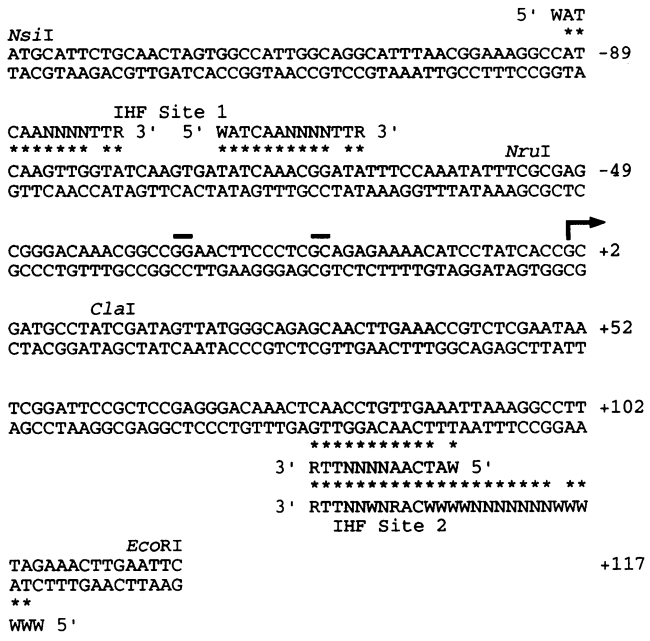


FIG. 1. DNA sequence of the 5′ region of *algD*. The arrow indicates the start of *algD* transcription (51). Sequences with similarity to RpoN promoters are overlined. Pertinent restriction sites are also shown. IHF-binding sites 1 and 2 are illustrated with sequences that have homology to consensus IHF-binding sites (WATCAANNNNTTR; W is dA or dT, N is any deoxynucleoside triphosphate, and R is purine). Site 2 is also similar (25 of 27 identical residues) to the consensus IHF-binding site described by Kur et al. (WWWWW WNNNNNNNWWWWCARNWNNTTR; 29). Asterisks indicate residues conserved between consensus IHF-binding sites and *algD* sequences. The numbers at the right are positions relative to the start of *algD* transcription.

## RESULTS

**Identification of two *algD* IHF-binding sites.** During routine gel band mobility shift assays with a labeled 300-bp *Nsi*I-*Eco*RI *algD* fragment (Fig. 1), a specific complex was formed when *E. coli* cell extracts were tested. Furthermore, a protein present in extracts of an IHF$^+$ *E. coli* strain bound to this fragment whereas no binding was observed when extracts from the isogenic IHF$^-$ *E. coli* strain were tested. The DNA sequence of this FRD1-derived *Nsi*I-*Eco*RI *algD* fragment was determined (Fig. 1) and found to be identical to a previously published *algD* sequence from another *P. aeruginosa* cystic fibrosis isolate (9). Upon examination of this sequence, three consensus binding sites for the histone-like DNA-binding-bending IHF protein were observed (Fig. 1). Two of these elements (IHF site 1) are separated by 8 bp and located approximately 80 bp upstream of the start of *algD* transcription (Fig. 1). Site 1 has previously been described by Mohr and Deretic (34). A third element (IHF site 2) is oriented opposite site 1 and located 104 bp downstream from the *algD* transcription start site (Fig. 1). The three sequences contained within sites 1 and 2 are similar to the consensus IHF-binding site (5′-WATCAANNNNTTR-3′) proposed by Friedman (16). Site 2 contains this core element but also has poly(dA) and poly(dT) sequences located upstream (Fig. 1) and is highly similar to the IHF consensus sequence described by Kur et al. (29).

To determine if IHF binds to these sequences, gel band mobility shift assays were utilized (Fig. 2). In these experiments, two labeled *algD* fragments were generated by PCR amplification of a plasmid containing *algD* and pairs of *algD*-specific primers. These fragments were designed such that one contained only site 2 while the second contained the two consensus IHF sequences within site 1. IHF purified from *E. coli* bound to both of these PCR-generated fragments, sites 1 (Fig. 2, lanes 6 to 8) and 2 (Fig. 2, lanes 2 to 4). However, at identical IHF concentrations, 70% of the fragment containing site 2 was found in the IHF-DNA complex (Fig. 2, lane 2) whereas only 3% of the fragment containing site 1 formed such a complex (Fig. 2, lane 6; percentages were determined by radioanalytical scanning of the gel). These results indicate different affinities for IHF (see below). This binding was specific, since 5′ *algD* PCR-generated fragments tested under

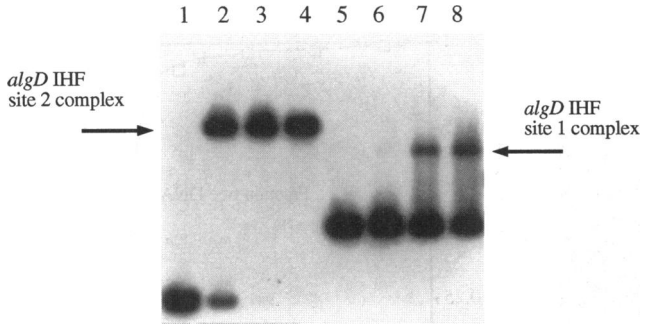


FIG. 2. Analysis of IHF binding to *algD* sequences by the gel band mobility shift assay. Approximately 1 fmol of a PCR-generated fragment containing site 1 (lanes 5 to 8) or 2 (lanes 1 to 4) was incubated with the following concentrations of IHF purified from *E. coli*: lanes 1 and 5, 0 M; lanes 2 and 6, 44 nM; lanes 3 and 7, 440 nM; lanes 4 and 8, 880 nM. Complexes were separated from free DNA by electrophoresis in nondenaturing polyacrylamide gels followed by autoradiography as described in Materials and Methods.

identical conditions failed to form such complexes (data not shown).

A protein present in extracts from both mucoid and nonmucoid *P. aeruginosa* FRD strains also bound to the fragment containing site 2 (Fig. 3, lanes 4 and 6, respectively). This binding was completely eliminated when the *P. aeruginosa* extracts were preincubated with antibodies raised against *E. coli* IHF (Fig. 3, lanes 5 and 7). In addition, the position of the complex formed with purified IHF from *E. coli* was identical to that formed with the protein present in extracts of *P. aeruginosa*. When extracts from mucoid and nonmucoid *P. aeruginosa* FRD strains were tested with a fragment containing upstream site 1, no IHF-specific complex was observed (data not shown).

**Comparison of IHF affinities for sites 1 and 2.** The gel band mobility shift experiment in Fig. 1 and the observation that proteins present in *P. aeruginosa* FRD extracts did not form an IHF-specific complex with a fragment containing site 1 indicate a lower affinity of IHF for this DNA. To more accurately

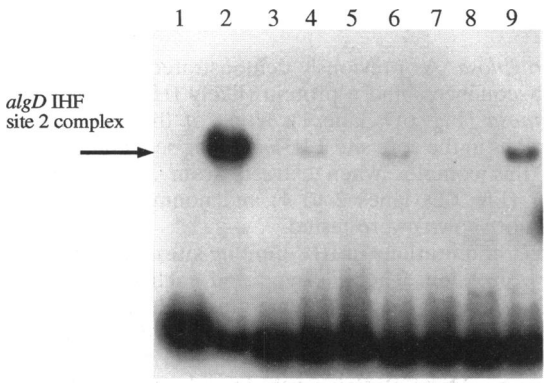


FIG. 3. Binding of IHF in extracts of *P. aeruginosa* to site 2. Approximately 100 fmol of a PCR-generated fragment containing site 2 was incubated with IHF, cell extracts, or combinations of the above with anti-IHF (0.5 μl) as follows: lane 1, 0 M IHF; lane 2, 440 nM IHF; lane 3, 440 nM IHF plus anti-IHF; lane 4, 2 μg of extract from $Alg^+$ *P. aeruginosa* FRD1; lane 5, 2 μg of extract from $Alg^+$ *P. aeruginosa* FRD1 plus anti-IHF; lane 6, 2 μg of extract from $Alg^-$ *P. aeruginosa* FRD2; lane 7, 2 μg of extract from $Alg^-$ *P. aeruginosa* FRD2 plus anti-IHF; lane 8, 500 ng of extract from $IHF^-$ *E. coli* MH10906; lane 9, 500 ng of extract from $IHF^+$ *E. coli* MH10900.

compare the affinity of IHF for sites 1 and 2, fragments containing either site were tested over a range of IHF concentrations (0.44 nM to 4.4 μM; Fig. 4). In this experiment, a low concentration of each PCR fragment (1 fmol) was incubated with increasing concentrations of IHF. The complexes were separated and subjected to autoradiography (Fig. 4A and B), and the fraction of DNA bound and unbound at each IHF concentration used was quantitated by radioanalytical scanning of the dried gel. The data in Fig. 4A and B for sites 1 and 2, respectively, were also subjected to Bjerrum plots in which the fraction of free DNA was plotted versus the log IHF concentration. These plots illustrate the different affinities of IHF for the different sites. At IHF concentrations which resulted in 50% saturation of site 2 (28 nM; Fig. 4B), no binding was detected with a fragment containing site 1 (Fig. 4A). Moreover, 50% saturation of site 1 occurred at 1.9 μM IHF (Fig. 4A), a concentration which completely saturated site 2. In addition, since the molar ratio of protein to DNA in the linear range of these plots was between $4.4 \times 10^4$ and $4.4 \times 10^6$ to 1, the effective IHF concentration was not decreased by subtracting the fraction of protein bound to DNA and an estimate of the thermodynamic binding constant ($K_b$) can thus be calculated by using a formula described elsewhere (40; see Materials and Methods). These results estimate a relative $K_b$ for site 1 of $5.33 \times 10^5\ M^{-1}$ and a relative $K_b$ for site 2 of $5.0 \times 10^7\ M^{-1}$. Thus, site 2 has an approximately 90-fold higher affinity for IHF than does site 1.

**Mapping of IHF site 2 binding by 1,10-phenanthroline–copper DNA footprinting.** In previous studies, DNase footprinting was utilized to demonstrate binding of IHF to low-affinity *algD* site 1 (spanning positions −50 to −85 relative to the *algD* mRNA start site; 34). The protection of high-affinity site 2 by IHF is shown in Fig. 5. IHF protection of site 2 extends from approximately +78 to +104 on the top strand (Fig. 5B) and from +77 to +105 on the bottom strand (Fig. 5C). This location is entirely consistent with the consensus IHF-binding sites (Fig. 1) and contains the core sequences whose mutation (see below) results in loss of in vitro IHF binding. The size of the region protected with IHF (approximately 25 to 30 bp) is smaller than the average 30- to 40-bp protection patterns observed in other IHF-binding sites of *E. coli* (16) and site 1 of *algD* (34).

**Mutational analysis of IHF sites 1 and 2.** To determine if IHF binding is required for *algD* transcription, oligonucleotide-directed mutagenesis was performed on the consensus IHF-binding core sequences of sites 1 and 2. These mutations (Fig. 6A) were based on changes which severely affect IHF binding to genes under IHF control in *E. coli* (30, 31) and are similar to mutations previously made in the consensus *algB* IHF-binding site (50). Five mutant *algD* alleles were constructed, i.e., *algD22* and *algD23*, with mutations in the consensus IHF-binding site 1, and *algD14*, *algD15*, and *algD17*, which had mutations in the site 2 core IHF-binding site (Fig. 6A). These mutations, which were verified by DNA sequencing (data not shown), did not change the spacing between critical *algD cis* elements (e.g., the promoter and AlgR-binding sites) and these IHF-binding sites.

PCR-generated fragments (top of Fig. 6) were prepared from plasmids containing wild-type sequences and from each of the mutant *algD* alleles described above. Gel band mobility shift assays were used to determine the effect of each mutation on IHF binding (Fig. 6B to D). As observed previously, when a high concentration (1.1 μM) of IHF was used, IHF bound to a fragment containing site 1 (Fig. 6B, lane 2). DNA from the *algD22* allele bound IHF at levels similar to those obtained with wild-type *algD* sequences (Fig. 6B, compare lanes 2 and

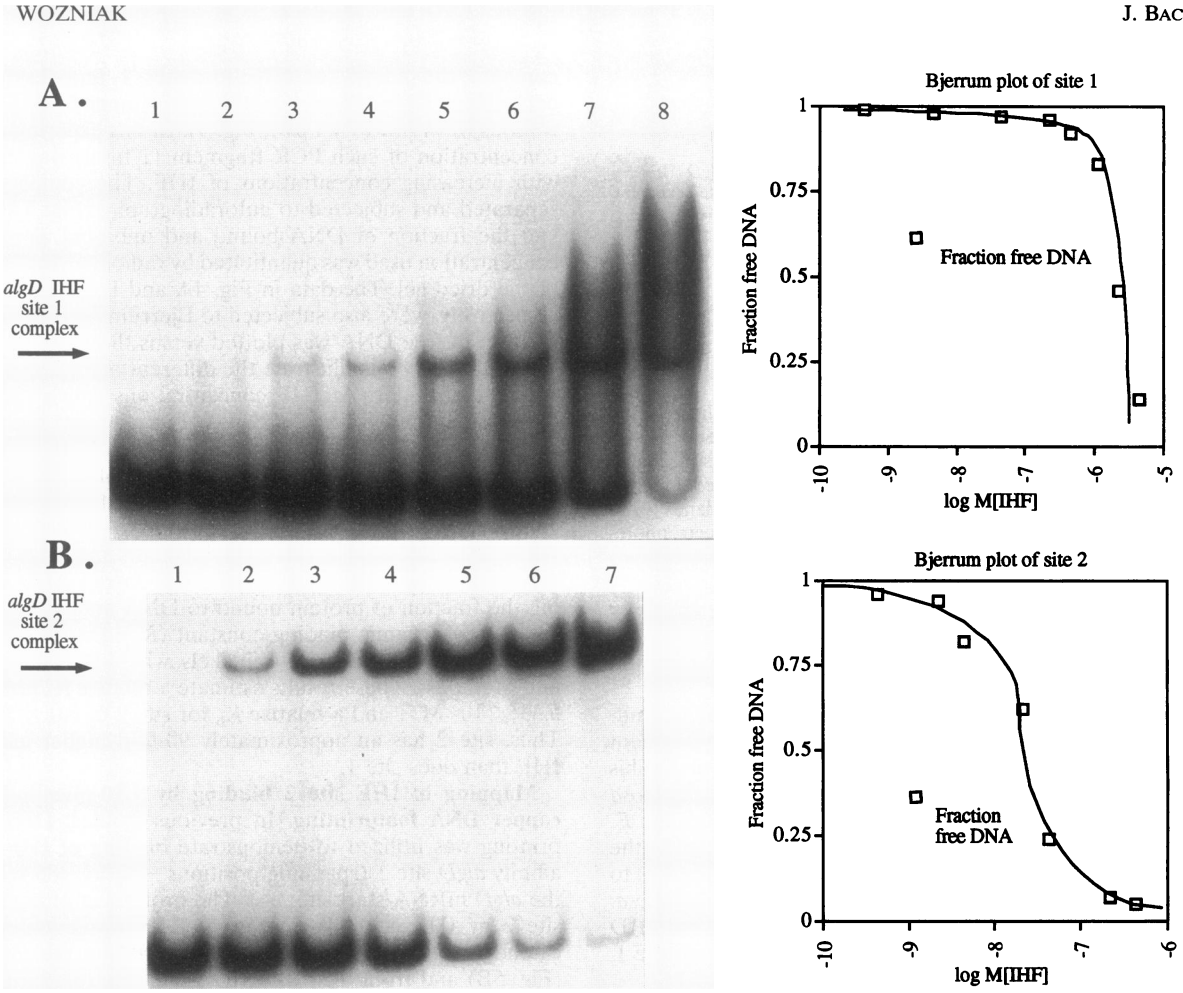


FIG. 4. Comparison of the affinity of IHF for fragments containing site 1 (A) or 2 (B). (A) Approximately 1 fmol of a PCR fragment containing site 1 was incubated with increasing amounts of IHF as follows: lane 1, 0.44 nM; lane 2, 4.4 nM; lane 3, 44 nM; lane 4, 220 nM; lane 5, 440 nM; lane 6, 1.1 μM; lane 7, 2.2 μM; lane 8, 4.4 μM. A Bjerrum plot (fraction of free DNA versus log IHF concentration) of these data is shown at the right. (B) Approximately 1 fmol of a PCR fragment containing site 2 was incubated with increasing amounts of IHF as follows: lane 1, 0.44 nM; lane 2, 2.2 nM; lane 3, 4.4 nM; lane 4, 22 nM; lane 5, 44 nM; lane 6, 220 nM; lane 7, 440 nM. A Bjerrum plot (fraction of free DNA versus log IHF concentration) of these data is shown at the right.

4). However, identical mutations (*algD23*) in the second core IHF consensus sequence of site 1 completely eliminated IHF binding to this fragment (Fig. 6B, lane 6). Thus, the mobility shift observed with high concentrations of IHF to a fragment containing the two consensus IHF sequences of site 1 (Fig. 1 and 5A) and that observed by others (34, 46) are likely due to IHF interaction at or within the vicinity of the 3′ core IHF consensus element.

Three sets of mutations were also introduced into high-affinity IHF-binding site 2, located downstream of the start of *algD* transcription (Fig. 5A), and wild-type versus mutant *algD* sequences were examined for IHF binding (Fig. 5C and D). In these assays, a concentration of 440 nM IHF was used, since this amount of IHF results in almost complete saturation of site 2 (Fig. 4B, lane 7; Fig. 6C, lane 2). DNA from the *algD14* allele demonstrated a dramatic (80%) reduction in IHF binding compared with wild-type *algD* sequences (Fig. 6C, lane 4). No detectable binding to a fragment containing the *algD15* or *algD17* allele was observed (Fig. 6B, lanes 6 and 8, respectively). In addition, fragments containing wild-type sequences and each of the mutations (*algD14*, *algD15*, and *algD17*) were examined for the ability to bind the IHF present in extracts of *P. aeruginosa*. As previously demonstrated (Fig. 3), wild-type site 2 sequences bind a protein (likely IHF) in extracts of *P. aeruginosa* (Fig. 6D, lane 1). None of the *algD* alleles with mutations in the core site 2 IHF-binding sequence were able to form this complex when extracts from $Alg^+$ *P. aeruginosa* FRD1 (Fig. 6D, lanes 2 to 4) and nonmucoid strain FRD2 (data not shown) were tested.

**Effect of mutations in IHF-binding sites 1 and 2 on *algD-cat* gene expression.** The wild-type and each of the *algD* IHF-binding site *algD-cat* alleles generated as described above were subcloned into the low-copy-number, broad-host-range vector pLAFR3 to monitor the effect of the mutations on *algD* expression. These plasmids were introduced into *P. aeruginosa* FRD1, a mucoid cystic fibrosis isolate (38), and CAT levels in cell extracts produced from wild-type and mutant *algD-cat* alleles were determined (Table 1). Both mutations in IHF-binding site 1 reduced *algD-cat* expression by three- to fourfold, indicating that IHF binding in this region is essential for *algD* transcription (Table 1). Mutations which reduced or eliminated IHF binding to high-affinity site 2 (*algD14*, *algD15*, and *algD17*) also caused a three- to fourfold decrease in *algD-cat* expression (Table 1). Thus, IHF binding downstream

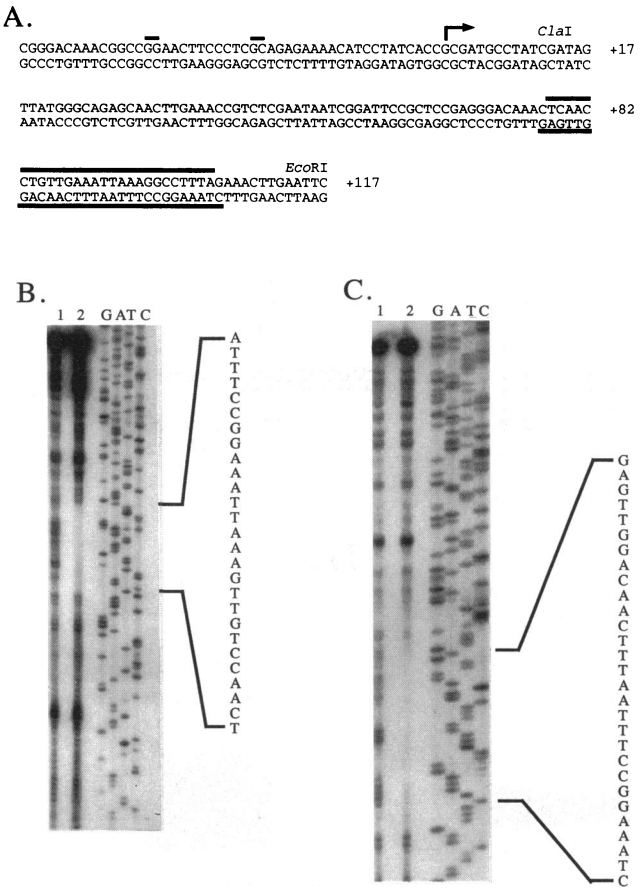


FIG. 5. 1,10-Phenanthroline–copper footprinting of site 2. (A) Sequence of *algD* near site 2 with the regions of the top and bottom strands protected by IHF overlined. The arrow indicates the start of *algD* transcription (9, 51). Sequences with similarity to RpoN promoters are overlined, and pertinent restriction sites are also shown. Approximately 1 pmol of a PCR fragment containing site 2 was specifically end labeled (23) on the top (B) or bottom (C) strand and left untreated (lanes 1) or incubated with 500 nM IHF (lanes 2). The free and complexed DNAs were separated by 4% polyacrylamide gel electrophoresis and subjected to in situ 1,10-phenanthroline–copper footprinting as described in Materials and Methods and reference 42. The IHF-binding site 2-complexed and free DNAs were excised from the gel, eluted from the gel slice, precipitated with ethanol, and electrophoresed adjacent to a sequencing ladder primed with the same *algD* oligonucleotide used to generate the end-labeled PCR product.

of the start of *algD* transcription is necessary for high-level *algD* expression.

**Evidence that *cis* sequences downstream of the *algD* transcription initiation site are required for expression.** In the *Klebsiella pneumoniae nifH* operon (24), IHF binds to sites upstream of the $\sigma^{54}$ promoter. This binding induces a bend which brings activators into contact with RNA polymerase at the promoter. Thus, IHF binding to downstream site 2 may promote loop formation with activators bound further downstream (3′) of site 2. To examine this possibility, the sequences located between +110 and +835 (3′ of site 2 but 5′ of *cat*; (Fig. 6) were deleted from a wild-type *algD-cat* plasmid to generate pDJW240 (*algD16*). This plasmid was mobilized into FRD1, and CAT levels in FRD1/pDJW240 cell extracts were quantitated (Table 1). Compared with CAT levels in cells containing a wild-type *algD-cat* allele, expression of *algD* from pDJW240 was reduced 10-fold. Thus, *cis* sequences located within the 730-bp *Eco*RI fragment located downstream of the start of *algD* transcription and IHF-binding site 2 are required for *algD* expression.

## DISCUSSION

In this study, a requirement for IHF in *algD* expression was examined. I identified two sequences which matched consensus IHF-binding sites of *E. coli* located approximately 75 bp upstream (site 1) and 90 bp downstream (site 2) of the start of *algD* transcription. These *cis* elements bind *E. coli* IHF with dramatically different affinities. Mutations in each of the sites resulted in reduction or loss of in vitro IHF binding and a three- to fourfold decrease in *algD-cat* expression, indicating that IHF binding is necessary for high-level *algD* transcription. The observation of a high-affinity IHF-binding site (site 2) located downstream of the *algD* transcription start site suggested that sequences further 3′ of site 2 may be involved in *algD* expression. When the *Eco*RI fragment located downstream of site 2 and upstream of the promoterless *cat* gene (+110 to +835) was deleted, *algD-cat* expression was reduced 10-fold, supporting the notion that 3′ *cis* elements are required for *algD* transcription.

The finding that sites 1 and 2 bind IHF with different affinities is likely important in the transcription of *algD* and, therefore, alginate biosynthesis. Data in Fig. 4 indicate that 50% saturation of a fragment containing site 1 occurs at 1.9 μM IHF, whereas site 2 is half saturated at 28 nM. In addition, analysis of the Bjerrum plots over a range of IHF concentrations with both sites indicated that site 2 has an approximately 90-fold higher affinity for IHF than does site 1. Although it is difficult to correlate results obtained with in vitro DNA-binding and in vivo studies, in *E. coli* the intracellular concentration of IHF was estimated to be 1.5 μM (28). Thus, if the concentrations of IHF in *E. coli* and *P. aeruginosa* are similar, the interactions with sites 1 and 2 observed in this study could occur in vivo. In addition, it is likely that site 2 would always be occupied, which may not be the case for site 1. As such, the intracellular concentration of IHF in *P. aeruginosa* may be a critical factor in determining the ultimate levels of *algD* transcription, particularly with respect to IHF binding at site 1, and this may represent an important point of *algD* control. The data presented in Fig. 4 regarding the affinity of IHF for sites 1 and 2 must be interpreted with caution, since two assumptions were made, that our IHF preparation was 100% active and that IHF from *E. coli* is similar in binding specificity and affinity to *P. aeruginosa* IHF. The following observations support the latter assumption. (i) A fragment containing site 2 bound *E. coli* IHF and a protein present in extracts from *P. aeruginosa*, and the complexes formed had identical mobilities (Fig. 3). (ii) Antibodies to *E. coli* IHF inhibit binding of the protein in *P. aeruginosa* (Fig. 3). These antibodies also cross-react with a protein in *P. aeruginosa* extracts which has a molecular mass similar to that of IHF (34, 47). (iii) Mutations in site 2 which affect *E. coli* IHF binding also affect the binding of a protein (likely IHF) in *P. aeruginosa* extracts. (iv) An IHF-like protein which binds to site 1 has been purified from *P. aeruginosa* (46).

To determine if IHF binding affects *algD* expression, mutations were introduced into the high- and low-affinity IHF-binding sites. The effects of these mutations were examined by comparing expression of plasmid-borne wild-type and mutant *algD-cat* alleles in mucoid *P. aeruginosa* FRD1. Mutations in either site 1 or 2 resulted in a three- to fourfold decrease in *algD-cat* expression. This may not be an entirely accurate representation of the effect of IHF on *algD* transcription.

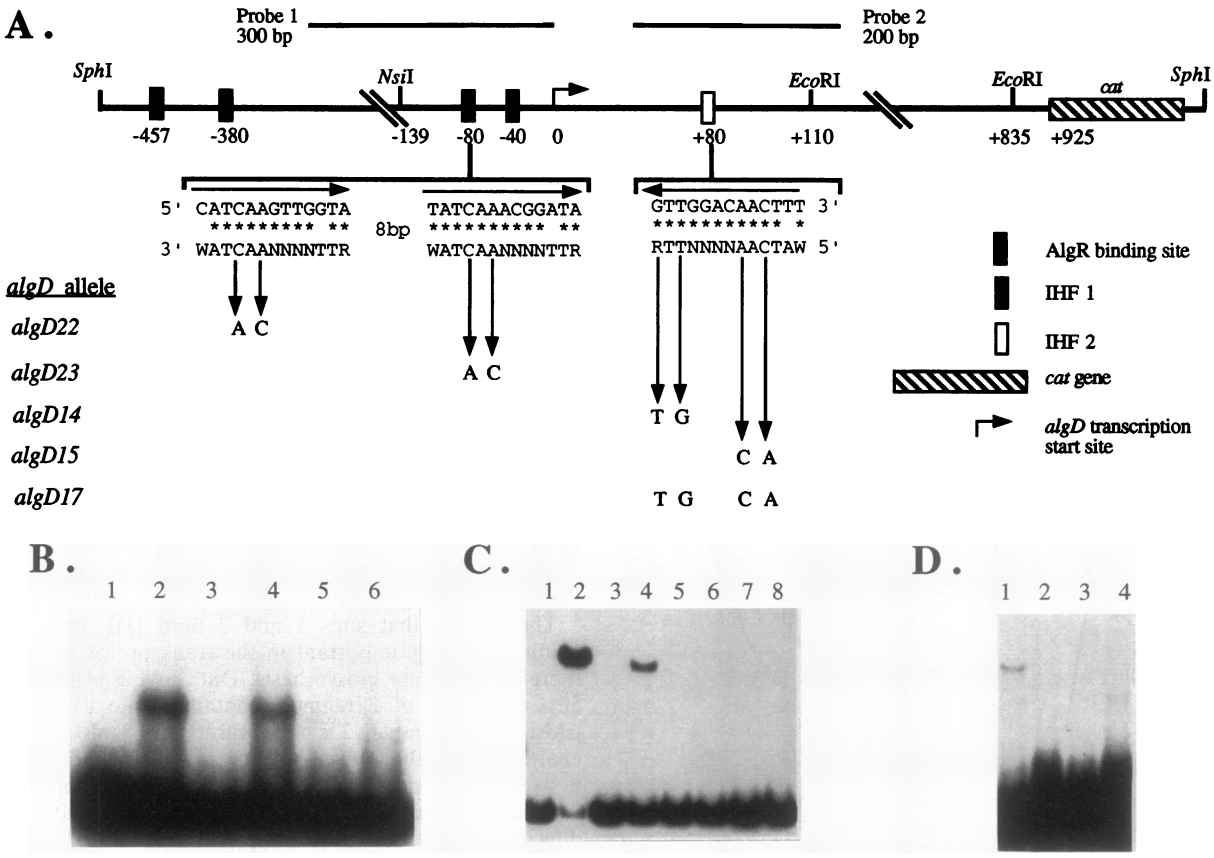


FIG. 6. Mutagenesis of *algD* IHF-binding sites. (A) Partial map of the 3.1-kb *Sph*I fragment containing *algD-cat*. The relative positions of the PCR fragments generated for panels B to D are illustrated at the top. IHF-binding sites 1 and 2 are indicated at −80 and +80, respectively. The core sequences of sites 1 and 2 with homology to a consensus IHF-binding site (WATCAANNNNTTR; see Fig. 1 legend for a description) are indicated. Asterisks indicate identical residues. The AlgR-binding sites are depicted. The horizontal arrow illustrates the start of *algD* transcription. Mutagenesis of the *algD* IHF-binding sites was performed, generating the five *algD* alleles shown (vertical arrows). (B) Gel band mobility shift assays of site 1 and mutations therein. Approximately 1 fmol of a PCR-generated fragment (probe 1) from the wild type or the *algD* site 1 mutants was incubated with 1.1 μM IHF. Lanes: 1, wild-type *algD*; 2, wild-type *algD* plus IHF; 3, *algD22* allele; 4, *algD22* allele plus IHF; 5, *algD23* allele; 6, *algD23* allele plus IHF. (C) Gel band mobility shift assays of site 2 and mutations therein. Approximately 1 fmol of a PCR-generated fragment (probe 2) from the wild type or the *algD* site 2 mutants was incubated with 440 nM IHF. Lanes: 1, wild-type *algD*; 2, wild-type *algD* plus IHF; 3, *algD14* allele; 4, *algD14* allele plus IHF; 5, *algD15* allele; 6, *algD15* allele plus IHF; 7, *algD17* allele; 8, *algD17* allele plus IHF. (D) Gel band mobility shift assays of site 2 and mutations therein with extracts from *P. aeruginosa* FRD1. Approximately 100 fmol of a PCR-generated fragment (probe 2) from the wild type or the *algD* site 2 mutants was incubated with 2 μg of cell extract. Lanes: 1, wild-type *algD*; 2, *algD14* allele; 3, *algD15* allele; 4, *algD17* allele.

Although these mutations result in a severe decrease or loss of IHF binding in vitro, it is possible that IHF is still capable of interacting with these mutant sequences in vivo (albeit with reduced affinity), resulting in a net decrease in *algD* expression. In addition, the role of IHF in *algD* expression may be to promote loop formation to allow activators bound far upstream or downstream access to the promoter (see below). The *algD* sequences surrounding sites 1 and 2 are unusually AT rich and contain runs of poly(A) sequences which may be involved in intrinsic (IHF-independent) DNA bending, resulting in low-level *algD* transcription. A mutation in the genes encoding *P. aeruginosa* IHF is necessary to determine the precise role that IHF plays in *algD* expression and alginate biosynthesis.

Of notable significance in this study is the observation that when the sequences located between IHF site 2 and the *cat* fusion were deleted, *algD-cat* expression was reduced 10-fold (Table 1). It should be emphasized that the *algD-cat* reporter system utilized in these studies is a transcriptional fusion; expression of *cat* is entirely under control of the upstream *algD* promoter and regulatory elements but utilizes the *cat* ribosome-binding site and AUG initiation codon for translational control. It therefore seems unlikely that these results were due to deletion of *algD* translation signals. Since this deletion is located downstream of site 2 and the start of *algD* transcription, this suggests that the sequences contained within this deleted element are essential for *algD* expression. This result is not unprecedented, since there are reports of transcriptional regulators (Lac and Gal repressors, fumarate nitrate reduction [FNR] protein, and the *Bordetella pertussis vag* gene product) which bind 3′ of the transcription start site of the genes they control. Perhaps the best analogous system might be the cell cycle-regulated *flaN* operon of *Caulobacter crescentus*. Transcription of *flaN* requires a $\sigma^{54}$ promoter and two *cis* elements, *ftr2* and *ftr3*, located downstream (positions +86 and +120, respectively) of the transcription start site (37). In addition, an IHF-binding site has been identified downstream of the *flaN* $\sigma^{54}$ promoter. Mutations in this 3′ IHF-binding site reduced

TABLE 1. Effects of IHF sites 1 and 2 and deletion mutations on *algD* expression in mucoid *P. aeruginosa* FRD1

| Plasmid[a] | *algD-cat* allele | Description[b] | Avg CAT level[c] ± SD | % of wild-type *algD-cat* level |
|---|---|---|---|---|
| pDJW222 | Wild type | Wild type | 5,200 ± 470 | 100 |
| pDJW241 | *algD22* | Site 1; C −97→A, A −95→C | 1,700 ± 120 | 33 |
| pDJW242 | *algD23* | Site 1; C −76→A, A −74→C | 1,320 ± 100 | 25 |
| pDJW232 | *algD14* | Site 2; C +77→A, A +79→C | 1,360 ± 120 | 26 |
| pDJW233 | *algD15* | Site 2; T +84→G, G +86→T | 1,870 ± 240 | 36 |
| pDJW267 | *algD17* | Site 2; C +77→A, A +79→C, T +84→G, G +86→T | 1,730 ± 190 | 33 |
| pDJW240 | *algD16* | Deletion of *Eco*RI fragment from +110 to +835 | 550 ± 30 | 11 |

[a] *P. aeruginosa* FRD1 ($Alg^+$) cells containing the indicated *algD-cat* alleles were cultured to an identical logarithmic-phase cell density ($A_{595}$ = 0.7) in LB medium.
[b] Descriptions and positions of the mutations relative to the start of *algD* transcription are given (see Fig. 5A).
[c] Cell extracts were prepared and assayed for CAT activity by using a sandwich enzyme-linked immunosorbent assay technique. CAT levels in dilutions of the cell extracts were determined by extrapolation from a standard curve and normalized for protein content. The values shown are in nanograms of CAT per milligram of protein in extracts and are average results of three experiments.

*flaN* transcription threefold (17), similar to the effect of mutations in the *algD* IHF-binding sites generated in this study (Table 1).

It has been proposed that formation of DNA loops is an efficient mechanism for incorporating several proteins and distant DNA sites into complex regulatory systems which require specificity, precision, and flexibility (41). Thus, a tempting speculation is that IHF binding at high-affinity site 2 induces a DNA loop which brings an activator bound to a 3′ element (likely contained within the *Eco*RI fragment; Fig. 6) into proximity to the *algD* promoter. We are currently examining these sequences for DNA binding. *algD* also has an unusually long (366-bp) 5′ leader RNA sequence which has several inverted repeats with a high potential for secondary structure. Therefore, another possible role for IHF binding in this region of *algD* might be to influence RNA structure by altering the rate of dissociation of RNA from the DNA template, as IHF appears to do for the *c*II gene of bacteriophage λ (32). Another possibility we cannot rule out is that the mutations in the downstream *algD* IHF site reduce *algD-cat* expression by destabilizing the mRNA in an IHF-independent fashion. These possibilities are also being examined.

The *P. aeruginosa algD* promoter is subject to a complex pattern of control. This involves response regulators AlgR (7) and AlgB (18, 49), the *algT* (*algU*) gene product (33, 51), AlgQ (27), and histone-like proteins AlgP ($H_p1$; 27) and IHF (34, 46; this study). In addition, several studies have shown that expression of *algD* or alginate biosynthesis is under the environmental control of factors such as sodium chloride and nitrogen availability (10), glucose repression (13), nutrient starvation (43), oxygen tension (3), components of lung surfactant, iron limitation, and the overall energy and redox state of the cell (21, 45). Moreover, the mucoid phenotype of *P. aeruginosa* is relatively unstable in the laboratory and mucoid cultures spontaneously convert to nonmucoidy at high frequency. However, stable mucoid derivatives can be isolated by mutagenesis or by treatment with nalidixic acid (6, 19), suggesting a role for DNA topology in alginate biosynthesis. It is known that DNA supercoiling in bacteria is influenced by environmental factors (22). Other factors, such as growth phase and nutrient limitation, have been shown to affect DNA topology (14). In addition, the strength of a number of bacterial promoters depends on the level of DNA supercoiling (2). It is quite possible that genes such as *algD*, which are regulated by multiple environmental factors, respond to local changes in superhelical density brought on by growth conditions. Most of the factors discussed above which are involved in the environmental regulation of *algD* or the maintenance of mucoidy also affect DNA supercoiling. The identification of a requirement for the histone-like IHF protein in *algD* expression would support this, as IHF may be necessary to establish a transcriptionally active nucleoprotein complex, a role proposed for IHF in other systems (15).

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