JOURNAL OF BACTERIOLOGY, Dec. 1994, p. 7532–7542
0021-9193/94/$04.00+0

Vol. 176, No. 24

The *vfr* Gene Product, Required for *Pseudomonas aeruginosa* Exotoxin A and Protease Production, Belongs to the Cyclic AMP Receptor Protein Family

SUSAN E. H. WEST,[1,2]* ALLEN K. SAMPLE,[1]† AND LAURA J. RUNYEN-JANECKY[1,2]

Department of Pathobiological Sciences, School of Veterinary Medicine,[1] and Program in Cell and Molecular Biology,[2] University of Wisconsin—Madison, Madison, Wisconsin 53706

Received 10 August 1994/Accepted 12 October 1994

The synthesis of exotoxin A (ETA) by *Pseudomonas aeruginosa* is a complex, regulated event. Several ETA putative regulatory mutants of *P. aeruginosa* PA103 have previously been characterized (S. E. H. West, S. A. Kaye, A. N. Hamood, and B. H. Iglewski, Infect. Immun. 62:897–903, 1994). In addition to ETA production, these mutants, PA103-15, PA103-16, and PA103-19, were also deficient in the production of protease and in *regA* P1 promoter activity. RegA is a positive regulator of ETA transcription. We cloned a gene, designated *vfr* for virulence factor regulator, that restored ETA and protease production to parental levels in these mutants. In addition, transcription from the *regA* P1 promoter was restored. In *Escherichia coli*, when *vfr* was overexpressed from a phage T7 promoter, a protein with an apparent molecular mass of 28.5 kDa was produced. Analysis of the deduced amino acid sequence of *vfr* revealed that the expected protein is 67% identical and 91% similar over a 202-amino-acid overlap to the *E. coli* cyclic AMP receptor protein (CAP or Crp). The cloned *vfr* gene complemented the β-galactosidase- and tryptophanase-deficient phenotypes of *E. coli* RZ1331, a *crp* deletion mutant. However, the *E. coli crp* gene under the control of the *tac* promoter did not complement the ETA-deficient or protease-deficient phenotype of PA103-15 or PA103-16. The ability of *vfr* to restore both ETA and protease production to these mutants suggests that *vfr* is a global regulator of virulence factor expression in *P. aeruginosa*.

Exotoxin A (ETA), encoded by the *toxA* gene, is an ADP-ribosyl transferase produced by the opportunistic pathogen *Pseudomonas aeruginosa*. ETA expression is not constitutive but is regulated by a variety of environmental stimuli including iron concentration, temperature, aeration, and calcium concentration (6, 33). The presence of >10 μM iron in the growth medium results in the repression of *toxA* transcription (34). However, in iron-deficient medium, transcription of *toxA* occurs in a biphasic manner (17). During early log phase, there is a peak in *toxA* mRNA accumulation which is followed by a decline in *toxA*-specific mRNA. This early phase of *toxA* expression is slightly repressed by iron. A second accumulation of *toxA* mRNA peaks at entry into stationary phase and is strongly repressed by iron. Several genes including *regA*, *regB*, *fur*, and *lasR* have been implicated in regulation of ETA production (21, 26, 40, 58).

regA is a positive regulatory factor that effects *toxA* transcription (26); however, the mechanism by which *regA* modulates *toxA* transcription has not been elucidated. There are two *regA* transcripts which are transcribed from two distinct promoters, designated P1 and P2 (17, 18, 50). The larger of the two transcripts (T1) is transcribed from the start site farther upstream during the moderately iron-regulated, early logarithmic phase (6 h) of *regA* mRNA accumulation. The smaller *regA* transcript (T2) is made during the strictly iron-regulated, late-logarithmic and stationary phases (10 h) of *regA* mRNA accumulation. The *regB* gene is required for maximal production of ETA in strain PA103 (58).

The *P. aeruginosa lasR* and *fur* genes have also been implicated in the regulation of *toxA* expression. LasR is a positive regulator of transcription of the *lasB* and *lasA* genes, which encode two distinct elastases (20). LasR has also been shown to slightly enhance *toxA* transcription by an unknown mechanism (21). The *P. aeruginosa fur* gene product represses expression of numerous iron-regulated genes, including *toxA* and those required for siderophore production (40). This repression occurs in iron-replete growth conditions. It is not clear whether *P. aeruginosa fur* acts directly on the *toxA* and *regAB* promoters or acts indirectly through another gene to repress transcription of these two genes (40).

Several genetic studies of ETA expression indicate that additional genes are involved in regulation of ETA production. Gray and Vasil (23) isolated and characterized two nitrosoguanidine-generated ETA putative regulatory mutants of PAO1. These mutations were designated *tox-1* and *tox-2* and were mapped at 26 and 23 min, respectively, on the *P. aeruginosa* PAO1 revised chromosomal map (23). The fact that these mutations map to locations on the *P. aeruginosa* chromosome different from those of *toxA*, *regAB*, *lasR*, and *fur* indicates that at least two other unidentified genes regulate ETA expression. *toxA*, *regAB*, *lasR*, and *fur* are located at 62, 65 to 70, 12 to 15, and 0 to 5 min, respectively, on the PAO1 chromosomal map (41, 46, 53a). Ohman et al. (38) isolated four ETA-deficient mutants of *P. aeruginosa* PA103, designated PA103-8, PA103-15, PA103-16, and PA103-19, which were further characterized by West et al. (57). These mutants were not complemented by the cloned *toxA* or *regAB* genes and were deficient in transcription from the *regA* P1 promoter. These mutants did not accumulate intracellular ETA, indicating that the mutations were not in genes required for the secretion of ETA. In

* Corresponding author. Mailing address: Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin—Madison, 2015 Linden Dr. West, Madison, WI 53706. Phone: (608) 263-7884. Fax: (608) 263-6573. Electronic mail address: wests@svm.vetmed.wisc.edu.

† Present address: U.S. Army Medical Research Institute for Infectious Diseases, Fort Dietrick, MD 21701.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant properties[a]	Source or reference
Strains		
P. aeruginosa		
PA103	Prototroph; ETA hyperproducer; elastase deficient	33
PA103-15	ETA- and protease-deficient NTG-generated mutant of PA103	38
PA103-16	ETA- and protease-deficient NTG-generated mutant of PA103	38
PA103-19	ETA- and protease-deficient NTG-generated mutant of PA103	38
PAO1	Prototroph	28
E. coli		
KM381	Host for the phage T7 expression system	S. Tabor
RZ1331	MG1655 *rspL* Δ*crp lacP1*$^+$	13
RZ1330	MG1655 *rspL* Δ*crp lacP1*$^+$ *ilv*::Tn*10* Δ*cya*	W. S. Reznikoff
Plasmids		
pLA2917	Broad-host-range cosmid cloning vector; Tc^r Km^r	1
pMO011925	pLA2917 carrying the 23-min region of the *P. aeruginosa* PAO chromosome; *trpC*; Tc^r	41
pMO012425	pLA2917 carrying the 23-min region of the *P. aeruginosa* PAO chromosome; *trpC trpD argC*; Tc^r	41
pRK2013	pMB1 derivative carrying the RK2 transfer functions; Km^r	16
pUCP18	Multicopy *E. coli-P. aeruginosa* shuttle vector; Ap^r Cb^r	44
pUCP19	Multicopy *E. coli-P. aeruginosa* shuttle vector; Ap^r Cb^r	44
pKF901	pUCP19 carrying the 7.0-kb *Pst*I fragment from pMO011925; Ap^r Cb^r	This study
pKF902	pUCP19 carrying the 4.2-kb *Pst*I fragment from pMO011925; Ap^r Cb^r	This study
pKF904	pUCP19 carrying the 2.5-kb *Pst*I-*Sac*I fragment from pKF902; Ap^r Cb^r	This study
pKF906	*Sal*I deletion of pKF902; Ap^r Cb^r	This study
pKF812	pUCP18 carrying the 1.2-kb *Xho*I fragment from pKF902; Ap^r Cb^r	This study
pKF417	pBluescript KS$^+$ carrying the 0.9-kb *Nar*I fragment from pKF906; Ap^r Cb^r	This study
pKF917	pUCP19 carrying the 0.9-kb *Nar*I fragment as a *Bam*HI-*Sal*I fragment from pKF417; Ap^r Cb^r	This study
pKF506	pLA2917 carrying the 2.6-kb *Sal*I-*Pst*I fragment as a *Hin*dIII-*Bam*HI fragment from pKF906; Tc^r	This study
pQF26	Transcriptional fusion vector containing a promoterless CAT gene; Cb^r	15
pP11	pQF26 carrying the *regA* P1 promoter from *P. aeruginosa* PA103; Ap^r Cb^r	50
pP21	pQF26 carrying the *regA* P2 promoter from *P. aeruginosa* PA103; Ap^r Cb^r	50
pT7-5	pBR322 carrying a MCS downstream of the strong gene 10 promoter of phage T7; Ap^r	S. Tabor
pT7-6	pT7-5 with MCS in the opposite orientation to pT7-5; Ap^r	S. Tabor
pGP1-2	pACYC177 carrying the phage T7 RNA polymerase gene under p_L control and *c*I857 repressor gene; Km^r	51
pT7-5-906	pT7-5 carrying the 2.6-kb *Sal*I-*Pst*I fragment containing *vfr* as an *Eco*RI-*Pst*I fragment from pKF906; Ap^r	This study
pT7-5-917	pT7-5 carrying the 0.9-kb *Nar*I fragment containing *vfr* as an *Eco*RI-*Pst*I fragment from pKF917; Ap^r	This study
pT7-6-906	pT7-6 carrying the 2.6-kb *Sal*I-*Pst*I fragment containing *vfr* as an *Eco*RI-*Pst*I fragment from pKF906; Ap^r	This study
pT7-6-917	pT7-6 carrying the 0.9-kb *Nar*I fragment containing *vfr* as an *Eco*RI-*Pst*I fragment from pKF917; Ap^r	This study
pMMB66HE	Broad-host-range *tac*P expression vector derived from RSF1010; *lacI*^{q}; Ap^r Cb^r	19
pMMB66EH	pMMB66HE with MCS in opposite orientation; Ap^r Cb^r	19
pRZ1306	pMMB66HE carrying the *E. coli crp* gene as a *Hin*dIII-*Bam*HI fragment; Ap^r Cb^r	W. S. Reznikoff
ptac917	pMMB66EH carrying the 0.9-kb *Nar*I fragment containing *vfr* as an *Eco*RI-*Pst*I fragment from pT7-5-917; Ap^r Cb^r	This study

[a] Ap^r, ampicillin resistance; Cb^r, carbenicillin resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance; MCS, multiple cloning site.

addition, these mutants did not produce protease. Therefore, West et al. (57) hypothesized that the lesion(s) in these mutants was a mutation in a regulatory gene or genes.

In this report, we describe the cloning and characterization of a gene which complements the deficiencies in ETA production, transcription from the *regA* P1 promoter, and production of protease in mutants PA103-15, PA103-16, and PA103-19. The cloned gene was designated *vfr* for virulence factor regulator and is a member of the *Escherichia coli crp* gene family (30). We also report that the cloned *vfr* gene is able to complement an *E. coli crp* deletion mutant; however, the *E. coli crp* gene is unable to substitute for *vfr* in *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 1. Mutants PA103-15 and PA103-16 were isolated in the same mutagenesis experiment and therefore could be siblings (38).

Culture conditions. All strains were routinely maintained on Luria-Bertani (LB) agar (36) at 37°C. The following amounts of antibiotics (per milliliter) were used: 100 μg of ampicillin and 25 μg of tetracycline for *E. coli* and 200 μg of carbenicillin and 100 μg of tetracycline for *P. aeruginosa*.

TSBD broth, an iron-depleted medium used for optimal production of ETA, was prepared as described by Ohman et al. (38). Briefly, trypticase soy broth (Difco Laboratories, Detroit,

Mich.) was treated with Chelex-100 (−400 mesh; Bio-Rad Laboratories, Richmond, Calif.) to remove iron and then was dialyzed. The dialysate was supplemented with 0.05 M monosodium glutamate and 1% glycerol. For iron-replete conditions, TSBD broth was supplemented with 37 μM $FeCl_3 \cdot 6H_2O$.

Genetic procedures. Purified plasmid DNA was introduced into *P. aeruginosa* by electroporation as described by Smith and Iglewski (48). Alternatively, recombinant plasmids were mobilized from *E. coli* to *P. aeruginosa* by using triparental matings with pRK2013 as the mobilizing plasmid (16).

Recombinant DNA techniques. Recombinant DNA techniques were performed as described by Sambrook et al. (42) and Ausubel et al. (2). *E. coli* SURE and XL1-Blue (Stratagene Cloning Systems, La Jolla, Calif.) and HB101 (42) were used as the host strains in all cloning experiments. The vectors pBluescript KS^+ and SK^+ (Stratagene Cloning Systems) were used as general-purpose cloning vectors.

ETA activity. For measurement of ETA production, TSBD broth (5 ml) was inoculated with 50 μl of an overnight TSBD culture and was incubated at 32°C with shaking for 18 h. The cultures were centrifuged for 10 min at 12,000 × *g*. The supernatant fractions were stored at −80°C until assayed for ETA activity. Thawed supernatants (10 μl) were activated by treatment with urea and dithiothreitol and were assayed for ADP-ribosyl transferase activity as described by Ohman et al. (38) and Chung and Collier (9). Partially purified elongation factor 2 from wheat germ (9) and [^{14}C]NAD (NEN Research Products, Boston, Mass.) were the substrates. ETA activity was expressed as counts per minute per 10 μl of culture supernatant.

Immunoblotting. Extracellular ETA protein was detected by immunoblotting as described by Burnette (8) and by Towbin et al. (52). Briefly, proteins from 10 μl of culture supernatant were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (31) on an 11.25% acrylamide gel, transferred to nitrocellulose, and probed with rabbit anti-ETA antibody and then with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Sigma Chemical Co., St. Louis, Mo.). Protein bands were visualized by using BCIP-NBT (5-bromo-4-chloro-3-indolylphosphate toluidinium–nitroblue tetrazolium) as described by Blake et al. (5). The rabbit anti-ETA antibody has been described previously (25).

Assay for proteolytic activity. Volumes (10 μl) of an overnight LB culture were spotted onto tryptic soy agar plates containing 1.5% (wt/vol) skim milk as described by Wretlind and Pavlovskis (61). The plates were incubated at room temperature for 48 h. Proteolytic activity was detected as zones of clearing around the colonies.

CAT assays. For measurement of chloramphenicol acetyltransferase (CAT) activity under the control of the *regA* promoters, TSBD broth (70 ml) was inoculated to an optical density at 600 nm of 0.02 with an overnight iron-replete (37 μM) TSBD culture. The cultures were incubated at 32°C with shaking for 24 h. At the indicated times, samples were taken and the optical density at 600 nm was determined. The samples were centrifuged at 12,000 × *g* for 10 min at 4°C. The cell pellets were washed with 100 mM Tris-HCl (pH 7.0) and stored at −80°C. CAT assays of cell pellet lysates were performed as described by Neumann et al. (37), using [^{14}C] acetyl coenzyme A (NEN Research Products). Samples were routinely incubated at room temperature for 60 min. Units of CAT were calculated from standard curves generated with purified *E. coli* CAT (Pharmacia LKB Biotechnology, Piscataway, N.J.).

DNA hybridizations. Genomic DNAs from *P. aeruginosa* PA103, PA103-15, PA103-16, PA103-19, and PAO1 were prepared as described by Hull et al. (29). The genomic DNA was digested with an appropriate restriction endonuclease, electrophoresed on a 0.7% agarose gel, and transferred to nylon filters (Hybond-N; Amersham Corp., Arlington Heights, Ill.) as described by Smith and Summers (49). Hybridization probes were the plasmid vector pT7-5 and recombinant plasmid pT7-5-917, which contained the 0.9-kb *Nar*I fragment encoding *vfr*. They were labeled with [α-^{32}P]dCTP (NEN Research Products) by nick translation with a kit from Promega Corp. (Madison, Wis.). Hybridizations were carried out in 4× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]) (42)–2× Denhardt solution (42)–20 mM sodium PP_i–0.2% SDS–100 μg of sheared denatured salmon sperm DNA per ml of hybridization fluid at 65°C for 12 to 15 h. The final washes were done in 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (42)–0.1% SDS–5 mM sodium PP_i (pH 7.5) at 65°C. Hybridization products were visualized by autoradiography.

DNA sequencing. Double-stranded DNA sequencing was accomplished by the dideoxy chain termination method (43) using the Sequenase 2.0 sequencing kit (U.S. Biochemical Corp., Cleveland, Ohio). The 1.2-kb *Xho*I fragment from pKF812 was subcloned into the vector pBluescript KS^+ (Stratagene Cloning Systems). Additional subclones were constructed on the basis of convenient restriction enzyme sites. Plasmid DNA was prepared by the acid-phenol miniprep procedure of Weickert and Chambliss (55). 7-Deaza-dGTP was routinely used in place of dGTP (3). To eliminate sequence artifacts caused by enzyme pausing, unlabeled dideoxy nucleotides and terminal transferase were added to the completed termination reaction mixture and the incubation was continued at 30°C for 30 min as described by Li and Schweizer (32). Sequence data were analyzed by using the Genetics Computer Group (Madison, Wis.) software (version 7). The TFASTA algorithm for protein homology was used to compare the sequence of the deduced protein product of *vfr* with sequences in the GenBank database (release 79.0, October 1993).

Expression of the Vfr protein in *E. coli*. To label selectively the Vfr protein, we used the phage T7-based expression system of Tabor and Richardson (51). The 2.6-kb *Sal*I-*Pst*I and 0.9-kb *Nar*I fragments containing the *vfr* gene were cloned into the T7 gene 10 promoter containing vectors pT7-5 and pT7-6, to generate pT7-5-906, pT7-5-917, pT7-6-906, and pT7-6-917. These plasmids were transformed into *E. coli* KM381/pGP1-2. Expression of polypeptides encoded on the cloned fragments was analyzed by labeling the induced gene products with 20 μCi of ^{35}S-labeled methionine (NEN Research Products) as described by Tabor and Richardson (51). The labeled polypeptides were separated by SDS-PAGE (12.5% [wt/vol] acrylamide) (31) and visualized by autoradiography.

β-Galactosidase and tryptophanase assays. For measurement of β-galactosidase production, 10 ml of LB medium containing 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and the appropriate antibiotics was inoculated with 100 μl of an overnight culture. The cultures were incubated at 37°C and 250 rpm until mid-log phase. β-Galactosidase activity was measured as described by Miller (36). Tryptophanase activity was determined by a modification of the procedure described by Bilezikian et al. (4). Specifically, 40 μl of an overnight LB culture was removed to chilled glass tubes containing 0.36 ml of LB medium and 0.2 ml of xylene. After induction of tryptophanase, Ehrlich's reagent (1.8 ml) was added to detect the production of indole. A pink-red color indicated trypto-

TABLE 2. Restoration of ETA activity in *P. aeruginosa* PA103-15, PA103-16, and PA103-19

Strain	ADP-ribosyl transferase activity[a] (cpm/10 μl of supernatant)					
	pLA2917	pMO011925	pMO012425	pUCP19	pKF906	pKF917
PA103	8,989	9,377	10,432	8,139	4,507	8,276
PA103-15	0	9,088	9,412	67	7,612	683
PA103-16	90	10,188	9,330	75	5,897	749
PA103-19	13	7,480	5,875	31	9,596	6,199

[a] Determined as described by Ohman et al. (38) and Chung and Collier (9) for supernatants from overnight TSBD cultures.

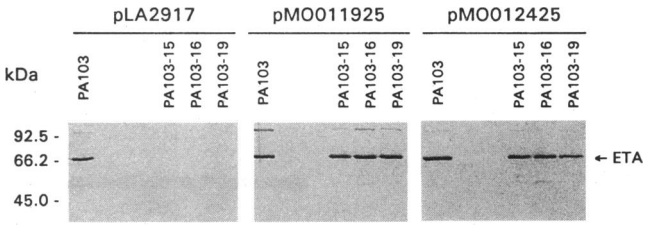

FIG. 1. Detection of ETA in culture supernatants from PA103, PA103-15, PA103-16, and PA103-19 by immunoblotting. pMO011925 and pMO012425 are cosmids containing the 23-min region of the *P. aeruginosa* PAO chromosome in vector pLA2917 (1, 41). The positions of the molecular mass standards (left) and the position of ETA (right) are indicated.

phanase activity, and a yellow color indicated no tryptophanase activity.

Detection of CAP in cell lysates. To detect expression of cyclic AMP (cAMP) receptor protein (CAP), *P. aeruginosa* PA103, PA103-15, and PA103-16 were grown in TSBD broth plus 1 mM IPTG at 32°C for 18 h. The cultures were centrifuged at 12,000 × *g* for 10 min, and lysates were prepared by sonication of the cell pellets. The cell lysates (25 μg of protein) were electrophoresed on an SDS–12.5% PAGE gel and immunoblotted as described above. Rabbit anti-CAP antibody, a gift of Agnes Ullmann, was used to detect CAP. CAP was purified by affinity chromatography on cAMP-agarose from *E. coli* RZ1330/pRZ1306. The purification was based on the procedure of Ghosaini et al. (22).

Nucleotide sequence accession number. The sequence data reported here have been submitted to GenBank and assigned accession number U16318.

RESULTS

Cloning of a DNA fragment which complements the ETA- and protease-deficient phenotypes of PA103-15, PA103-16, and PA103-19. Ohman et al. (38), using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) mutagenesis, generated several ETA- and protease-deficient mutants of the ETA-hyperproducing strain *P. aeruginosa* PA103. They were designated PA103-15, PA103-16, and PA103-19. These mutants were further characterized by West et al. (57), who hypothesized that they were ETA regulatory mutants. From these previous studies, it was not clear whether the ETA- and protease-deficient phenotypes were linked. We hypothesized that the lesion or lesions in PA103-15, PA103-16, or PA103-19 might map to the same regions of the chromosome as the *tox-1* and *tox-2* putative ETA regulatory mutations (23). Therefore, we obtained from Ratnaningshi et al. (41) six cosmid clones containing DNA fragments from the 23- or 26-min region of the PAO1 chromosome. These clones were introduced into PA103, PA103-15, PA103-16, and PA103-19, and the resulting exconjugates were assayed for ETA and protease activities. Two of the clones, pMO011925 and pMO012425, restored ETA activity to parental levels in PA103-15 and PA103-16 and to 80 and 56% of parental levels in PA103-19 (Table 2). To verify that restoration of ETA activity was accompanied by the production of an ETA-specific protein band, culture supernatant from the mutants containing pMO011925 and pMO012425 were analyzed by immunoblotting with anti-ETA antibody. Results are shown in Fig. 1. ETA was not detected in culture supernatants from the mutants containing the vector pLA2917; however, ETA was detected in culture supernatants of PA103-15, PA103-16, and PA103-19 containing either pMO011925 or pMO012425. Since these mutants were also deficient in protease production, we assayed PA103-15, PA103-16, and PA103-19 containing pMO011925 and pMO012425 for protease production on skim milk plates. Both cosmids restored protease production in all three mutants (data not shown).

Since the complementing cosmid clones contained fragments of DNA that were approximately 20 kb in size, it was possible that two genes were independently responsible for the deficiencies in ETA production and protease production. To localize the ability to complement these phenotypes, recombinant plasmids containing fragments of pMO011925 and pMO012425 were constructed and tested for their ability to complement the ETA- or protease-deficient phenotype of PA103-15, PA103-16, and PA103-19 (Fig. 2). The two *Pst*I fragments that were present in both cosmids were subcloned into pUCP19 (44) to generate pKF901 and pKF902. pKF902, but not pKF901, restored ETA and protease activity in PA103-19, indicating that the 4.2-kb *Pst*I fragment contained the complementing gene or genes (Fig. 2). Further subclones were generated by cloning smaller fragments of pKF902 into pUCP18 or pUCP19 as shown in Fig. 2. pKF906 and pKF812 restored both ETA and protease activity to PA103-15, PA103-16, and PA103-19 (Table 2 and Fig. 2 and 3); however, pKF917 did not restore ETA to full parental levels. This inability of pKF917 to complement fully the ETA-deficient phenotype was due to loss of the plasmid, even with carbenicillin selection. In TSBD medium containing 200 μg of carbenicillin per ml, 100% of the cells in an overnight culture of PA103-15/pKF917 and 60% of the cells in a PA103-19/pKF917 culture had lost the plasmid as measured by sensitivity to carbenicillin, while 96 to 100% of the cells in overnight cultures of these strains containing pKF906 were still carbenicillin resistant. Thus, for reasons that are not understood, pKF917 is unstable in these mutants.

To determine if the cloned fragment was of *P. aeruginosa* origin and was present in more than one copy on the PA103 and PAO1 chromosomes, Southern hybridizations were done with *Xho*I-, *Sal*I-, *Cla*I-, or *Bam*HI-digested genomic DNA isolated from PAO1, PA103, PA103-16, and PA103-19. The digested DNA was separated by agarose gel electrophoresis and probed with ^{32}P-labeled pT7-5-917, which contains the 0.9-kb *Nar*I fragment, and with the vector pT7-5. With all four strains, hybridization to 1.2-kb *Xho*I, 3.2-kb *Sal*I, 2.6-kb *Cla*I, and >8.0-kb *Bam*HI chromosomal fragments was detected (data not shown). From this analysis, we concluded that the cloned fragment was of *P. aeruginosa* origin, was present in one copy, and was located on the same DNA fragment in *P. aeruginosa* PAO1 and PA103.

On the basis of the results described above, we concluded that the gene (or genes) that restored both ETA and protease production was located on the 0.9-kb *Nar*I fragment. The small

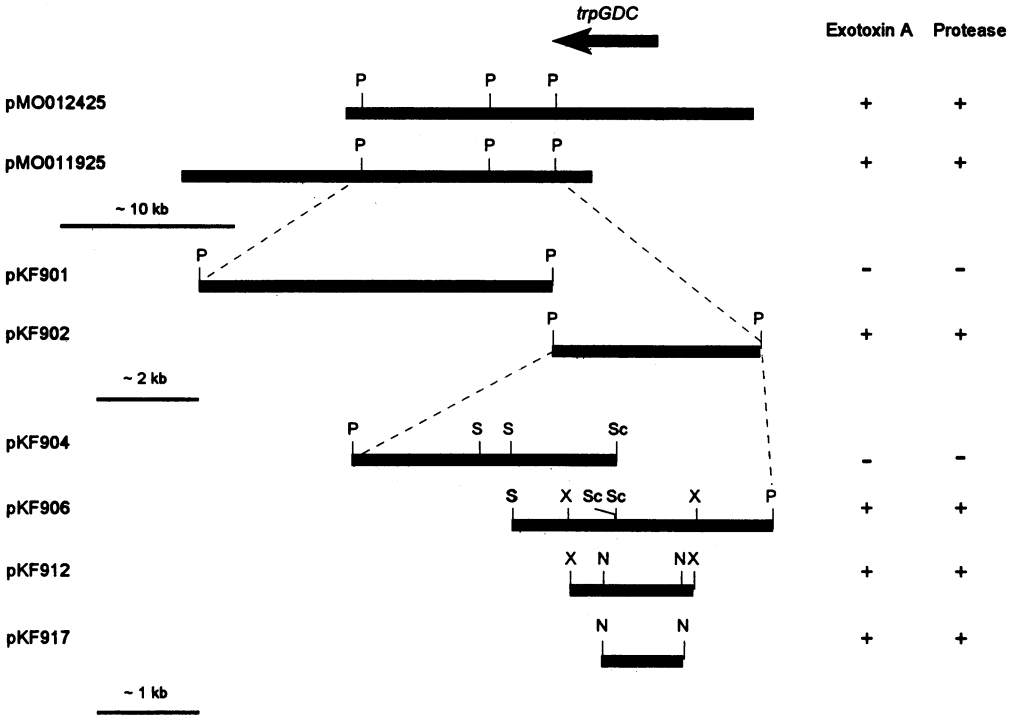

FIG. 2. Subcloning of *vfr*. pMO011925 and pMO012425 are cosmids containing the 23-min region of the *P. aeruginosa* PAO chromosome in vector pLA2917 (1, 41). pMO011925 and pMO012425 share a common 11.2-kb DNA fragment; this fragment was subcloned into pUCP19 (44) as 7.0- and 4.2-kb *Pst*I fragments to generate pKF901 and pKF902, respectively. The 4.2-kb *Pst*I fragment was further subcloned as a 2.6-kb *Pst*I-*Sal*I fragment, a 1.2-kb *Xho*I fragment, and a 0.9-kb *Nar*I fragment into pUCP18 or pUCP19 to generate pKF906, pKF812, and pKF917, respectively. The abilities of these plasmids to restore ADP-ribosyl transferase and protease activities to PA103-15, PA103-16, and PA103-19 are indicated on the right. Restriction endonucleases: N, *Nar*I; P, *Pst*I; S, *Sal*I; Sc, *Sca*I; X, *Xho*I.

size of the complementing fragment suggested that one gene complemented both phenotypes. In addition, because recombinant plasmids carrying the 0.9-kb *Nar*I fragment complemented both the ETA- and the protease-deficient phenotypes of PA103-15, PA103-16, and PA103-19, we concluded that the lesion responsible for these deficiencies was in the same gene in each mutant. We have designated this gene *vfr*.

Restoration of *regA* P1 promoter activity by *vfr*. In addition to being deficient in ETA and protease production, the *P. aeruginosa* mutants PA103-15, PA103-16, and PA103-19 were deficient in transcription from the *regA* P1 promoter but not from the *regA* P2 promoter (57). Therefore, to determine if *vfr* would restore transcription from the *regA* P1 promoter, we quantitated *regA* P1 promoter activity in the presence and the absence of the cloned *vfr* gene. For this analysis, we used the reporter plasmids pP11 and pP21, which contained transcriptional fusions of the *regA* P1 or P2 promoter, respectively, to the CAT gene (50), and pKF506, a recombinant plasmid that carries *vfr* as a 2.6-kb *Sal*I-*Pst*I fragment cloned into the vector pLA2917. Throughout the growth curve, significantly less CAT activity was detected in PA103-16 containing both pP11 and pLA2917 than in the parent strain containing the same plasmids (Fig. 4). However, addition of the cloned *vfr* gene on pKF506 restored CAT activity in PA103-16/pP11 to parental levels (Fig. 4). CAT activities directed by the *regA* P2 promoter on pP21 in the mutant PA103-16 and its parent PA103 in the presence and the absence of the cloned *vfr* gene were similar (data not shown). These data indicated that *vfr* could restore transcription from the *regA* P1 promoter in PA103-16 and that *regA* P2 was unaffected by *vfr*. Similar results were obtained with PA103-15 and PA103-19 (data not shown).

Nucleotide sequence of the *vfr* gene. The nucleotide sequence of the 1.2-kb *Xho*I fragment containing *vfr* was determined (Fig. 5). A search for open reading frames (ORFs) revealed that the 1.2-kb *Xho*I fragment contained a single 813-bp ORF beginning at nucleotide 330. A ribosome binding site was not found immediately 5′ of the ATG translational start codon for this ORF. However, a putative ribosome binding site, GGGA, was found 8 bp 5′ of a putative ATG translational start codon located at nucleotide 498 in the same reading frame. The codon usage of the ORF from bp 330 to 498 was not typical of codon usage patterns found in *P.*

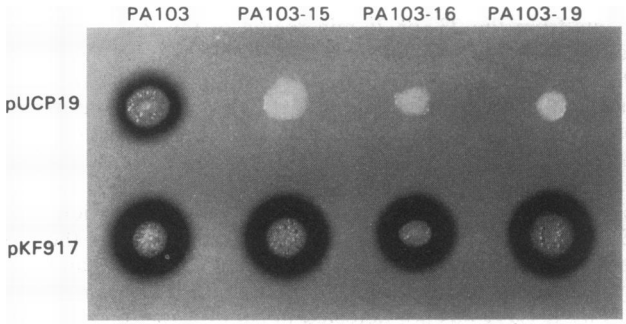

FIG. 3. Restoration of protease production in PA103-15, PA103-16, and PA103-19 containing pKF917. Protease activity was detected on tryptic soy agar plates containing 1.5% (wt/vol) skim milk as described by Wretlind and Pavlovskis (61). Zones of clearing around the colonies indicate proteolysis.

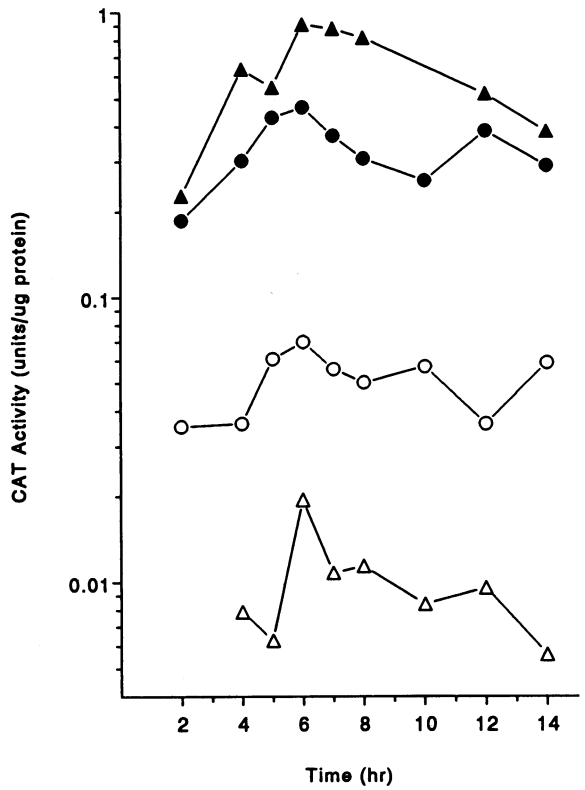

FIG. 4. Restoration of transcription from the *regA* P1 promoter in PA103-16. pKF506 is a recombinant plasmid which contains a 2.6-kb *SalI-PstI* fragment encoding *vfr*; the vector is pLA2917. pP11 is the *regA* P1 promoter fused to the *cat* gene on pQF26 (50). CAT activity was determined as described in Materials and Methods. Symbols: ●, PA103/pP11/pKF506; ○, PA103/pP11/pLA2917; ▲, PA103-16/pP11/pKF506; △, PA103-16/pP11/pLA2917.

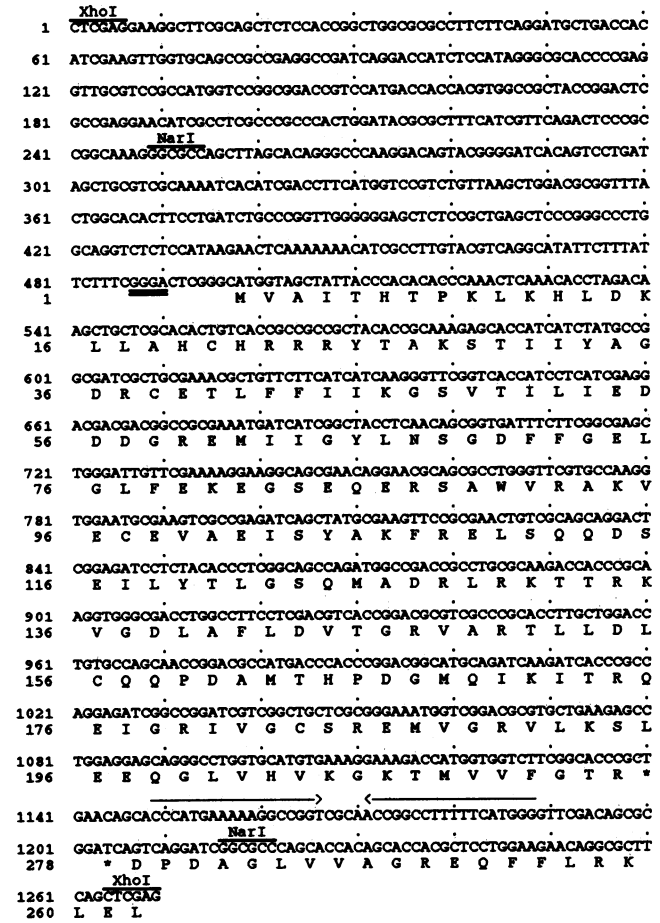

FIG. 5. Nucleotide sequence of the *vfr* gene and the 3′ end of the *trpC* gene. The derived amino acid sequences of *vfr* and *trpC* are shown below the coding sequences. The putative ribosome binding site (double underline), putative stop codons for *vfr* and *trpC* (asterisks), a putative transcriptional terminator (convergent arrows), and *Xho*I and *Nar*I restriction sites are indicated.

aeruginosa; however, from bp 498 to 1142, the codon usage agreed with typical *P. aeruginosa* codon usage patterns (56). A recombinant plasmid carrying bp 413 to 1269 under the control of the *tac* promoter restored protease production to PA103-15, PA103-16, and PA103-19 (data not shown). This result supports the hypothesis that the 645-bp ORF which begins at bp 498 encodes the *vfr* gene. A perfect inverted repeat, which may form a stem-loop structure and thus may function as a transcriptional terminator, was identified 9 bp 3′ of the putative stop codon by sequence analysis. The predicted amino acid sequence revealed that Vfr is 214 amino acids in length. The predicted molecular mass was 24,225 Da, and the pI was 8.08. Sequencing downstream of the *vfr* gene revealed that the C terminus of the *trpC* gene (14) is located 60 bp downstream of the C terminus of *vfr*.

Expression of the *vfr* gene in *E. coli*. To verify that a translated protein is produced from the 645-bp putative Vfr ORF, the *vfr* gene product was expressed from an inducible phage T7 promoter in *E. coli* (51). We cloned the 2.6-kb *SalI-PstI* fragment containing the *vfr* gene in two different orientations relative to the phage T7 promoter to generate pT7-5-906 and pT7-6-906. In addition, the 0.9-kb *NarI* fragment was cloned into pT7-5 and pT7-6 to generate pT7-5-917 and pT7-6-917, respectively. A unique protein with an apparent molecular mass of approximately 28.5 kDa was produced in cells containing pT7-5-906 and pT7-5-917 but not in cells containing the pT7-6 derivatives, which carried the *vfr* gene in the opposite orientation (Fig. 6). The apparent molecular mass of Vfr is higher than its predicted molecular mass of 24.2 kDa but lower than that of the 30.6-kDa polypeptide predicted for an 813-bp ORF. The higher molecular mass is probably due to aberrant migration of Vfr on an SDS-PAGE gel. Two proteins of approximately 20 and 6 kDa were induced in cells containing pT7-6-906 and presumably are encoded by ORFs on the strand opposite to *vfr*. These proteins were not produced from the smaller construct pT7-6-917 and therefore are not responsible for the complementing activity.

Similarity of Vfr to *E. coli* CAP and members of the CAP family of regulatory proteins. The deduced amino acid sequence of *vfr* was used to conduct a TFASTA search of the GenBank database. The Vfr sequence was 67% identical and 91% similar over a 202-amino-acid overlap to *E. coli* CAP (Fig. 7) and to the *Klebsiella aerogenes*, *Salmonella typhimurium*, and *Shigella flexneri* CAPs. Less similarity to other members of the CAP family (30), including the *Haemophilus influenzae* Crp protein (59%), the *Xanthomonas campestris* catabolite activator-like protein Clp (48.3%), the *E. coli* Fnr protein (23%), and the *Rhodopseudomonas palustris* AadR protein (17.9%), was found. CAP is a transcriptional regulator of numerous

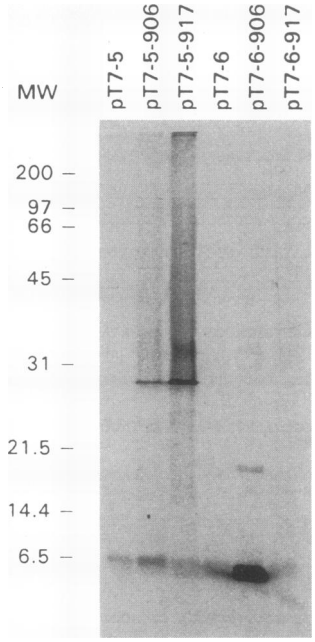

FIG. 6. Autoradiogram showing expression of the *vfr* gene product in *E. coli*. Expression of *vfr* was induced from the inducible phage T7 promoter as described in Materials and Methods. Plasmid constructs are indicated above the lanes. pT7-5-906 and pT7-6-906 are pT7-5 and pT7-6 carrying the 2.6-kb *SalI-PstI* fragment containing the *vfr* gene. pT7-5-917 and pT7-6-917 are pT7-5 and pT7-6 carrying the 0.9-kb *NarI* fragment containing the *vfr* gene. The locations of the molecular weight (MW) standards (in thousands) are indicated on the left.

genes in *E. coli*, most notably those involved in utilization of various carbon sources (53). Other genes regulated by CAP include those involved in pH-regulated gene expression, flagellum synthesis, iron acquisition, enterotoxin production, transfer of the F plasmids, and the heat shock response (7). The residues important for CAP activity were conserved in Vfr (Fig. 7); these residues included those involved in DNA binding, interaction with RNA polymerase, and cAMP binding (11–13, 54, 59, 62).

```
Vfr   MVAITHTPKLKHLDKLLAHCHRRRYTAKSTIIYAGDRCETLFFIIKGSVTILIEDDDGRE  60
CAP     VLGKPQTDPTLEWFLSHCHIHKYPSKSTLIHQGEKAETLYYIVKGSVAVLIKDEEGKE  58

Vfr   MIIGYLNSGDFFGELGLFEKEGSEQERSAWVRAKVECEVAEISYAKFRELSQQDSEILYT  120
CAP   MILSYLNQGDFIGELGLFE.EG..QERSAWVRAKTACEVAEISYKKFRQLIQVNPDILMR  115

Vfr   LGSQMADRLRKTTRKVGDLAFLDVTGRVARTLLDLCQQPDAMTHPDGMQIKITRQEIGRI  180
CAP   LSAQMARRLQVTSEKVGNLAFLDVTGRIAQTLLNLAKQPDAMTHPDGMQIKITRQEIGQI  175

Vfr   VGCSREMVGRVLKSLEEQGLVHVKGKTMVVFGTR  214
CAP   VGCSRETVGRILKMLEDQNLISAHGKTIVVYGTR  209
```

FIG. 7. Alignment of the deduced amino acid sequence of the *P. aeruginosa vfr* gene with *E. coli* CAP. Identical amino acids (vertical lines) and gaps inserted to optimize alignment (dots) are indicated. Regions important for CAP activity are indicated below the CAP sequence as follows: ★, residues involved in cAMP binding (54); ◆, residues which stabilize the cAMP binding pocket (54); ▽, residues which form the hinge between the two domains of cAMP (54); single underline, residues which are involved in CAP-RNA polymerase interactions (13, 59); double underline, residues which form the helix-turn-helix motif involved in DNA binding (54); and ◇, residues in the HTH motif which make direct contact with nucleotides in the CAP binding site (11, 12, 62).

TABLE 3. Complementation of an *E. coli crp* mutation by *vfr*

Strain	β-Galactosidase activity[a] (Miller units) with:			
	No added glucose	1% Glucose	No added cAMP	3 mM cAMP
RZ1331/pMMB66HE	2.5	1.1	ND	ND
RZ1331/pRZ1306	478	98	ND	ND
RZ1331/ptac917	226	65	ND	ND
RZ1330/pMMB66HE	ND	ND	10	10
RZ1330/pRZ1306	ND	ND	71	490
RZ1330/ptac917	ND	ND	15	398

[a] Determined as described by Miller (36) for mid-log-phase cultures grown in LB broth. ND, not determined.

Complementation of an *E. coli crp* mutation with the *P. aeruginosa vfr* gene. The fact that Vfr was 67% identical to CAP suggested that Vfr possesses similar functions. Therefore, we introduced the cloned *vfr* gene into the *E. coli crp* deletion mutant RZ1331 (13) to determine whether Vfr could functionally substitute for CAP. CAP is required for transcription of the genes coding for β-galactosidase and tryptophanase production (53). We assayed *E. coli* RZ1331 for the ability to produce β-galactosidase or tryptophanase in the presence and absence of the cloned *vfr* or *E. coli crp* gene. ptac917 was constructed by cloning the 0.9-kb *NarI* fragment containing *vfr* into pMMB66EH. pRZ1306 contains the cloned *crp* gene under the control of the *tac* promoter on pMMB66HE. *E. coli* RZ1331/pRZ1306 produced 200-fold more β-galactosidase than RZ1331 containing the vector pMMB66HE (Table 3). Likewise, *E. coli* RZ1331 containing ptac917 produced approximately 100-fold more β-galactosidase than RZ1331/pMMB 66HE. Tryptophanase was not detected in *E. coli* RZ1331/pMMB66HE; however, both RZ1331/pRZ1306 and RZ1331/ptac917 produced tryptophanase, as indicated by the production of a red color when Ehrlich's reagent was added to overnight LB cultures (data not shown). Thus, these results indicate that the cloned *vfr* gene can substitute for *crp* to regulate production of β-galactosidase and tryptophanase in *E. coli*.

In *E. coli*, glucose represses the synthesis of β-galactosidase and tryptophanase through a process known as catabolite repression (53). *E. coli* responds to exogenous glucose by decreasing the intracellular level of cAMP, which is required for CAP activity. To determine if Vfr could mediate catabolite repression in *E. coli* RZ1331, we compared the levels of β-galactosidase produced by RZ1331/ptac917 and RZ1331/pRZ1306 grown in media with and without glucose. Both strains produced approximately three- to fivefold less β-galactosidase in medium supplemented with 1% glucose than in medium lacking glucose (Table 3). To determine if cAMP modulates Vfr activity in *E. coli*, we introduced ptac917, pRZ1306, and pMMB66HE into RZ1330, a *crp cya* mutant. In this mutant, both CAP and cAMP must be supplied for β-galactosidase production. In the absence of cAMP, RZ1330 containing either pRZ1306 or ptac917 produced low levels of β-galactosidase (Table 3). However, addition of 3 mM cAMP to the culture restored β-galactosidase levels to those observed in the *E. coli crp* mutant RZ1331 containing either pRZ1306 or ptac917. These data indicate that Vfr, like CAP, is able to mediate cAMP-modulated catabolite repression in *E. coli*.

***E. coli crp* does not complement a *vfr* mutation.** To determine whether CAP could functionally substitute for Vfr in *P. aeruginosa*, we introduced the cloned *crp* gene under the control of the inducible *tac* promoter on pRZ1306 into

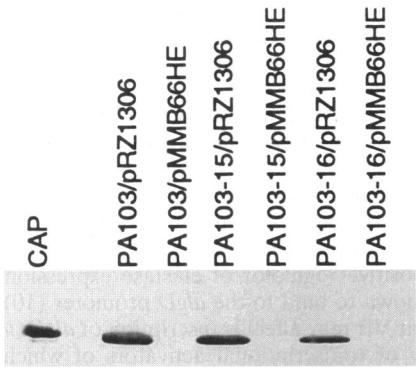

FIG. 8. Expression of CAP in *P. aeruginosa* PA103/pRZ1306, PA103-15/pRZ1306, and PA103-16/pRZ1306. pRZ1306 contains the *E. coli crp* gene under the control of the *tac* promoter in the vector pMMB66HE. CAP was detected by immunoblotting with rabbit anti-CAP antisera as described in Materials and Methods. Purified CAP (approximately 0.78 μg) was used as a positive control.

PA103-15 and PA103-16 and assayed for ETA activity and protease production. The *tac* promoter is functional in *P. aeruginosa* (19) and, in fact, immunoblotting of cell lysates from *P. aeruginosa* containing pRZ1306 with rabbit anti-CAP antisera showed that CAP was indeed expressed (Fig. 8). The levels of ETA activity in PA103-15 and PA103-16 carrying pRZ1306 were the same as the levels observed with PA103-15 and PA103-16 carrying the vector pMMB66HE (Table 4). In PA103, overexpression of CAP from the *tac* promoter inhibited ETA production. PA103/pRZ1306 produced approximately sixfold less ETA than PA103/pMMB66HE. We suspect that this is due to the formation of nonfunctional heterodimers of CAP and Vfr. As with ETA activity, CAP did not restore protease activity in PA103-15 or PA103-16, as indicated by the failure of these strains containing pRZ1306 to produce zones of clearing around colonies on skim milk plates (data not shown). Therefore, we concluded that the cloned *crp* gene under the control of the *tac* promoter could not functionally substitute for *vfr* in regulating either ETA or protease production in *P. aeruginosa*.

DISCUSSION

The production of ETA is a complex and highly regulated event. Many environmental stimuli, such as iron concentration and temperature, affect ETA production. However, until now, only the *regAB*, *lasR*, and *fur* gene products have been shown to regulate ETA production. In this report, we describe the identification of another gene involved in ETA production which we have designated *vfr*. *vfr* was cloned by complementing the defect in ETA production in three NTG-generated mutants derived from PA103. In addition to regulating ETA production, *vfr* was required for transcription from the *regA* P1 promoter and for production of protease.

TABLE 4. Failure of *crp* to complement the ETA-deficient phenotype of PA103-15 and PA103-16

Strain	ADP-ribosyl transferase activity[a] (cpm/10 μl of supernatant)	
	pMMB66HE	pRZ1306[b]
PA103	8,848	543
PA103-15	36	44
PA103-16	73	68

[a] Determined as described by Ohman et al. (38) and Chung and Collier (9) for supernatants from 18-h TSBD cultures.
[b] *crp* expression was induced with 1 mM IPTG.

The deduced amino acid sequence of *vfr* is 67% identical to the sequence for *E. coli* CAP, a global regulator of gene expression in *E. coli*. CAP regulates the expression of over 100 genes, most notably the genes involved in carbon source utilization whose expression is subject to catabolite repression by glucose (7, 30, 53). However, we suspect that Vfr is not involved in catabolite repression in *P. aeruginosa*, for several reasons. In *P. aeruginosa*, tricarboxylic acid cycle intermediates, not glucose, cause repression of enzymes for utilization of alternative carbon sources (39, 47). Additionally, the intracellular levels of cAMP do not change in response to various carbon sources; thus, cAMP does not appear to be the modulator of catabolite repression (39, 47). MacGregor et al. (35) and Wolff et al. (60) have recently identified and cloned the *crc* gene, which is the mediator of catabolite repression in *P. aeruginosa*. The *crc* gene is located at 11 min on the PAO chromosome (60).

The predicted Vfr ORF encoded a 214-amino-acid protein that was approximately the same size as the 23-kDa CAP monomer. The amino acid residues which constitute the structural features of CAP that are associated with cAMP binding, contact with RNA polymerase, and DNA binding are identical or conserved in Vfr (Fig. 7). The extensive amino acid identity between CAP and Vfr suggests that Vfr should be able to functionally substitute for CAP in *E. coli*. In fact, *vfr* was able to complement the β-galactosidase- and tryptophanase-deficient phenotypes of an *E. coli crp* deletion mutant. This observation suggests that Vfr can recognize the CAP binding sites in the *E. coli lacZ* and tryptophanase promoters and can interact productively with *E. coli* RNA polymerase to activate transcription of these genes. In addition, cAMP was required for Vfr activity in *E. coli*, indicating that Vfr can bind cAMP.

The crystal structure of the CAP-cAMP complex has been determined at 2.5-Å (0.25-nm) resolution and has revealed numerous features of the CAP-cAMP complex (54). The C terminus of CAP contains a helix-turn-helix (HTH) motif which is responsible for specific DNA binding (30). With the exception of a substitution of arginine at residue 179 in Vfr for glutamine 174 in CAP, the first helix of the HTH DNA binding domain of CAP is identical in Vfr (Fig. 7). The second helix (CAP residues 180 to 191), which recognizes and binds to nucleotides in one half of the CAP binding site, is identical to Vfr in 9 of 12 residues. The residues in CAP which make specific contact with nucleotides in the CAP binding site are arginine 180, glutamic acid 181, and arginine 185 (11, 12, 62) and are identical in Vfr (Fig. 7).

It has been suggested that CAP activates transcription of target promoters via interactions with RNA polymerase (13, 59). CAP residues 52 to 56 and 156 to 162 form surface-exposed loops which are hypothesized to contact RNA polymerase (13, 59). The residues forming the 156-to-162 loop are identical in Vfr; however, in the 52-to-56 loop, only two of the five residues are identical (Fig. 7).

The crystal structure analysis of CAP identified six residues which are predicted to be important in binding cAMP: glycine 71, glutamic acid 72, arginine 82, serine 83, threonine 127, and serine 128 (54). These residues are identical in Vfr with the exception of serine 128, which has been changed to threonine (Fig. 7). However, cAMP binding studies (24) with CAP mutants in which alanine was substituted for various residues indicated that serine 128 may not be essential for cAMP binding. Two residues of CAP, tyrosine 99 and arginine 123,

are important in stabilizing the cAMP binding pocket (54); these residues are identical in Vfr (Fig. 7). Thus, it appears that the cAMP binding region of Vfr is very similar to the same region of CAP. However, in Vfr three additional amino acid residues, lysine 80, serine 83, and glutamic acid 84, are present in the region corresponding to β strand 7 of CAP, which forms part of the cAMP binding pocket (54). This difference could allow Vfr to bind an allosteric effector other than cAMP. Because CAP lacks these extra amino acids, it may not be able to bind this effector.

The N-terminal cAMP binding region of CAP is connected to the C-terminal DNA binding region by a 3-amino-acid hinge (residues 135 to 137) (54). These amino acids are identical in Vfr (Fig. 7). These observations support our genetic data indicating that Vfr can recognize the CAP binding site in *E. coli*, can interact with *E. coli* RNA polymerase to facilitate transcription of CAP-responsive genes, and can bind cAMP.

Surprisingly, although the cloned *P. aeruginosa vfr* gene is able to complement a *crp* mutation in *E. coli*, the cloned *crp* gene did not complement the *vfr* mutations in PA103-15 or PA103-16, as assayed by restoration of ETA or protease production. There are several possible explanations for the inability of *crp* to complement a *vfr* mutation. One possibility is that in *P. aeruginosa*, a modulator other than cAMP activates Vfr and CAP is unable to bind this modulator. However, even if CAP does not bind the Vfr modulator, if sufficient levels of cAMP are present CAP should be activated. Siegel et al. (47) report cAMP levels in *P. aeruginosa* that are comparable to those in *E. coli*. However, Phillips and Mulfinger (39) report cAMP levels that are 10-fold lower. Therefore, there exists the possibility that the levels of cAMP in *P. aeruginosa* are insufficient to activate CAP. Identification of the modulator in *P. aeruginosa* is needed to test this hypothesis. A second reason why *crp* cannot complement a *vfr* mutation is that CAP may not interact properly with *P. aeruginosa* RNA polymerase to activate transcription of Vfr-dependent genes. This may be because the spacing between the CAP or Vfr binding site and the RNA polymerase binding site in *P. aeruginosa* promoters is such that CAP cannot interact appropriately with *P. aeruginosa* RNA polymerase. Alternatively, the region of Vfr which interacts with *P. aeruginosa* RNA polymerase may be different from the corresponding region of CAP. In addition, *P. aeruginosa* RNA polymerase may be sufficiently different from *E. coli* RNA polymerase that CAP cannot interact with it. A third reason why *crp* may not complement a *vfr* mutation is that CAP may not recognize the *P. aeruginosa* Vfr binding site, even though Vfr appears to recognize CAP binding sites in *E. coli*. There are two residues in the second helix of the Vfr HTH motif that are not identical or even conserved in CAP and may be involved in the binding of Vfr to a putative alternative binding site in *P. aeruginosa*. These two residues in Vfr are methionine 187 and serine 194 and correspond to threonine 182 and methionine 189, respectively, in CAP. Additionally, amino acid changes occur at two additional sites; these are arginine 179 for glutamine 173 of CAP in the first helix and valine 191 for isoleucine 186 of CAP in the second helix (Fig. 7). In fact, a CAP binding site was not found in the *toxA* and *regAB* promoter regions, and regions of dyad symmetry were not found immediately upstream of the *toxA* transcriptional start site (41a). Thus, it seems likely that CAP cannot bind to the *toxA* or *regA* P1 promoter. The possibility of a putative DNA binding site which differs from the CAP binding site implies that Vfr may be able to bind two different sites. The ability to bind two apparently different sequences has been described for the *Vibrio cholerae* ToxR protein (27). Identification of the putative Vfr binding site in the *toxA* and *regA* P1 promoters is needed to test this hypothesis.

The ability of *vfr* to complement the β-galactosidase-deficient phenotype of an *E. coli crp* mutant suggests that Vfr can recognize the CAP binding site and therefore should be able to activate genes containing this sequence appropriately positioned in their promoters. There are two *P. aeruginosa* virulence factor genes that have CAP-like binding sites in their promoters; therefore, they may be regulated by Vfr. These genes are *algD*, a key enzyme in alginate biosynthesis (10), and *lasR*, the positive regulator of elastase expression (20). CAP has been shown to bind to the *algD* promoter (10). Thus, it is possible that Vfr may affect transcription of *algD*. *lasR* belongs to a family of transcriptional activators of which the *Vibrio fischeri luxR* gene is the prototype and is regulated by CAP (20). CAP has also been shown to bind to a CAP binding site within the *luxR* promoter (45). *lasR*, like *luxR*, has a CAP binding site in its promoter; therefore, *vfr* should also be able to regulate expression of *lasR* and *lasR*-regulated genes. Studies to identify additional genes that are regulated by Vfr are in progress.

ACKNOWLEDGMENTS

This study was supported by grants from the University of Wisconsin—Madison Graduate School and the Cystic Fibrosis Foundation and by Public Health Service grant AI31477-0171 from the National Institute of Allergy and Infectious Diseases awarded to S.E.H.W. L.J.R.-J. is a trainee under Public Health Service grant T32 GM 07215.

Plasmids pMO011925 and pMO012425 were the kind gifts of B. W. Holloway, Monash University, Clayton, Victoria, Australia. We thank B. H. Iglewski, University of Rochester, for *P. aeruginosa* PAO1, PA103, PA103-15, PA103-16, and PA103-19 and for rabbit anti-ETA antisera; W. S. Reznikoff, University of Wisconsin—Madison, for *E. coli* mutants RZ1331 and RZ1330 and for plasmid pRZ1306, which carries the cloned *crp* gene; and A. Ullmann, Pasteur Institute, for the rabbit anti-CAP antisera. We acknowledge W. Hendrickson, University of Illinois, and N. A. Zielinski for their helpful discussions concerning the purification of CAP and Ellen B. Cook, Cristine J. Dall, and Janet Roggy for their excellent technical assistance.

REFERENCES

1. **Allen, L. N., and R. S. Hanson.** 1985. Construction of broad-host-range cosmid cloning vectors: identification of genes necessary for growth of *Methylobacterium organophilum* on methanol. J. Bacteriol. **161**:955–962.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.** 1990. Current protocols in molecular biology. Greene Publishing and Wiley-Interscience, New York.
3. **Barr, P. J., R. M. Thayer, P. Laybourn, R. C. Najarian, F. Seela, and D. R. Tolan.** 1986. 7-Deaza-2′-deoxyguanosine-5′-triphosphate: enhanced resolution in M13 dideoxy sequencing. BioTechniques **4**:428–432.
4. **Bilezikian, J. P., R. O. R. Kaempfer, and B. Magasanik.** 1967. Mechanism of tryptophanase induction in *Escherichia coli*. J. Mol. Biol. **27**:495–506.
5. **Blake, M. S., K. H. Johnston, G. J. Jones, and E. C. Gotschlich.** 1984. A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. Anal. Biochem. **136**:175–179.
6. **Blumentals, I. I., R. M. Kelly, M. Gorziglia, J. B. Kaufman, and J. Shiloach.** 1987. Development of a defined medium and two-step culturing method for improved ETA yields from *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. **53**:2013–2020.
7. **Botsford, J. L., and J. G. Harman.** 1992. Cyclic AMP in prokaryotes. Microbiol. Rev. **56**:100–122.
8. **Burnette, W. H.** 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112**:195–203.

9. **Chung, D. W., and R. J. Collier.** 1977. Enzymatically active peptide from the adenosine diphosphate-ribosylating toxin of *Pseudomonas aeruginosa*. Infect. Immun. **16:**832–841.
10. **DeVault, J. D., W. Hendrickson, J. Kato, and A. M. Chakrabarty.** 1991. Environmentally regulated *algD* promoter is responsive to the cAMP receptor protein in *Escherichia coli*. Mol. Microbiol. **5:** 2503–2509.
11. **Ebright, R. H., P. Cossart, B. Gicquel-Sanzey, and J. Beckwith.** 1984. Molecular basis of DNA sequence recognition by the catabolite gene activator protein: detailed inferences from three mutations that alter DNA sequence specificity. Proc. Natl. Acad. Sci. USA **81:**7274–7278.
12. **Ebright, R. H., A. Kolb, H. Buc, T. A. Kunkel, J. S. Krakow, and J. Beckwith.** 1987. Role of glutamic acid-181 in DNA-sequence recognition by the catabolite gene activator protein (CAP) of *Escherichia coli*: altered DNA-sequence-recognition properties of [Val^{181}]CAP and [Leu^{181}]CAP. Proc. Natl. Acad. Sci. USA **84:** 6083–6087.
13. **Eschenlauer, A. C., and W. S. Reznikoff.** 1991. *Escherichia coli* catabolite gene activator protein mutants defective in positive control of *lac* operon transcription. J. Bacteriol. **173:**5024–5029.
14. **Essar, D. W., L. Eberly, C.-Y. Han, and I. P. Crawford.** 1990. DNA sequences and characterization of four early genes of the tryptophan pathway in *Pseudomonas aeruginosa*. J. Bacteriol. **172:**853–866.
15. **Farinha, M. A., and A. M. Kropinski.** 1989. Construction of broad host-range vectors for general cloning and promoter selection in *Pseudomonas* and *Escherichia coli*. Gene **77:**205–210.
16. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. USA **76:** 1648–1652.
17. **Frank, D. W., and B. H. Iglewski.** 1988. Kinetics of *toxA* and *regA* mRNA accumulation in *Pseudomonas aeruginosa*. J. Bacteriol. **170:**4477–4483.
18. **Frank, D. W., D. G. Storey, M. S. Hindahl, and B. H. Iglewski.** 1989. Differential regulation by iron of *regA* and *toxA* transcript accumulation in *Pseudomonas aeruginosa*. J. Bacteriol. **171:**5304–5313.
19. **Fürste, J. P., W. Pansegrau, R. Frank, H. Blöcker, P. Scholz, M. Bagdasarian, and E. Lanka.** 1986. Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tacP* expression vector. Gene **48:**119–131.
20. **Gambello, M. J., and B. H. Iglewski.** 1991. Cloning and characterization of the *Pseudomonas aeruginosa lasR* gene, a transcriptional activator of elastase expression. J. Bacteriol. **173:**3000–3009.
21. **Gambello, M. J., S. Kaye, and B. H. Iglewski.** 1993. LasR of *Pseudomonas aeruginosa* is a transcriptional activator of the alkaline protease gene (*apr*) and an enhancer of exotoxin A expression. Infect. Immun. **61:**1180–1184.
22. **Ghosaini, L. R., A. M. Brown, and J. M. Sturtevant.** 1988. Scanning calorimetric study of the thermal unfolding of catabolite activator protein from *Escherichia coli* in the absence and presence of cyclic mononucleotides. Biochemistry **27:**5257–5261.
23. **Gray, G. L., and M. L. Vasil.** 1981. Isolation and genetic characterization of toxin-deficient mutants of *Pseudomonas aeruginosa* PAO1. J. Bacteriol. **147:**275–281.
24. **Gronenborn, A. M., R. Sandulache, S. Gärtner, and G. M. Clore.** 1988. Mutations in the cyclic AMP binding site of the cyclic AMP receptor protein of *Escherichia coli*. Biochem. J. **253:**801–807.
25. **Hamood, A. N., J. C. Olson, T. S. Vincent, and B. H. Iglewski.** 1989. Regions of toxin A involved in toxin A excretion in *Pseudomonas aeruginosa*. J. Bacteriol. **171:**1817–1824.
26. **Hedstrom, R. C., C. R. Funk, J. B. Kaper, O. R. Pavlovskis, and D. R. Galloway.** 1986. Cloning of a gene involved in regulation of exotoxin A expression in *Pseudomonas aeruginosa*. Infect. Immun. **51:**37–42.
27. **Higgins, D. E., and V. J. DiRita.** 1994. Transcriptional control of *toxT*, a regulatory gene in the *toxR* regulon of *Vibrio cholerae*. Mol. Microbiol. **14:**17–29.
28. **Holloway, B. W., V. Krishnapillai, and A. F. Morgan.** 1979. Chromosomal genetics of *Pseudomonas*. Microbiol. Rev. **43:**73–102.
29. **Hull, R. A., R. E. Gill, P. Hsu, B. H. Minshew, and S. Falkow.** 1981. Construction and expression of recombinant plasmids encoding type 1 or D-mannose-resistant pili from a urinary tract infection *Escherichia coli* isolate. Infect. Immun. **33:**933–938.
30. **Kolb, A., S. Busby, H. Buc, S. Garges, and S. Adhya.** 1993. Transcriptional regulation by cAMP and its receptor protein. Annu. Rev. Biochem. **62:**749–795.
31. **Laemmili, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:** 680–685.
32. **Li, M., and H. P. Schweizer.** 1993. Resolution of common DNA sequencing ambiguities of GC-rich DNA templates by terminal deoxynucleotide transferase without dGTP analogues. Focus **15:** 19–20.
33. **Liu, P. V.**1973. Exotoxins of *Pseudomonas aeruginosa*. I. Factors that influence the production of exotoxin A. J. Infect. Dis. **128:**506–513.
34. **Lory, S.** 1986. Effect of iron on accumulation of exotoxin A-specific mRNA in *Pseudomonas aeruginosa*. J. Bacteriol. **168:**1451–1456.
35. **MacGregor, C. H., J. A. Wolff, S. K. Arora, and P. V. Phibbs, Jr.** 1991. Cloning of a catabolite repression control (*crc*) gene from *Pseudomonas aeruginosa*, expression of the gene in *Escherichia coli*, and identification of the gene product in *P. aeruginosa*. J. Bacteriol. **173:**7204–7212.
36. **Miller, J. H.** 1992. A short course in bacterial genetics. A laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37. **Neumann, J. R., C. A. Morency, and K. O. Russian.** 1987. A novel rapid assay for chloramphenicol acetyltransferase gene expression. BioTechniques **5:**444–447.
38. **Ohman, D. E., J. C. Sadoff, and B. H. Iglewski.** 1980. Toxin A-deficient mutants of *Pseudomonas aeruginosa* PA103: isolation and characterization. Infect. Immun. **28:**899–908.
39. **Phillips, A. T., and L. M. Mulfinger.** 1981. Cyclic adenosine 3′,5′-monophosphate levels in *Pseudomonas putida* and *Pseudomonas aeruginosa* during induction and carbon catabolite repression of histidase synthesis. J. Bacteriol. **145:**1286–1292.
40. **Prince, R. W., C. D. Cox, and M. L. Vasil.** 1993. Coordinate regulation of siderophore and exotoxin A production: molecular cloning and sequencing of the *Pseudomonas aeruginosa fur* gene. J. Bacteriol. **175:**2589–2598.
41. **Ratnaningshi, E., S. Dharmsthiti, V. Krishnapillai, A. Morgan, M. Sinclair, and B. W. Holloway.** 1990. A combined physical and genetic map of *Pseudomonas aeruginosa* PAO. J. Gen. Microbiol. **136:**2351–2357.

41a. **Runyen-Janecky, L. J., and S. E. H. West.** Unpublished observations.

42. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
43. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:** 5463–5467.
44. **Schweizer, H. P.** 1991. *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19. Gene **97:**109–121.
45. **Shadel, G. S., J. H. Devine, and T. O. Baldwin.** 1990. Control of the *lux* regulon of *Vibrio fischeri*. J. Biolumin. Chemilumin. **5:**99–106.
46. **Shortridge, V. D., M. L. Pato, A. I. Vasil, and M. Vasil.** 1991. Physical mapping of virulence-associated genes in *Pseudomonas aeruginosa* by transverse alternating-field electrophoresis. Infect. Immun. **59:**3596–3603.
47. **Siegel, L. S., P. B. Hylemon, and P. V. Phibbs, Jr.** 1977. Cyclic adenosine 3′,5′-monophosphate levels and activities of adenylate cyclase and cyclic adenosine 3′,5′-monophosphate phosphodiesterase in *Pseudomonas* and *Bacteroides*. J. Bacteriol. **129:**87–96.
48. **Smith, A. W., and B. H. Iglewski.** 1989. Transformation of *Pseudomonas aeruginosa* by electroporation. Nucleic Acids Res. **17:**10509.
49. **Smith, G. E., and M. D. Summers.** 1980. The bidirectional transfer of DNA and RNA to nitrocellulose or diazobenzyloxymethyl paper. Anal. Biochem. **109:**123–129.
50. **Storey, D. G., D. W. Frank, M. A. Farinha, A. M. Kropinski, and**

B. H. Iglewski. 1990. Multiple promoters control the regulation of the *Pseudomonas aeruginosa regA* gene. Mol. Microbiol. **4**:499–503.

51. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82**:1074–1078.
52. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
53. **Ullmann, A., and A. Danchin.** 1983. Role of cyclic AMP in bacteria. Adv. Cyclic Nucleotide Res. **15**:1–53.

53a. **Vasil, M. L.** Personal communication.

54. **Weber, I. T., and T. A. Steitz.** 1987. Structure of a complex of catabolite gene activator protein and cyclic AMP refined at 2.5 Å resolution. J. Mol. Biol. **198**:311–326.
55. **Weickert, M. J., and G. H. Chambliss.** 1989. Acid-phenol minipreps make excellent sequencing templates. U.S. Biochemical Corp. Editorial Comments **16**:5–6.
56. **West, S. E. H., and B. H. Iglewski.** 1988. Codon usage in *Pseudomonas aeruginosa*. Nucleic Acids Res. **16**:9323–9335.
57. **West, S. E. H., S. A. Kaye, A. N. Hamood, and B. H. Iglewski.** 1994. Characterization of *Pseudomonas aeruginosa* mutants that are deficient in exotoxin A synthesis and are altered in expression of *regA*, a positive regulator of exotoxin A. Infect. Immun. **62**:897–903.
58. **Wick, M. J., D. W. Frank, D. G. Storey, and B. H. Iglewski.** 1990. Identification of *regB*, a gene required for optimal exotoxin A yields in *Pseudomonas aeruginosa*. Mol. Microbiol. **4**:489–497.
59. **Williams, R., A. Bell, G. Sims, and S. Busby.** 1991. The role of two surface exposed loops in transcription activation by the *Escherichia coli* CRP and FNR proteins. Nucleic Acids Res. **19**:6705–6712.
60. **Wolff, J. A., C. H. McGregor, R. C. Eisenberg, and P. V. Phibbs, Jr.** 1991. Isolation and characterization of catabolite control mutants of *Pseudomonas aeruginosa* PAO. J. Bacteriol. **173**:4700–4706.
61. **Wretlind, B., and O. R. Pavlovskis.** 1984. Genetic mapping and characterization of *Pseudomonas aeruginosa* mutants defective in the formation of extracellular proteins. J. Bacteriol. **158**:801–808.
62. **Zhang, X., and R. H. Ebright.** 1990. Identification of a contact between arginine-180 of the catabolite gene activator protein (CAP) and base pair 5 of the DNA site in the CAP-DNA complex. Proc. Natl. Acad. Sci. USA **87**:4717–4721.