fleQ, the Gene Encoding the Major Flagellar Regulator of *Pseudomonas aeruginosa*, Is σ⁷⁰ Dependent and Is Downregulated by Vfr, a Homolog of *Escherichia coli* Cyclic AMP Receptor Protein

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The flagellar transcriptional regulator FleQ appears to be the highest-level regulator in the hierarchical regulatory cascade of flagellar biogenesis in *Pseudomonas aeruginosa*. Except for the posttranslational downregulation of FleQ activity by FleN, an antiactivator, not much is known about the regulation of the *fleQ* gene or its gene product. Some FleQ homologs in other bacterial species either are positively regulated by another regulator (e.g., CtrA, the master regulator regulating FlbD in *Caulobacter crescentus*) or are expressed from a σ⁷⁰-dependent promoter (e.g., FlgR of *Helicobacter pylori*). In this study we demonstrated that Vfr, an *Escherichia coli* CRP homolog known to function as an activator for various genes, including *lasR*, *regA*, and *toxA*, in *P. aeruginosa*, is capable of repressing *fleQ* transcription by binding to its consensus sequence in the *fleQ* promoter. In a DNase I footprint assay, purified Vfr protected the sequence 5′-AATTGACTAATCGTTCACA TTTG-3′. When this putative Vfr binding site in the *fleQ* promoter was mutated, Vfr was unable to bind the *fleQ* promoter fragment and did not repress *fleQ* transcription effectively. Primer extension analysis of the *fleQ* transcript revealed two transcriptional start sites, t1 and t2, that map within the Vfr binding site. A putative −10 region (TAAAAT) for the t2 transcript, with a five-of-six match with the *E. coli* σ⁷⁰ binding consensus, overlaps with one end of the Vfr binding site. A 4-bp mutation and an 8-bp mutation in this −10 region markedly reduced the activity of the *fleQ* promoter. The same mutations led to the disappearance of the 203-nucleotide *fleQ* transcript in an in vitro transcription assay. Vfr probably represses *fleQ* transcription by binding to the Vfr binding site in the *fleQ* promoter and preventing the sigma factor from binding to the −10 region to initiate transcription.

Flagellar biogenesis in *Pseudomonas aeruginosa* is an intricate process in which various transcriptional activators (FleQ, FleR) and alternative sigma factors (RpoN/σ⁵⁴, RpoF/σ²⁸) participate in the transcriptional regulation of the flagellar genes and operons (3, 23, 31, 33). Of the two transcriptional activators identified in our laboratory, FleQ appears to be the highest-level positive regulator of flagellar biogenesis in *P. aeruginosa*.

FleQ is homologous to the NtrC group of response regulators, which are part of a two-component signal transduction system, that works in concert with the alternative sigma factor RpoN (3). Typically, a two-component system consists of a phosphorelay between a sensor kinase and a response regulator pair (5). The sensor kinase is activated by autophosphorylation when an environmental stimulus is sensed, and the response regulator is subsequently phosphorylated at the receiver domain. The response regulators are usually DNA binding proteins, and phosphorylation activates them to promote activation or repression of the downstream genes (5). However, FleQ and several of its homologs that regulate flagellar biogenesis in other bacterial species, including FlrA of

Vibrio cholerae (16), FlaK of *Vibrio parahaemolyticus* (15), and FlgR of *Helicobacter pylori* (30), do not contain the hallmark residues in their receiver domains for phosphorylation and do not have a cognate sensor kinase encoded as a part of the same operon. Therefore, phosphorelay signaling does not appear to be the modulating mechanism that regulates the activities of these response regulators. Attempts to detect phosphorylation of FleQ in vitro were not successful in our laboratory, thus raising further doubts about the existence of a putative sensor kinase for FleQ (unpublished data). One of the mechanisms employed to accomplish posttranslational modulation of FleQ activity in *P. aeruginosa* involves direct protein-protein interactions with FleN, an antiactivator that represses FleQ-dependent transcriptional activation (8). Whether *fleQ* is subject to transcriptional activation or repression in addition to posttranslational modulation has been a question. Preliminary data have demonstrated that FleQ synthesis occurs from the early log phase to the stationary phase of growth in shaken cultures of *P. aeruginosa* PAK (unpublished data), which suggests that constitutive expression occurs under these conditions. Whether the constitutive expression observed was driven by a constitutively expressed activator or merely by the housekeeping RNA polymerase holoenzyme is not clear.

The flagellar regulon is subject to hierarchical regulation in most of the flagellated bacteria. Among the flagellar regulators of monoflagellate bacteria, *flgR*, the *fleQ* homolog of *H. pylori*,

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has been proposed to be transcribed from a σ^{70}-like promoter (30). No information has been published concerning the transcriptional regulation of the *fleQ* homolgs *flrA* and *flaK* in *V. cholerae* and *V. parahaemolyticus*, respectively. However, *flbD*, the *fleQ* homolog of *Caulobacter crescentus*, along with the gene encoding its cognate sensor kinase, *flbE*, is positively regulated by CtrA, the master regulator which controls the initiation of DNA replication, DNA methylation, cell division, and flagellar biogenesis (10, 35). In the peritrichous organisms *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, transcriptional activators encoded by *flhC* and *flhD* are at the top of the hierarchy and are essential for the expression of flagellar genes (6). The *flhDC* operon is controlled by a σ^{70} promoter and a number of global regulatory factors (17). There is evidence which suggests that the cyclic AMP receptor protein (CRP) activates and the histone-like nucleoid-structuring protein (H-NS) represses transcription of *flhDC* in *E. coli* (28). In *S. enterica* serovar Typhimurium it has been demonstrated that the *flhDC* operon carries six possible transcriptional start sites, one of which is CRP dependent and one of which is CRP repressible (36). Thus, flagellar biogenesis may be under both positive regulation and negative regulation in some bacteria.

Based on the known mechanisms of transcriptional regulation of FleQ homologs described above, *fleQ* could be envisaged to be regulated by one or more similar mechanisms. In this report, we provide evidence that *fleQ* and thus flagellar biogenesis in *P. aeruginosa* are σ^{70} dependent and are negatively regulated by Vfr. Vfr is a homolog of *E. coli* CRP that functions as an activator for many genes, including *lasR*, that have been implicated in virulence and quorum sensing (2, 32, 34). The physiological implications are discussed below.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Strains and plasmids used in this paper are listed in Table 1. All strains were routinely maintained by using Luria-Bertani (LB) (25) medium at 37°C. Antibiotics were used as necessary at the following concentrations: 100 μg of ampicillin per ml and 20 μg of tetracycline per ml for *E. coli* and 300 μg of carbenicillin per ml and 100 μg of tetracycline per ml for *P. aeruginosa*.

Homology searches. The Vfr binding sequence (AAATGTGATCTAGATCACATTT) from the *lasR* promoter was used to search the complete *P. aeruginosa* genome (www.pseudomonas.com) with the program FindPattern in the Genetics Computer Group package (Accelrys, San Diego, Calif.). Nine mismatches were allowed.

Site-directed mutagenesis. Primers Qpmut1 and Qpmutcomp2 (Table 1) (Genomechanix Inc., Alachua, Fla.) were used with a Quick-change site-directed mutagenesis kit (Stratagene, La Jolla, Calif.) to mutate the Vfr binding site in the *fleQ* promoter according to the supplier's protocol. Primers Qp70mut1 and Qp70mutcomp2 and primers Qp70mut3 and Qp70mutcomp4 (Table 1) (Genomechanix Inc.) were similarly used to introduce 8- and 4-bp mutations in the putative −10 region of the *fleQ* promoter. Briefly, 20 ng of column-purified (Plasmid mini kit; Qiagen, Valencia, Calif.) plasmid template (pBS-*fleQ*p) was used in a 50-μl amplification reaction mixture consisting of 1 μl of a solution containing each deoxynucleoside triphosphate at a concentration of 10 mM, 1 μl of *Pfu* polymerase, 1.25 μl of a 20 μM solution of each primer, and 5 μl of 10× reaction buffer. The mixture was subjected to a cycling profile consisting of initial denaturation for 30 s at 95°C, followed by 13 cycles (Qpmut1 and Qpmitcomp2) or 18 cycles (Qp70mut1 and Qp70mutcomp2 or Qp70mut3 and Qp70mutcomp4) of denaturation at 95°C for 30 s, annealing at 60°C for 1 min, and extension at 68°C for 10 min. The reaction mixture contents were then treated with *Dpn*I to digest the original plasmid template. One microliter of the postdigestion amplification reaction mixture was used to transform *E. coli* XL1 Blue cells, and transformants were selected on LB agar plates containing ampicillin. Several clones were sequenced, and a clone with the desired site-specific mutation was

subsequently used for further subcloning. The plasmid with the mutated Vfr binding site (ACA → CGC) was designated pBS-*fleQ*pmutV. The plasmids with the 8-bp (ATAAAATT → GCGCGCGC) and 4-bp (AAAT → GCGC) mutations in the −10 region were designated pBS-*fleQ*pmutS8 and pBS-*fleQ*pmutS4, respectively. The inserts from pBS-*fleQ*pmutS8 and pBS-*fleQ*pmutS4 were subcloned into pDN19lacΩ, generating placΩmutS8 and placΩmutS4, respectively.

Transformation and electroporation. Unless indicated otherwise, *E. coli* DH5α was the strain used for transformation (25). Electroporation of *P. aeruginosa* was carried out as described elsewhere (7).

PCR amplification of fragments for EMSAs and DNase I footprint assays. For electrophoretic mobility shift assays (EMSAs), 88-bp DNA fragments containing the *fleQ* promoter and the mutant *fleQ* promoter (ACA → CGC) were generated by PCR amplification by using oligonucleotide primers fleQ1F and fleQ1R (Table 1) (Integrated DNA Technologies, Inc., Coralville, Iowa). placΩ and pBS-*fleQ*pmutV (Table 1) served as the templates. The high-fidelity polymerase *Pfx* (Stratagene) was used in the amplification reactions, which were performed according to the manufacturer's specifications. Radiolabeling of the DNA fragments was accomplished by inclusion of [α-^{32}P]dCTP (800 Ci/mmol; Perkin-Elmer Life Sciences Inc., Boston, Mass.) in the PCR mixtures. Following PCR amplification, the wild-type and mutant *fleQ* promoter fragments were purified by using the Promega Wizard DNA Cleanup system (Promega Corp., Madison, Wis.).

For the DNase I footprint assays, oligonucleotide primers fleQ2F and fleQ2R (Table 1) (Integrated DNA Technologies) were used to PCR amplify a 158-bp fragment containing the *fleQ* promoter region by using placΩ as a template. The first 10 bp of each primer contained either a *Sal*I (fleQ2F) or *Xho*I (fleQ2R) restriction site. PCR amplification and fragment purification were carried out as described above. The amplified fragment was cut with either *Sal*I or *Xho*I to generate a 5′ overhang that was end labeled with a single 5′ [α-^{32}P]TTP (800 Ci/mmol; Perkin-Elmer Life Sciences Inc.) by using T7 polynucleotide kinase (Amersham Biosciences, Piscataway, N.J.) according to the manufacturer's directions.

Vfr purification. Vfr purification from *P. aeruginosa* PA103(pWNP28) was based on cAMP affinity chromatography and additional procedures described by Albus et al. (2), with modifications. Briefly, cultures were grown to an optical density at 600 nm (OD$_{600}$) of ~0.5, and then isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce overexpression of Vfr. After an additional 6 h of incubation, the cells were harvested by centrifugation at 5,000 × g for 15 min. The cell pellet was resuspended in a solution containing 100 mM Tris-HCl (pH 8.0), 50 mM KCl, 2 mM EDTA, 5 mM β-mercaptoethanol, and 1 mM sodium azide. A crude lysate was prepared by incubation of the solution with 200 μg of lysozyme per ml for 20 min at room temperature. The lysate was adjusted to 125 mM Tris-HCl (pH 8.0), 25 mM MgSO$_4$, and 1% Brij 35, incubated at 4°C for 10 min, and then passed through a 40K SLM-Aminco French pressure cell (Thermo Electron Corp., Waltham, Mass.) at a pressure of approximately 15,000 lb/in². Cellular debris was removed by centrifugation at 16,000 × g for 30 min at 4°C. An ammonium sulfate fractionation step was performed next; most of the Vfr protein precipitated between 50 and 65% ammonium sulfate saturation. This fraction was centrifuged at 27,000 × g for 30 min at 4°C. The pellet was resuspended in a solution containing 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 5% glycerol, and 5 mM β-mercaptoethanol and dialyzed against the same buffer to remove the ammonium sulfate. The resulting solution was then applied to a cAMP-agarose column (Sigma Chemical Co., St. Louis, Mo.) as described by Ghosaini et al. (12). Eluted fractions containing Vfr were pooled and dialyzed into a solution containing 10 mM sodium phosphate (pH 7.2), 2 mM EDTA, 5 mM β-mercaptoethanol, and 5% glycerol. The resulting solution was applied to an SP-Sephadex cation-exchange column. Vfr-containing fractions were pooled and dialyzed into a solution containing 50 mM Tris-HCl (pH 7.2), 500 mM KCl, 0.2 mM EDTA, 0.2 mM dithiothreitol (DTT), and 50% glycerol for storage.

EMSAs. Binding reactions were performed as described by Devault et al. (9), with minor modifications. Briefly, the 20-μl reaction mixtures contained the radiolabeled DNA fragments, with the radioactivity standardized to approximately 1,000 cpm (15 to 40 pM), and various concentrations of purified Vfr in a solution containing 10 mM Tris-HCl (pH 7.4), 100 mM KCl, 1 mM EDTA, 5% glycerol, 1 mM DTT, 50 μg of bovine serum albumin per ml, 20 μM cAMP, and 10 μg of poly(dI-dC)-poly(dI-dC) (Amersham Biosciences Inc.) per ml. The reaction mixtures were incubated for 20 min at room temperature. Samples were electrophoresed on a preequilibrated 4% acrylamide–45 mM Tris-HCl–45 mM borate gel at 10 V/cm and 4°C. The upper buffer chamber contained 45 mM Tris-HCl, 45 mM borate (pH 8.0), and 20 μM cAMP. The gels were dried and

TABLE 1. Strains, plasmids, and primers

Strain, plasmid, or primer	Relevant information	Source or reference
E. coli strains		
DH5α	*hsdR recA lacZYA* ϕ80 *lacZ*Δ*M15*	GIBCO-BRL
XL1 Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lac1*^{q} *Z*Δ*M15* Tn*10* (Tet^r)]	Stratagene
S17-1	RP4-2 Tc::Mu Km::Tn7 Tp^r Sm^r Pro Res$^-$ Mod$^+$	27
P. aeruginosa strains		
PAK	Wild-type clinical isolate	D. Bradley
PAK *fleQ*p-*lacZ*	PAK with *fleQ*p-*lacZ* in the *attB* site	This study
PAK *fleQ*pmutV-*lacZ*	PAK with *fleQ*pmutV-*lacZ* in the *attB* site	This study
PAK *lacZ*	PAK with *lacZ* in the *attB* site	This study
PAO1	Wild type	14
PAO *fleQ*p-*lacZ*	PAO with *fleQ*p-*lacZ* in the *attB* site	This study
PAO *fleQ*pmutV-*lacZ*	PAO with *fleQ*pmutV-*lacZ* in the *attB* site	This study
PAO *lacZ*	PAO with *lacZ* in the *attB* site	This study
PA103	Prototroph, exotoxin A hyperproducer, elastase deficient	18
PAO9001	Δ*vfr*::Gent^r derivative of strain PAO1	2
PAO9001 *fleQ*p-*lacZ*	PAO9001 with *fleQ*p-*lacZ* in the *attB* site	This study
Plasmids		
pMMB66HE/EH	Broad-host-range *tacP* expression vector, *lac1*^{q} Ap^r Cb^r	11
pWNP28 (p*tac*917Δp)	pMMB66HE carrying the 0.9-kb *Nar*I fragment containing *vfr* region from positions −77 to +999, Ap^r Cb^r	2
pDN19*lac*Ω	Promoterless *lacZ oriV oriT* Tet^r Str^{r}Ω fragment	7
p*lac*ΩQ	pDN19*lac*Ω containing the *fleQ* promoter region as a 720-bp *Eco*RI-*Bam*HI insert	3
p*lac*ΩQmutS8	p*lac*ΩQ with 8 bp in the −10 region of the *fleQ* promoter mutated	This study
p*lac*ΩQmutS4	p*lac*ΩQ with 4 bp in the −10 region of the *fleQ* promoter mutated	This study
pBluescript II/KS(+)	General-purpose cloning vector, Ap^r	Stratagene
pBS-*fleQ*p	pBluescript II/KS(+) containing the *fleQ* promoter region as a 720-bp *Eco*RI-*Bam*HI nsert	This study
pBS-*fleQ*pmutV	pBS-*fleQ*p with an ACA→CGC mutation in the Vfr binding site	This study
mini-CTX*lacZ*	Integration-proficient vector for single-copy chromosomal *lacZ* fusion	4
CTX *fleQ*p-*lacZ*	Insert from pBS-*fleQ*p cloned into mini-CTX*lacZ*	This study
CTX *fleQ*pmutV-*lacZ*	Insert from pBS-*fleQ*pmutV cloned into mini-CTX*lacZ*	This study
pFLP2	Plasmid encoding the FLP recombinase, Cb^r	4
Primers[a]		
fleQ2F	5′ ATCGGTCGACTTTTTTATTGCCTCT 3′	
fleQ2R	5′ CGATCTCGAGATCGAATGGGTCTCGCTC 3′	
fleQ1F	5′ CAGCAAAAAAAAACACGCC 3′	
fleQ1R	5′ GCCTATTTCTAATAAGCAGCC 3′	
Qpmut1	5′ CCGCCATAAATTGACTAATCGTTC<u>CGC</u>TTTGACTTAACTAGTGGCC 3′	
Qpmutcom2	5′ GGCCACTAGTTAAGTCAAA<u>GCG</u>GAACGATTAGTCAATTTTATGGCGTG 3′	
Qp70mut1	5′ GAAACAGCAAAAAAAACACGCC<u>GCGCGCGC</u>GACTAATCGTTCACATTTGACTTA 3′	
Qp70mutcom2	5′ TAAGTCAAATGTGAACGATTAGT<u>CGCGCGCG</u>CGGCGTGTTTTTTTTGCTGTTTC 3′	
Qp70mut3	5′ CAGCAAAAAAAACACGCCCATA<u>GCGC</u>TGACTAATCGTTCACATTTGAC 3′	
Qp70mutcom4	5′ GTCGTTTTTTTTTGTGCGGTAT<u>CGCG</u>ACTGATTAGCAAGTGTAAACTG 3′	
RT2	5′ CAGCTGCCTTGCATCGAATGGGTCTCGC 3′	

[a] Bases mutated in the primers are underlined.

exposed to Kodak X-Omat AR5 film overnight at −80°C with an intensifying screen.

DNase I footprint assay. The DNase I footprint assay was based on the procedure of Ross et al. (24). Binding reactions were performed by incubating purified Vfr with the radiolabeled DNA fragments in a solution containing 10 mM Tris-HCl (pH 7.4), 100 mM KCl, 10 mM MgCl$_2$, 5% glycerol, 1 mM DTT, 50 μg of bovine serum albumin per ml, and 20 μM cAMP for 20 min at room temperature. DNase I (0.75 to 1.5 μg/ml) was then added to the samples. The DNase I digestion reaction mixtures were incubated for 30 s at room temperature, and then the reactions were terminated by adding 12.5 mM (final concentration) EDTA, 200 mM (final concentration) sodium acetate, and 30% (vol/vol) (final concentration) phenol. The digestion products were ethanol precipitated and then resuspended in 0.5× TBE buffer (45 mM Tris-HCl, 45 mM borate) containing 8 M urea, 0.5% bromophenol blue, and 0.5% xylene cyanol. The samples were analyzed on a 7 M urea–8% polyacrylamide gel to determine the regions protected by Vfr. Maxam-Gilbert sequencing reactions (19) were also performed with the radiolabeled DNA fragments to obtain sequence ladders for comparison with the footprint reaction mixtures.

Construction of promoter-*lacZ* fusions in the mini-CTX *lacZ* plasmid and chromosomal insertion of these fusions into the *attB* site. The wild-type and mutated *fleQ* promoter-containing inserts in pBS-*fleQ*p and pBS-*fleQ*pmutV were cloned as *Eco*RI-*Bam*HI fragments in mini-CTX *lacZ* (4), generating CTX-*fleQ*p*lacZ* and CTX-*fleQ*pmutV*lacZ*. *E. coli* S17-1 was transformed with these plasmids and the vector control mini-CTX*lacZ*. Matings were set up

with the transformed *E. coli* S17-1 strain as the donor and *P. aeruginosa* PAK or PAO1 as the recipient. The exconjugants were selected on tetracycline-containing plates, and one colony was cultured further to electroporate the pFLP2 plasmid into it. Transformants were selected on carbenicillin-containing plates. Two colonies from each strain background were propagated in LB broth, and serial dilutions of the cultures were plated on LB medium and LB medium containing sucrose (5%). A few colonies from the sucrose-containing plate were examined for tetracycline sensitivity and carbenicillin sensitivity to ensure excision of the plasmid backbone and curing of the pFLP2 plasmid, respectively. The chromosomal integrants with the *fleQ*p-*lacZ* fusion in PAK and PAO1 were designated PAK *fleQ*p-*lacZ* and PAO *fleQ*p-*lacZ*, respectively. The *fleQ*pmutV-*lacZ* derivatives were designated PAK *fleQ*pmutV-*lacZ* and PAO *fleQ*pmutV-*lacZ*. The controls with a promoterless *lacZ* integrated were designated PAK *lacZ* and PAO *lacZ*.

Motility assay. Plain 0.3% LB agar plates were used to test the motility of *P. aeruginosa* PAO9001, a *vfr* deletion mutant. To examine the effect of Vfr overexpression on the motility of strain PAK harboring either pMMB66EH or pWNP28, 0.3% LB agar plates containing carbenicillin without or with various concentrations of IPTG were used. The plates were stabbed with the strains and incubated at 37°C.

β-Galactosidase assays. *P. aeruginosa* cultures were grown in LB broth with the appropriate antibiotic (if needed) to an OD$_{600}$ of ~0.8. The β-galactosidase assay was performed as described previously (20). To study the effect of Vfr overexpression on the *fleQ* promoter activity, cultures were grown in LB broth

Sca I

CCCAAAACCC CTGCC<u>AGTAC TGC</u>TTTCCAT GCGGTCTGTA GGGGTTTTTT ATTGCCTCTC   60

pBS-*fleQ*pmutS8   **G   CGCGCGC**
pBS-*fleQ*pmutS4   **GCGC**   t1   t2 (Transcriptional start sites)

CCTGTCGGAA ACAGCAAAAA AAACACGCCA TA AAATTGAC TAATCGTTCA CATTTG ACTT   120

<u>-35</u>   N(17)   <u>-10</u>   **(Vfr binding site)   C GC** pBS-*fleQ*pmutV

σ 70 binding region

AACTAGTGGC CAAATGCTGG CTGCTTATTA GAAATAGGCA TTAGTCCCAG CCCTCTTTCG   180

GGTCGAGCGA GACCCATTCG ATGCAAGGCA GCTGATCAAA **ATG TGG CGC GAA ACC AAA**   238
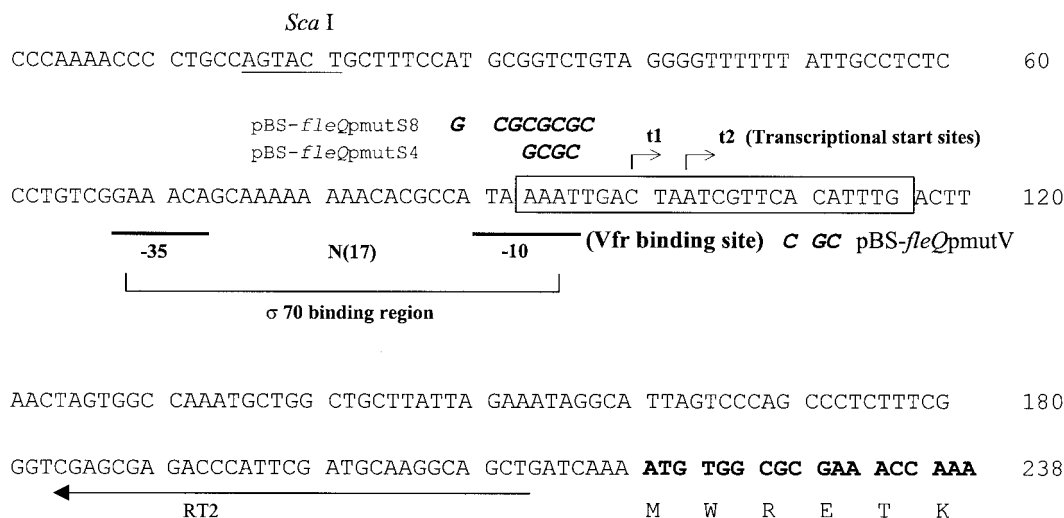   RT2   M   W   R   E   T   K

FIG. 1. Schematic representation of the *fleQ* promoter region: 238-bp region including the upstream region of *fleQ* and the first six codons (boldface type) of its coding region. The deduced amino acid sequence is shown under the codons. The Vfr binding site is enclosed in a box. The putative −10 and −35 sequences for the σ[70] binding site are underlined. Transcriptional start sites t1 and t2, as determined by primer extension analysis, are shown. The bases mutagenized in the Vfr binding site and the −10 region are italicized and in boldface type, and the designations of the plasmids in which they were generated are indicated. The location of the RT2 primer used for primer extension is indicated by an arrow. The *Sca*I site is underlined.

with carbenicillin to an OD$_{600}$ of ~0.2, induced with 1 mM IPTG, and grown for an additional 6 h at 37°C with shaking.

SDS-polyacrylamide gel electrophoresis and Western analysis. Bacteria were pelleted from 1 ml of each culture grown for 6 h (noninduced and IPTG induced). The cells were denatured by boiling in 2% sodium dodecyl sulfate (SDS)–1% β-mercaptoethanol–50 mM Tris-Cl (pH 7.5). Aliquots (5 μl) of the samples were resolved on a 12.5% polyacrylamide gel, and the proteins were transferred to a polyvinylidene difluoride membrane by Western blotting. Following blocking in 3% skim milk for 2 h, the blot was treated with either anti-FleQ (1:2,000) or anti-Vfr (1:5,000) antibody as the first antibody and anti-rabbit immunoglobulin G-alkaline phosphatase conjugate as the second antibody. The blot was developed by using nitroblue tetrazolium and BCIP (5-bromo-4-chloro-3-indolylphosphate) as the color substrate (8).

RNA extraction. A 100-ml LB broth culture of *P. aeruginosa* PAK was grown at 37°C to an OD$_{600}$ of 0.6 with shaking, and the cells were harvested by centrifugation at 3,000 rpm for 20 min at 4°C (JA-10 rotor; Beckman). Total RNA was isolated from the cells by using the TRIZOL reagent (Invitrogen Life Technologies, Carlsbad, Calif.) and the supplier's protocol for isolating total bacterial RNA.

Primer extension analysis. Oligonucleotide RT2 was 5′ end labeled in the presence of [γ-^{32}P]ATP (3,000 Ci/mmol; Perkin-Elmer Life Sciences) and T4 polynucleotide kinase (Invitrogen Life Technologies). The labeled oligonucleotide (1 × 10^6 cpm) was used in a primer extension reaction mixture (total volume, 20 μl) consisting of 30 μg of denatured (incubation at 65°C for 20 min) total RNA, 5 μl of 5× first-strand buffer, 1 μl of a solution containing each deoxynucleoside triphosphate at a concentration of 10 mM, 1 μl of a 100 mM DTT solution, 1 μl of RNaseOUT, and 200 U of Superscript II RT. The reaction mixture was incubated at 42°C for 1 h, treated with 20 U of RNase H, phenol-chloroform extracted, and ethanol precipitated. The precipitate was resuspended in 6 μl of sequencing loading buffer, denatured at 95°C for 2 min, and subjected to 8% urea–polyacrylamide gel electrophoresis along with a sequencing ladder. The sequencing ladder was generated by Sanger's dideoxy sequencing method by using a Sequenase version 2.0 kit (U.S. Biochemical Corp., Cleveland, Ohio) with pBS-*fleQ*pmutV as the template and RT2 as the primer. The gel was dried and subsequently autoradiographed.

In vitro transcription assay with *E. coli* RNA polymerase σ^{70} holoenzyme. In vitro single-round runoff transcription analysis was performed by using *E. coli* RNA polymerase holoenzyme saturated with σ^{70} (Epicentre Technologies, Madison, Wis.) and a DNA fragment containing the *fleQ* promoter. *Sca*I-*Bam*HI fragments (289 bp) excised from p*lac*ΩQ and p*lac*ΩQmutS4 served as templates for the transcription assays. Briefly, 0.1 pmol of the DNA template was incubated with 0.2 U of the RNA polymerase holoenzyme, 10 μCi of [α-^{32}P]CTP (3,000 Ci/

mmol; Perkin-Elmer Life Sciences), 0.4 mM ATP, 0.4 mM GTP, 0.4 mM UTP, 11 μM CTP, 1× transcription buffer (40 mM Tris-HCl [pH 7.9], 6 mM MgCl$_2$, 2 mM spermidine, 10 mM NaCl), and 1 μl of RNaseOUT at 37°C for 1 h in a 10-μl (total volume) mixture. Subsequently, the samples were treated with 1 U of RNase-free DNase for 15 min at 37°C, phenol-chloroform extracted, and ethanol precipitated. Each pellet was dissolved in RNA gel loading dye, and samples were electrophoresed in a 6% denaturing polyacrylamide gel containing 7 M urea and analyzed by autoradiography. A 50-bp DNA ladder served as the molecular weight marker.

RESULTS

Vfr binds to the *fleQ* promoter. Vfr has been shown to bind to specific sequences in the *lasR*, *regA*, and *toxA* promoters (K. J. Kanack, E. P. Ferrell, L. J. Runyen-Janecky, and S. E. H. West, Abstr. 100th Gen. Meet. Am. Soc. Microbiol., abstr. D140, p. 258, 2000; L. J. Runyen-Janecky, A. M. Albus, B. H. Iglewski, and S. E. H. West, Abstr. 96th Gen. Meet. Am. Soc. Microbiol. 1996, abstr. B-35, p. 160, 1996). To identify additional Vfr binding sites, homology searches of the *P. aeruginosa* genome were conducted by using the Vfr binding site present in the *lasR* promoter. These searches revealed the putative Vfr binding site sequence AATTGACTAATCGTTCACATTT centered 116.5 bp upstream of the *fleQ* translational start (Fig. 1). This sequence is 62.5% similar to the *lasR* Vfr binding site sequence. In order to authenticate the putative Vfr binding site identified in the *fleQ* promoter, EMSAs were performed by using an 88-bp fragment containing this sequence and purified Vfr. The mobility of the 88-bp *fleQ* promoter fragment was retarded. The retardation was partial in the presence of 72.2 nM Vfr (Fig 2A) and complete in the presence of 722 nM Vfr (data not shown). This demonstrated that the promoter region did indeed contain a Vfr binding site. Inclusion of a 100-fold excess of the unlabeled *fleQ* promoter fragment in the binding reaction mixture inhibited the retardation of the labeled frag-
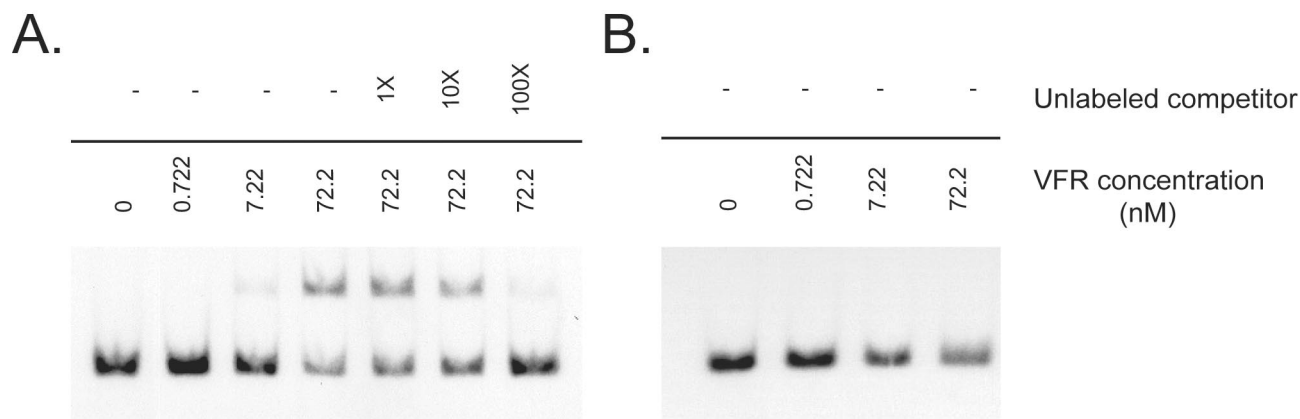
A.

B.

FIG. 2. EMSA of Vfr on the *fleQ* promoter. (A) An 88-bp radiolabeled *fleQ* promoter fragment was incubated either with different concentrations of Vfr or with a fixed amount (72.2 nM) of Vfr and different amounts of the unlabeled *fleQ* promoter fragment as described in Materials and Methods. (B) The mutated *fleQ* promoter fragment in which CGC was substituted for ACA in the putative Vfr binding site was incubated with different concentrations of Vfr. The amounts of the unlabeled competitor *fleQ* DNA are indicated above the bars. The concentrations of purified Vfr are indicated below the bars.

ment (Fig. 2A). This result indicated that the observed binding of Vfr to the *fleQ* promoter fragment was specific.

To confirm the identity of the Vfr DNA binding site in the *fleQ* promoter, DNase I footprinting was performed for a 158-bp *fleQ* promoter fragment that included the 88-bp region used in the EMSAs (Fig. 1 and 3). In the presence of Vfr, the 5′-AAATTGACTAATCGTTCACATTTG-3′ site was protected from DNase I cleavage. This region contains hypersensitive sites located at positions 11 and 19 on the coding strand and at positions 9 and 10 on the antisense strand of the protected sequence. This observation is similar to previous observations of DNase I hypersensitive sites in the promoters of other Vfr-regulated genes (Kanack et al., Abstr. 100th Gen. Meet. Am. Soc. Microbiol.).

Of the eight known and putative Vfr binding sites identified in the *P. aeruginosa* genome by subsequence search analysis, the CACA motif was highly conserved. To further confirm that the AAATTGACTAATCGTTCACATTTG site is the Vfr DNA binding site in the *fleQ* promoter, we substituted CGC for ACA by performing site-directed mutagenesis of pBS-*fleQ*p. EMSAs were performed for a 88-bp *fleQ* promoter fragment containing this mutation. At a Vfr concentration of 72.2 nM (Fig. 2B), Vfr failed to bind to the *fleQ* promoter, probably because of its reduced affinity for the mutated site.

***fleQ* promoter activity is not downregulated in a PAO1 *vfr* mutant.** Vfr activates transcription of *toxA*, *regA*, and *lasR* (2, 34). To test whether the same was true for *fleQ*, the promoter activity of *fleQ* was assessed in PAO1 and the *vfr* deletion mutant PAO9001. The CTX-*fleQ*placZ plasmid containing the *fleQ* promoter cloned upstream of a promoterless *lacZ* gene and the mini-CTX*lacZ* plasmid were integrated into the *attB* site of the chromosome of each strain, and their β-galactosidase activities were quantified (Table 2). The *fleQ* promoter activities were comparable in the wild-type and *vfr* mutant strains. The absence of Vfr in PAO9001 had no effect on the activity of the promoter, indicating that the *fleQ* promoter was not dependent on Vfr for transcriptional activation. To further confirm that the activation of *fleQ* transcription was not Vfr

dependent, PAO1 and the *vfr* mutant PAO9001 were tested in a motility plate assay (data not shown). There was no motility defect in *vfr* mutant PAO9001.

***vfr* overexpression from a plasmid renders *P. aeruginosa* nonmotile.** As Vfr binds to the *fleQ* promoter in vitro and does not activate *fleQ* transcription, we explored the possibility that Vfr may function as a repressor. To test whether overexpression of Vfr from an IPTG-inducible promoter would repress flagellar motility through *fleQ*, the *vfr* overexpression plasmid pWNP28 and the vector control pMMB66EH were electroporated into PAK and PAO1. The carbenicillin-resistant transformants were tested on motility agar plates containing carbenicillin and various concentrations of IPTG (0, 0.01, 0.1, and 1 mM). At IPTG concentrations of 0.1 and 1 mM, the PAK and PAO1 strains containing the inducible Vfr expression plasmid pWNP28 initially appeared to be nonmotile (6 h) (data not shown), but they displayed small motility zones upon further incubation (22 h), which were markedly reduced compared to that of the vector control. The motility profile of PAK under these conditions is shown in Fig. 4A.

FleQ production is downregulated following Vfr overproduction. To examine whether the repressive effect of Vfr on motility was due to repression of *fleQ*, a semiquantitative Western analysis of FleQ production was performed. Lysates of equivalent numbers of bacterial cells from IPTG-induced cultures of PAK containing either pWNP28, the *vfr* overexpression plasmid, or pMMB66HE, the vector control, were electrophoresed on an SDS-polyacrylamide gel and subjected to Western analysis with anti-FleQ (Fig. 4B) and anti-Vfr (Fig. 4C) polyclonal antibodies. The specificities of the anti-FleQ and anti-Vfr antibodies were examined prior to being used (data not shown). FleQ was barely detectable in PAK (pWNP28) (0.1 mM and 1.0 mM IPTG) compared to the control strain containing the vector. This indicated that downregulation of *fleQ* was one possible reason for the reduced motility observed with PAK(pWNP28). The amount of Vfr produced increased as the IPTG concentration increased (Fig. 4C). For
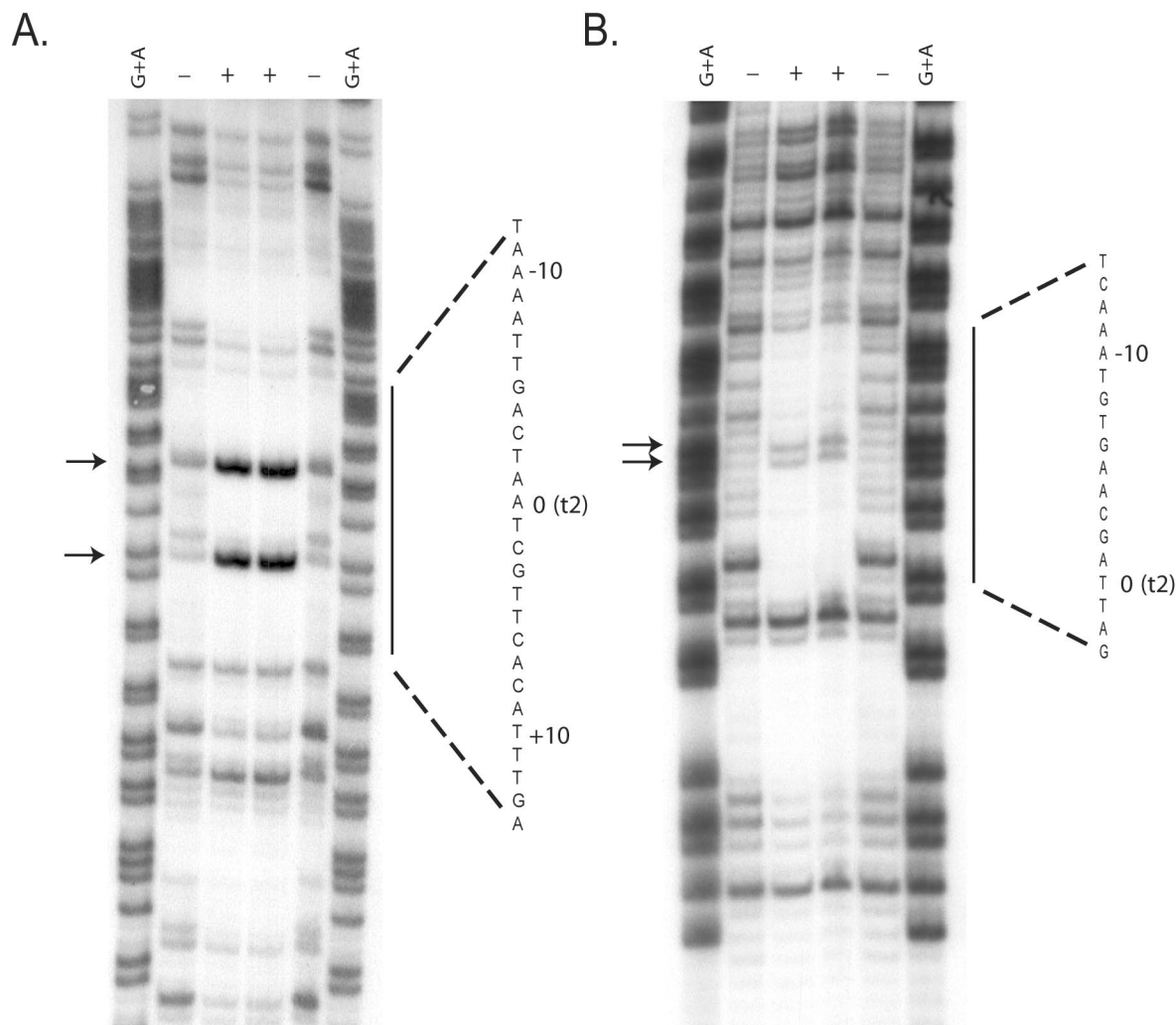
FIG. 3. DNase I footprint of Vfr bound to the *fleQ* promoter. The *fleQ* promoter radiolabeled on the coding (A) or antisense (B) strand was incubated with or without purified Vfr and was treated with DNase I as described in Materials and Methods. Lanes G+A, sequencing ladder of the *fleQ* promoter; lanes +, Vfr present; lanes −, Vfr absent. The region protected from DNase I digestion by Vfr is indicated by a bar and the sequence of the protected region. The DNase I hypersensitive sites are indicated by arrows. The numbers indicate the positions of the nucleotides with respect to the t2 transcriptional start site.

further experiments under Vfr overexpression conditions we used 1 mM as the inducing concentration of IPTG.

To ascertain that the observed lower level of expression of FleQ in the *vfr* overexpression strain PAK(pWNP28) was due to transcriptional repression and not due to instability of FleQ under *vfr*-overexpressing conditions, the activities of the *fleQ* promoter-*lacZ* fusions integrated into the *attB* sites in strains PAK *fleQ*p-*lacZ* and PAO *fleQ*p-*lacZ* were assessed by per-

TABLE 2. Activity of the *fleQ* promoter in PAO1 and its isogenic *vfr* mutant, PAO9001

Promoter-*lacZ* fusion (chromosomal insertion)	β-Galactosidase activity (Miller units)	
	PAO1	PAO9001 (*vfr* mutant)
*fleQ*p-*lacZ* (wild type)	1,981 ± 59[a]	1,992 ± 36
Promotrless *lacZ*	69 ± 16	99 ± 20

[a] The values are means ± standard deviations.

forming a β-galactosidase reporter assay with *vfr* overexpression from pWNP28. PAK *lacZ* and PAO *lacZ* with promoterless *lacZ* inserted into the *attB* site served as controls. Strains overexpressing *vfr* repressed transcription from the wild-type *fleQ* promoter four- and twofold in PAK(pWNP28) and PAO1(pWNP28), respectively (Table 3). The basal level of activity of the wild-type promoter in PAO1(pMMB66EH) was higher than that in PAK(pMMB66EH).

If downregulation of the *fleQ* promoter was due to repression by Vfr, an ACA-to-CGC mutation in the Vfr binding site of the *fleQ* promoter with reduced affinity for Vfr in an EMSA would be expected to be unresponsive or less responsive to Vfr-dependent downregulation. Therefore, we assessed the activity of an *fleQ*pmutV-*lacZ* fusion that had been integrated into the *attB* sites of PAK and PAO1 containing the *vfr* overexpression plasmid pWNP28 (Table 3). The activity of *fleQ*p-mutV was higher than the activity of the wild-type *fleQ* promoter in both PAK and PAO1 carrying either the *vfr*

A

No IPTG

a　　　b

0.01 mM

a　　　b

0.1 mM

a　　　b

1.0 mM

a　　　b

B

1 2 3 4 5 6 7 8
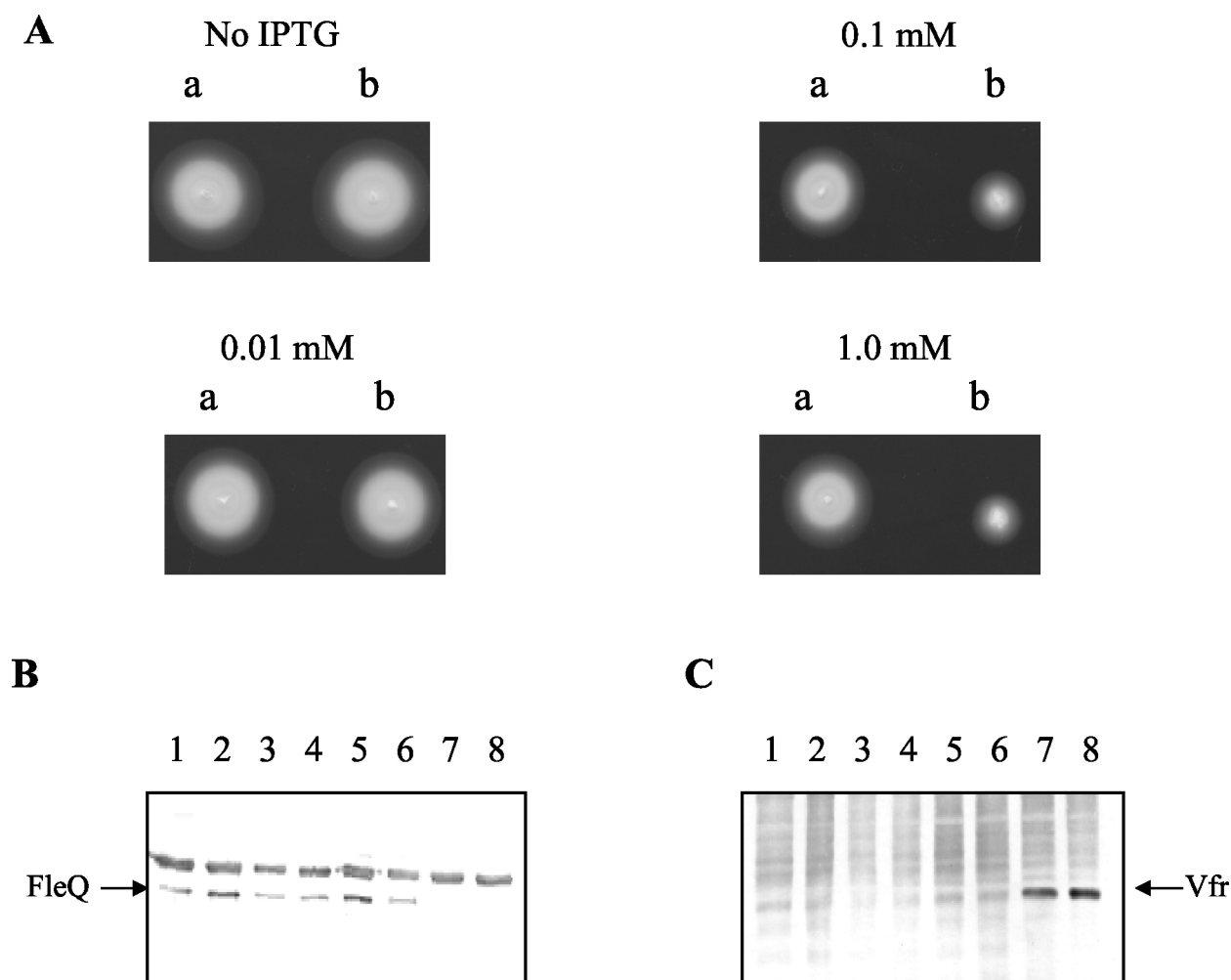

FleQ →

C

1 2 3 4 5 6 7 8

← Vfr

FIG. 4. Vfr overexpression downregulates FleQ, thereby reducing motility. (A) Motility assay conducted on 0.3% agar plates containing carbenicillin and various IPTG concentrations, as indicated. Spots a and b, PAK harboring the vector control pMMB66EH and the *vfr* overexpression plasmid pWNP28, respectively. (B and C) Western analysis of the strains used in the motility assay with different IPTG concentrations and with anti-FleQ (B) and anti-Vfr (C) antibodies. Lanes1 to 4 contained lysates from cultures of PAK harboring pMMB66EH grown with 0, 0.01, 0.1, and 1.0 mM IPTG, respectively. Lanes 5 to 8 contained lysates from cultures of PAK harboring pWNP28 grown with 0, 0.01, 0.1, and 1.0 mM IPTG, respectively.

overexpression plasmid pWNP28 or the vector pMMB66EH. The activity of the mutated promoter was not repressed by overproduction of Vfr in PAO1, indicating that Vfr was not able to bind to the *fleQ* promoter in vivo in strain PAO1. However, in PAK, the *fleQ*pmutV promoter activity still displayed a twofold reduction under *vfr* overexpression conditions, indicating that the 3-bp ACA-to-CGC mutation in the Vfr binding site with reduced affinity for purified Vfr in vitro

(Fig. 2B) was still responsive to partial repression by Vfr in vivo.

Vfr binding site overlaps the putative −10 region and the transcriptional start sites of *fleQ*. We used primer extension analysis with PAK total RNA to determine the transcriptional start sites of *fleQ*. The transcriptional start sites mapped 121 bp (t1) and 118 bp (t2) upstream of the ATG initiation codon (Fig. 1 and 5), and both of these sites mapped within the Vfr

TABLE 3. Effect of *vfr* overexpression on the activity of the wild-type *fleQ* promoter and its mutagenized derivative

Promoter-*lacZ* fusion (chromosomal insertion)	β-Galactosidase activities (Miller units) in the following strains:			
	PAK(pMMB66EH) (vector)	PAK(pWNP28) (*vfr*)	PAO(pMMB66EH) (vector)	PAO(pWNP28) (*vfr*)
*fleQ*p-*lacZ*	1,007 ± 15[a]	253 ± 7	2,649 ± 37	1,267 ± 6
*fleQ*pmutV-*lacZ*	1,339 ± 4	680 ± 49	3,341 ± 76	3,005 ± 59
Promoterless *lacZ*	33 ± 3	54 ± 1	92 ± 60	150 ± 32

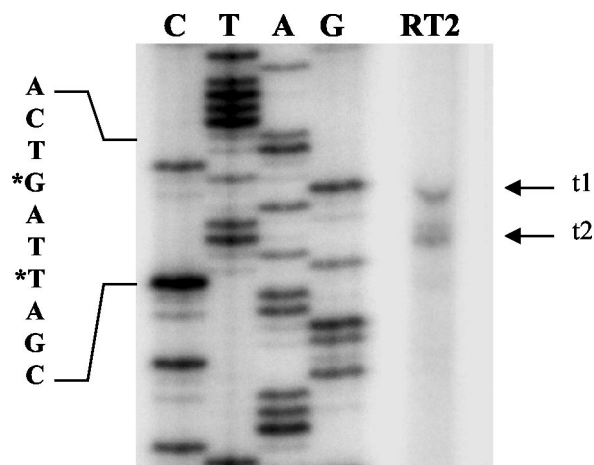[a] The values are means ± standard deviations.

FIG. 5. Primer extension analysis of *fleQ*. Thirty micrograms of total RNA isolated from PAK was subjected to primer extension analysis with the RT2 primer and loaded in lane RT2. A sequencing reaction mixture (lanes C, T, A, and G) containing the same primer with pBS-*fleQ*pmutV as the template was loaded and run on a denaturing urea-polyacrylamide gel simultaneously. The sequence of the region spanning the transcriptional start site is shown on the left. The residues marked with an asterisk are the probable start sites determined in this experiment, designated t1 and t2.

TABLE 4. Effects of mutations in the putative −10 region of the *fleQ* promoter on promoter activity

Plasmid	β-Galactosidase activity (Miller units)
p*lac*ΩQ	253 ± 21[a]
p*lac*ΩQmutS8	41 ± 4
p*lac*ΩQmutS4	39 ± 21
p*lac*Ω	38 ± 1

[a] The values are means ± standard deviations.

vitro transcription assay. Promoter sequences cloned in p*lac*ΩQ and p*lac*ΩQmutS4 were excised as *Sca*I-*Bam*HI fragments and used in a cell-free in vitro transcription assay with the *E. coli* RNA polymerase holoenzyme saturated with σ^{70} (Fig. 6). The presence of a major transcript migrating at the expected position of the *fleQ* transcript (203 nucleotides) (Fig. 6, lane 1) and the absence of this transcript in the reaction performed with the −10 region mutated template (Fig. 6, lane 2) confirmed that σ^{70} was capable of transcribing *fleQ*. A smaller transcript (lane 2) specific to the mutated template could have resulted from an alternative transcription initiation site.

DISCUSSION

fleQ appears to be the highest-level activator in the hierarchical regulatory cascade involved in the flagellar biogenesis pathway of *P. aeruginosa*. It is essential for flagellar motility as an *fleQ* mutant does not assemble a flagellum nor does it synthesize detectable amounts of flagellin (3). Moreover, at least eight of the various flagellar genes and operons studied to date appear to be directly and positively regulated by FleQ (7).
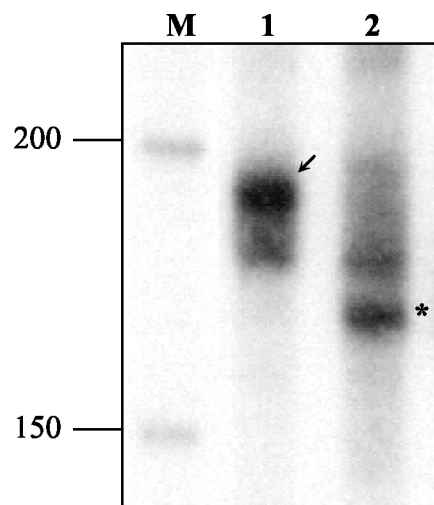
binding site. Analysis of the nucleotide sequence upstream of the transcriptional start sites revealed a −10 region centered 9.5 bp upstream of the t2 transcript and a −35 region centered 34 bp upstream of the t2 transcript with five of six and three of six matches with the *E. coli* σ70 consensus sequence, respectively (Fig. 1). Four of the six bases comprising the −10 region were also part of the Vfr binding consensus region. These findings were consistent with a model in which the t2 transcript of *fleQ* is σ^{70} regulated and occupancy of the Vfr binding site by Vfr could sterically hinder binding of the sigma factor (σ^{70}) in the −10 region and deny access to the RNA polymerase to initiate transcription at either t1 or t2, thereby repressing *fleQ* transcription. However, it was uncertain whether *fleQ* was σ^{70} regulated.

Mutations in the putative −10 region of the *fleQ* promoter result in significant reductions in its promoter activity. Two mutated derivatives of the *fleQ* promoter carrying an 8-bp mutation (ATAAAATT → GCGCGCGC) and a 4-bp mutation (AAAT → GCGC) in the −10 region were cloned into pDN19lacΩ, generating p*lac*ΩQmutS8 and p*lac*ΩQmutS4, respectively. p*lac*ΩQmutS8 and p*lac*ΩQmutS4 were electroporated into PAK, and their β-galactosidase activities were measured and compared. Both the mutated derivatives displayed a sixfold reduction in promoter activity compared to the wild-type promoter activity (Table 4). Moreover, the residual β-galactosidase activities of the 8- and 4-bp mutated promoters were comparable to the residual activity of the promoterless vector control, indicating that there was total loss of transcriptional activity even with the less extensive 4-bp mutation.

***E. coli* RNA polymerase σ^{70} holoenzyme initiates transcription from the *fleQ* promoter in an in vitro transcription assay.** In order to determine whether transcription from the *fleQ* promoter could be initiated solely by the σ^{70}-associated RNA polymerase, the *fleQ* promoter region was subjected to an in

FIG. 6. In vitro transcription analysis of *fleQ*. DNA templates containing the wild-type *fleQ* promoter (lane 1) and the 4-bp mutation in its −10 region (lane 2) were analyzed in a transcription reaction by using the *E. coli* RNA polymerase σ^{70} holoenzyme. ^{32}P-labeled DNA size markers were electrophoresed in lane M. The sizes of the individual DNA fragments (in bases) are indicated on the left. The arrow indicates the position of the σ^{70}-dependent *fleQ* transcript in lane 1. The asterisk indicates the position of the smaller transcript visible exclusively in lane 2.

Any mechanism that downregulates FleQ activity would have a negative impact on flagellar biogenesis and thereby motility and chemotaxis in this pathogen. We have identified FleN, an antiactivator for FleQ which posttranslationally downregulates FleQ activity by direct binding (8). Whether in addition to this level of regulation there is any other mechanism that upregulates or downregulates the transcription of *fleQ* remains to be determined.

Based on sequence homology, we found a putative Vfr binding site in the *fleQ* promoter, and we set out to ascertain its functional significance. The data presented here indicate that the Vfr binding site is an authentic site, as purified Vfr was able to bind to it, as demonstrated in the EMSAs (Fig. 2), and to protect it from DNase I (Fig. 3). The sequence of the protected region on both strands was consistent with the predicted consensus binding site.

Vfr is known to function as a transcriptional activator for the quorum-sensing regulatory protein *lasR* (2). To determine the consequences of Vfr binding to the *fleQ* promoter, the promoter activity of FleQ was tested in PAO1 and a *vfr* mutant derivative of this strain, PAO9001 (Table 2). As there was no appreciable decline in the promoter activity in the *vfr* mutant, activation of *fleQ* transcription was not dependent on Vfr. We next explored whether repression by Vfr was a possibility. Since *fleQ* promoter activity in the *vfr* mutant was not greater than *fleQ* promoter activity in the wild-type strain, there was no evidence that suggested a repressor function for Vfr. We hypothesized that the amount of Vfr synthesized under our culture conditions was probably not sufficient for repression. Overexpression of *vfr* from pWNP28 under IPTG induction conditions in PAK (Fig. 4) and PAO1 (data not shown) repressed motility and downregulated the transcription of *fleQ* (Table 3), indicating the ability of Vfr to repress *fleQ* as speculated. A comparison of the promoter activities in PAK and PAO1 (Table 3) revealed that although the *fleQ* promoter regions analyzed in strains PAK and PAO1 were identical, the basal activity of the promoter appeared to be greater in PAO1. Repression of the *fleQ* wild-type promoter by Vfr appears to be more effective in PAK (fourfold downregulation) than in PAO1 (twofold downregulation). As the sources of Vfr and the Vfr binding site sequences in the two strains tested are the same, involvement of an accessory factor (corepressor) that makes Vfr a better repressor in PAK than in PAO1 remains a possibility. The effector molecule that regulates Vfr activity in *P. aeruginosa* has not been identified. cAMP is one possibility and is required for Vfr activity in *E. coli* (32, 34). Not much is known about the levels of cAMP in *P. aeruginosa* (21, 26) and specifically about quantitative differences (if any) in the cAMP levels in PAK and PAO1 which would enable us to explain our observations.

To understand how Vfr represses *fleQ* transcription, we mapped the *fleQ* transcriptional start sites. Two transcriptional start sites, t1 and t2, mapped within the Vfr binding site in PAK (Fig. 5). Inspection of the sequences upstream of the transcriptional start sites revealed a σ^{70} binding consensus (Fig 1) for the t2 transcript, indicating that it may be σ^{70} dependent. In order to determine whether transcription of *fleQ* was σ^{70} dependent in vivo, a 4-bp mutation and an 8-bp mutation were generated in the putative −10 region of the *fleQ* promoter, and the activities of the mutated promoters were assessed with a β-galactosidase assay by using a plasmid-based *lacZ* reporter (Table 4). Both of the mutated derivatives showed low promoter activity comparable to that of the promoterless *lacZ* construct. Thus, the AAAT-to-GCGC substitution probably eliminated *fleQ* promoter activity due to the inability of the σ^{70} RNA polymerase holoenzyme to bind to the −10 region of the promoter and to initiate transcription effectively in *P. aeruginosa*. In an in vitro transcription assay (Fig. 6), the ability of the σ^{70}-saturated RNA polymerase holoenzyme of *E. coli* to initiate transcription from the *fleQ* promoter suggested the presence of an authentic σ^{70}-recognizing sequence in the *fleQ* promoter. The same holoenzyme was unable to generate the 203-nucleotide *fleQ*-specific transcript from a DNA template with a 4-bp mutation in the −10 region of the *fleQ* promoter, which is predicted to be important in σ^{70} binding. Another, smaller transcript seen in the assays probably arose from an alternative start site in the mutated template but did not appear to contribute to any residual β-galactosidase activity in a reporter plasmid carrying the same mutation (p*lac*ΩQmutS4) (Table 4). The results of the primer extension analysis, β-galactosidase assay, and in vitro transcription assay are consistent with involvement of σ^{70} in the transcription of *fleQ*. Possible involvement of σ^{54} in the transcriptional regulation of *fleQ* in another pseudomonad (29) has been suggested. However, in *P. aeruginosa*, the *fleQ* promoter retains activity in an *rpoN* (σ^{54}) mutant (3). These differences suggest that the promoters of *fleQ* and its homologs are probably complex structures with multiple regulatory mechanisms functioning under different environmental conditions; repression by Vfr is probably one of these mechanisms. The involvement of σ^{70} in transcriptional control of *fleQ* suggests that *fleQ* could either be constitutively expressed in a manner similar to the housekeeping genes using the σ^{70} holoenzyme or be activated by an activator that works in concert with σ^{70} (13).

Introduction of mutations into the Vfr binding site to eliminate Vfr binding and thereby repress *fleQ* would add credence to the proposed role of Vfr as a repressor of *fleQ*. As the Vfr binding site overlaps the proposed −10 region and transcriptional start sites, the sites that could be mutated that would eliminate Vfr binding but not transcription of *fleQ* were limited. As the CACA motif appeared to be conserved in eight putative Vfr binding sites, CGC was substituted for ACA and tested in an EMSA (Fig. 2B) for Vfr binding in vitro. The mutated Vfr binding site did not exhibit binding in an EMSA with purified Vfr. The same mutation was integrated into the chromosomes of PAK and PAO1 as an *fleQ*pmutV-*lacZ* fusion, and the activity of the *fleQ* promoter was measured under *vfr*-overexpressing conditions with a β-galactosidase assay (Table 3). In PAO1 repression by Vfr was apparently eliminated as there was no significant reduction in the promoter activity when Vfr was overproduced. In PAK, the same mutation in the Vfr binding site still resulted in twofold downregulation of the mutated promoter (compared to 4-fold downregulation with the wild-type promoter), indicating that this mutation only partially eliminated repression in this strain. A detailed analysis of various mutations is needed to determine the best possible combination that completely eliminates repression of the *fleQ* promoter by Vfr in PAK without compromising the initiation of *fleQ* transcription.

Whether any environmental or cultural conditions enhance

vfr expression from its own promoter enough to result in substantial repression of *fleQ*, like that seen in the overexpression experiments, is difficult to predict. Nevertheless, as there are at least eight flagellar genes or operons that are FleQ dependent (7, 8), even a small quantitative reduction in the free FleQ pool within the cell could potentially retard the process of flagellar biogenesis and have significant physiological implications.

Repression of flagellar biogenesis by Vfr is new to *Pseudomonas* research because to the best of our knowledge our report is the first evidence that a nonflagellar regulator (Vfr) affects the topmost regulator (FleQ) in the flagellar biogenesis hierarchy by downregulating its transcription. Moreover, previously, Vfr has been shown to be a transcriptional activator of *lasR* and *toxA*, which are involved in quorum sensing (2) and exotoxin A production, respectively (34). Evidence obtained in this study suggests that like CRP, its *E. coli* homolog (13), Vfr is also capable of functioning as a repressor, depending on the position of its binding site in the promoter region with respect to the transcriptional start site. In general, in σ^{70}-dependent promoters activator binding sites do not appear in positions downstream from the −30 region, whereas repressor binding sites are very common in this region (13). For example, in the *malT* promoter CRP binds around the −70 region and functions as an activator (22), whereas in the *cya* promoter the CRP binding site encompasses the +1 site and functions as a repressor instead (1). Similarly, in the *lasR* promoter of *P. aeruginosa*, where the Vfr binding site is located around position −47, Vfr is reported to function as an activator (2), whereas in the *fleQ* promoter, where Vfr binds to DNA from position −10 to position +14 with respect to the t2 transcript (Fig. 1), it functions as a repressor (this study).

Induction of many virulence genes occurs in *P. aeruginosa* at a time after quorum sensing has been initiated. We hypothesize that in order for Vfr to promote quorum sensing, it would be necessary to inhibit flagellar motility through repression of *fleQ* in order for bacteria to reach the appropriate cell density. Appropriate environmental cues could induce Vfr production and simultaneously downregulate flagellar biogenesis to prevent the bacteria from swimming away from a site of infection. If quorum-sensing mechanisms are important for virulence in specific infections, then a similar mechanism should be initially operative to prevent motility-dependent dissemination of *P. aeruginosa* from infection sites until the full repertoire of virulence genes is induced.

ACKNOWLEDGMENTS

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