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Characterization of the *Caulobacter crescentus flbF* Promoter and Identification of the Inferred FlbF Product as a Homolog of the LcrD Protein from a *Yersinia enterocolitica* Virulence Plasmid

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We have investigated the organization and expression of the *Caulobacter crescentus flbF* gene because it occupies a high level in the flagellar gene regulatory hierarchy. The nucleotide sequence comprising the 3′ end of the *flaO* operon and the adjacent *flbF* promoter and structural gene was determined, and the organization of transcription units within this sequence was investigated. We located the 3′ ends of the *flaO* operon transcript by using a nuclease S1 protection assay, and the 5′ end of the *flbF* transcript was precisely mapped by primer extension analysis. The nucleotide sequence upstream from the 5′ end of the *flbF* transcript contains −10 and −35 elements similar to those found in promoters transcribed by σ^{28} RNA polymerase in other organisms. Mutations that changed nucleotides in the −10 or −35 elements or altered their relative spacing resulted in undetectable levels of *flbF* transcript, demonstrating that these sequences contain nucleotides essential for promoter function. We identified a 700-codon open reading frame, downstream from the *flbF* promoter region, that was predicted to be the *flbF* structural gene. The amino-terminal half of the FlbF amino acid sequence contains eight hydrophobic regions predicted to be membrane-spanning segments, suggesting that the FlbF protein may be an integral membrane protein. The FlbF amino acid sequence is very similar to that of a transcriptional regulatory protein called LcrD that is encoded in the highly conserved low-calcium-response region of virulence plasmid pYVO3 in *Yersinia enterocolitica* (A.-M. Viitanen, P. Toivanen, and M. Skurnik, J. Bacteriol. 172:3152–3162, 1990).

The *Caulobacter crescentus* flagellum has been used as a model for investigating cell differentiation because its morphogenesis is under strict spatial and temporal control in the cell division cycle (for reviews, see references 35 and 48). Flagellum biosynthesis and function in *C. crescentus* is a complex process that requires at least 48 genes, and about half of these are contained in three major gene clusters (11). The *C. crescentus* flagellar genes (6, 7, 36, 38, 55), like those in *Escherichia coli* (23, 24) and *Salmonella typhimurium* (26), can be arranged in a regulatory hierarchy in which *trans*-acting genes at one level are required for expression of genes at lower levels. The *flbD* and *flbF* genes occupy high levels in the *C. crescentus* regulatory hierarchy because they are required in *trans* for the expression of genes needed for the synthesis of the flagellar hook and filament (30, 32, 36, 39). These genes that depend upon *flbD* and *flbF* for their transcription contain conserved *cis*-acting sequences that include σ^{54}-type promoters, a sequence called *ftr* (30, 32, 33, 37), and another sequence that matches the consensus binding site for *E. coli* integration host factor protein (15). *flbD* is a member of the *flaO* operon (39), whose transcription in vivo depends upon a sequence similar to that of the −10 box of *E. coli* promoters that are transcribed by σ^{32} RNA polymerase (34, 52). It is evident that the promoters and other *cis*-acting sequences in *C. crescentus* flagellar genes at different levels of the regulatory hierarchy have different sequences.

We have undertaken an analysis of the *flbF* gene to investigate its role in flagellar gene transcription. The transcriptional map and nucleotide sequence of a 2.3-kb DNA fragment that includes the 3′ end of the *flaO* operon and the *flbF* promoter and structural gene were determined. Sequences similar to the −10 and −35 boxes conserved in σ^{28} promoters (1, 3, 18) were identified near the *flbF* transcription start site, and evidence is presented that these sequences are required for promoter function. The deduced FlbF amino acid sequence shows striking similarity to that of LcrD, a transcription regulatory protein encoded in the low-calcium-response region of *Yersinia enterocolitica* virulence plasmid pYVO3 (53). These proteins also share inferred structural features, and we propose that they are homologs.

MATERIALS AND METHODS

Strains and culture conditions. The bacterial strains, plasmids, and phages used in this work are listed in Table 1. *C. crescentus* CB15 and mutant derivatives were grown at 30°C in peptone-yeast extract medium (PYE) (41). Plasmid-containing *C. crescentus* strains were grown in PYE broth supplemented with 2 μg of tetracycline per ml. The motilities of merodiploid *C. crescentus* strains were tested by stabbing them into motility agar (21). The extent of swarming was compared with those of the wild-type *fla*$^+$ strain and the *fla*::Tn*5* parent. Plasmid-containing *E. coli* strains were grown at 37°C in yeast extract-tryptone medium (19) supplemented with appropriate antibiotics, as follows: ampicillin, 50 μg/ml; kanamycin sulfate, 50 μg/ml; tetracycline hydrochloride, 10 μg/ml.

Plasmid and phage construction. Plasmid and phage DNAs were introduced into *E. coli* by using a calcium chloride transformation method (27). Plasmids were introduced into *C. crescentus* by electroporation with a Bio-Rad electroporator (33).

Bacteriophage λ Hk1 (57) is an EMBL3 derivative isolated

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TABLE 1. Bacterial strains, plasmids, and phages

Strain, plasmid, or phage	Relevant characteristic	Source or reference
Strains		
C. crescentus		
CB15	Wild type	ATCC 19089
SC1052	*flaO172*::Tn*5*	39
SC1032	*flaD198*::Tn*5*	39
SC1061	*flbF177*::Tn*5*	39
SC1132	*flbF608*::Tn*5*	39
E. coli		
HB101		5
DH5α	Km^r male	BRL[a]
RZ1032	Tc^r *dut ung* male	25
Plasmids and phages		
pUC18 and 19	Ap^r	56
pUC19-LS1	Ap^r	This work
pUC19-1.6	Ap^r	This work
pUC19-3.0	Ap^r	This work
pUC19-3.2	Ap^r	This work
pUC19-4.5	Ap^r	This work
pRK2L1-1.6	Ap^r	This work
pRK2L1-3.0	Ap^r	This work
pRK2L1-3.2	Ap^r	This work
pRK2L1-LS1	Tc^r	This work
pRK2L1-LS2	Tc^r	This work
pRK2L1	Tc^r	33
M13mp18 and -19		56
M13mp19-LS1		This work
λ Hk1		57

[a] BRL, Bethesda Research Laboratories Life Sciences, Inc.

from a genomic library of the *C. crescentus* genome (Fig. 1A); it was used as a source of DNA for cloning fragments from the *flaO* and *flbF* region. pUC19-1.6 was constructed by filling in the *Afl*II end of the 1.6-kb *Afl*II-*Bam*HI(e) (Fig. 1B) fragment by using Klenow polymerase, and the resulting fragment was ligated to *Bam*HI- and *Hinc*II-digested pUC19. pRK2L1-1.6 was constructed by ligating the 1.6-kb *Hin*dIII-*Bam*HI(e) fragment from pUC19-1.6 to *Bam*HI- and *Hin*dIII-digested pRK2L1. pUC19-3.0 was constructed by ligating the 3-kb *Bam*HI(e)-*Bam*HI(f) fragment (Fig. 1B) to *Bam*HI-cleaved pUC19. pRK2L1-3.0 was constructed by ligating the 3-kb *Bam*HI fragment from pUC19-3.0 to *Bam*HI-digested pRK2L1. pUC-4.5 was constructed by ligating the 3-kb *Bam*HI fragment from pUC19-3.0 into the *Bam*HI site of pUC19-1.6. The 3-kb *Bam*HI fragment is oriented so that the *flbF* gene is reconstituted. pRK2L1-3.2 was constructed by ligating the 3.2-kb *Hin*dIII fragment from pUC-4.5 to *Hin*dIII-cleaved pRK2L1.

Isolation of RNA and nuclease S1 mapping. Stationary-phase *C. crescentus* cells were diluted 1:10 into 100 ml of PYE broth and grown with aeration at 30°C to an optical density at 650 nm of 0.8. The cells were collected by centrifugation, lysed by vigorous pipetting in 100°C lysis buffer (10 mM Tris-HCl [pH 8.0], 7 M urea, 2% sodium dodecyl sulfate [SDS], and 1 mM EDTA), and phenol extracted three times, and nucleic acids were ethanol precipitated. The nucleic acid pellet was dissolved in 2.5 ml of diethylpyrocarbonate (DEPC)-treated water containing 1 g of CsCl, layered over a 2.5-ml cushion of 5.7 M CsCl–0.1 M EDTA (pH 8.0), and spun at 36,000 rpm for 16 h at 25°C in a Beckman SW50.1 rotor. The CsCl and DNA were aspirated, and the RNA pellet was dissolved in DEPC-treated water at a concentration of about 1 mg/ml.

DNA restriction fragments from hybrid plasmids were electroeluted from agarose gels (29). DNA probes were 5′ ^{32}P labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase after the cold 5′ phosphates were removed with calf intestinal alkaline phosphatase. Probes used in nuclease S1 mapping assays are shown in Fig. 1C and 6B. The 500-bp *Not*I-*Eco*RI(d) fragment, ^{32}P labeled at both 5′ ends (Fig. 1C), was recut with *Bgl*I, and the 354-bp *Bgl*I-*Eco*RI(d) fragment, single-end labeled at the *Eco*RI end, was used as a probe for genomic *flbF* transcript. The 682-nucleotide (nt) *Bam*HI-*Hin*dIII fragment labeled at both 5′ ends (Fig. 6B) was recut with *Not*I, and the 510-bp *Not*I-*Bam*HI fragment single-end labeled at the *Bam*HI end was used as a probe for *flbF* mRNA expressed from plasmids. Probes for mapping the 3′ end of the *flaO* operon transcripts were labeled with the Klenow fragment of DNA polymerase I and [α-^{32}P]dATP to fill in the ends of the 497-bp *Afl*II-*Eco*RI(d) fragment (Fig. 1C). This double-end-labeled probe was recut with *Hin*fI, and the 474-nt *Afl*II-*Hin*fI fragment 3′ ^{32}P single-end labeled at the *Afl*II end (Fig. 1C) was purified by electroelution from a 5% native acrylamide gel. Nuclease S1 protection assays (4) contained 10^5 cpm of probe DNA and 100 μg of RNA, and, after denaturing at 95°C for 5 min, the nucleic acids were allowed to hybridize at 55°C for 3 h. The products of the nuclease S1 protection reactions were denatured by heating to 90°C in a formamide-containing marker dye solution and fractionated by electrophoresis in 6% polyacrylamide gels that contained 8 M urea.

Primer extension mapping. The method of Inouye et al. (20) was used for primer extension. Five picomoles of 5′ ^{32}P-labeled synthetic oligonucleotide primer (5′-TCTCGC CGCGCATCAACCCGTCGAG-3′) was mixed with 10 μg of RNA isolated from strain CB15 and heated in a water bath, first at 95°C for 2 min and then at 42°C for 1 h. Primer extension was carried out for 1 h at 37°C with 20 U of avian myeloblastosis virus reverse transcriptase and 0.5 mM (each) deoxynucleoside triphosphates. Nucleic acids were phenol extracted, ethanol precipitated, and fractionated by electrophoresis in a 4% polyacrylamide gel next to a dideoxy nucleotide sequencing ladder generated with M13mp19-LS1 single-stranded DNA, and the end-labeled oligonucleotide primer was used for primer extension.

Nucleotide sequencing. Dideoxy nucleotide sequencing (46) was performed with native T7 DNA polymerase (Pharmacia), [α-^{35}S]dATP or [α-^{32}P]dATP as the radiolabel, and 7-deazaguanosine 5′-triphosphate in place of guanosine 5′-triphosphate to reduce compression artifacts (2, 31). Sequencing reaction products were fractionated by electrophoresis on 4 or 6% polyacrylamide gels with 8 M urea.

Clones for sequencing the 1,610-bp *Afl*II-*Bam*HI(e) fragment were obtained from pUC19-1.6 by using nuclease *Bal* 31 to generate a nested set of deletions (42), and we sequenced across the *Bam*HI(e) site on the *flbF* antisense strand by using λ Hk1 template DNA and a synthetic oligonucleotide primer. Clones for sequencing from *Bam*HI(e) to the end of the sequence were obtained by using a strategy that utilized restriction endonucleases with 4-bp recognition sites to cleave pUC19-3.0 under conditions in which a single-site cleavage (SSC) was the predominant reaction. We chose restriction enzymes with only G or C in the recognition site so that the linearized *C. crescentus* DNA (67% G+C) (54) would be cleaved at one of the numerous possible sites. The linearized DNAs were purified by electroelution from agarose gels and recut with *Eco*RI or *Hin*dIII, which cleaved each molecule once near the end of the fragment to be sequenced. The released DNA fragments extend from *Eco*RI or *Hin*dIII cleavage sites to various endpoints, depending on where the SSC reaction occurred.

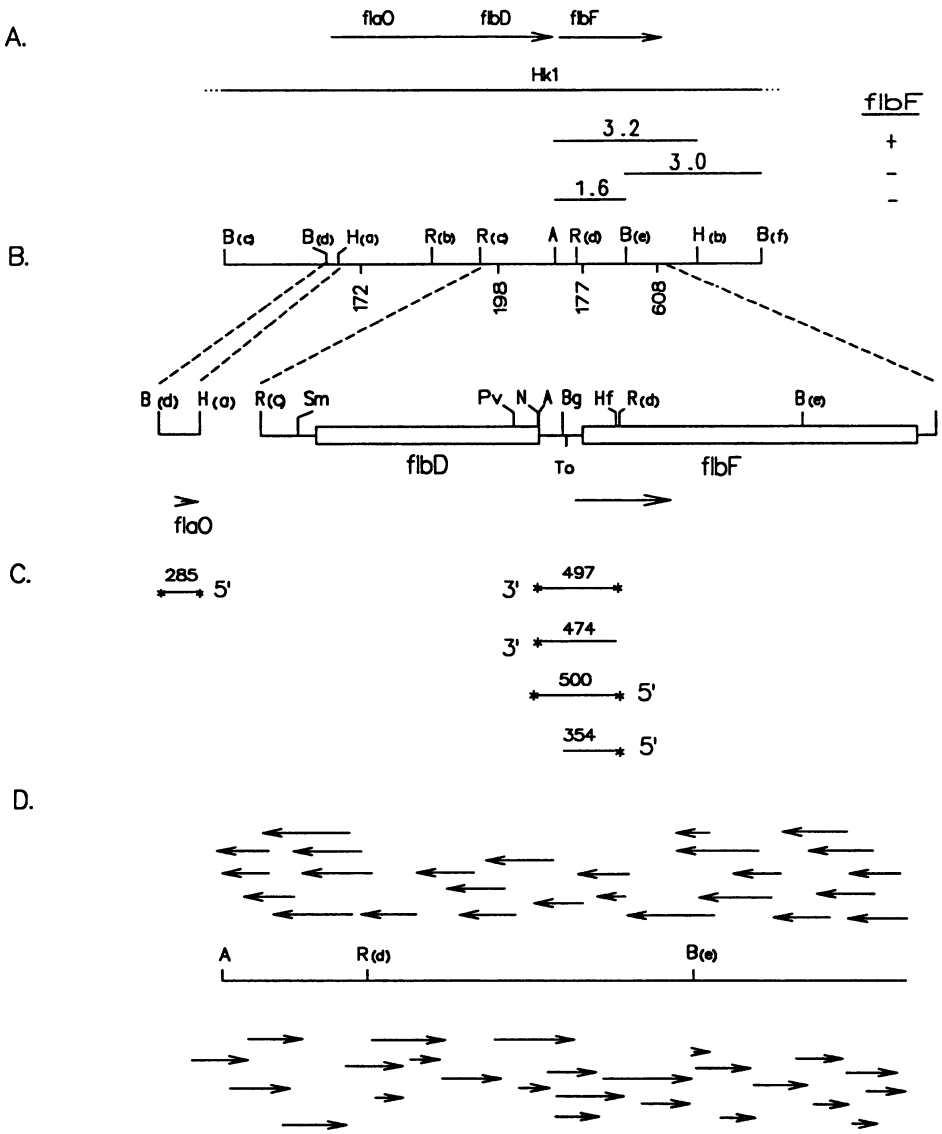

FIG. 1. Genetic and physical map of the *flaO-flbF* region, and sequencing strategy. (A) The organization of transcription units and of *fla* or *flb* genes has been described previously (32, 36, 39). Sequences carried by recombinant λ EMBL3 phage Hk1 and recombinant plasmids pRK2L1-3.2, -3.0, and -1.6 are indicated by solid lines. + and − indicate ability and inability, respectively, to correct the motility defect in *flbF* mutant strains SC1061 and SC1132. (B) Restriction endonuclease map. Relevant restriction sites are shown. Abbreviations: A, *Afl*II; B, *Bam*HI; Bg, *Bgl*I; H, *Hin*dIII; Hf, *Hin*fI; N, *Not*I; Pv, *Pvu*II; R, *Eco*RI. Letters in parentheses were used to designate restriction sites with more than one occurrence. To marks the major 3′ end of the *flaO* operon transcript. (C) Nuclease S1 probes. Asterisks indicate ^{32}P-labeled ends. (D) DNA sequencing strategy. Arrows indicate the direction and extent of sequencing.

Fragments produced by *Hin*PI or *Hpa*II SSC and released by *Eco*RI were ligated to M13mp18 DNA cleaved with *Acc*I and *Eco*RI. Fragments produced by *Hin*PI or *Hpa*II SSC and released by *Hin*dIII were ligated to M13mp19 DNA cleaved with *Acc*I and *Hin*dIII. Fragments produced by *Hae*III or *Bst*UI SSC and released by *Eco*RI were ligated to M13mp18 RFI (replicative form I) DNA cleaved with *Eco*RI and *Sma*I. Fragments produced by *Hae*III or *Bst*UI SSC and released by *Hin*dIII were ligated to M13mp19 RFI DNA cleaved with *Hin*dIII and *Sma*I. Following transfection of *E. coli* DH5α, clear plaques were transferred to nitrocellulose and screened for inserts by using nick-translated (45) 3-kb *Bam*HI(e)-*Bam*HI(f) DNA fragment (Fig. 1B) as a hybridization probe. The sequencing strategy is summarized in Fig. 1D.

Oligonucleotide mutagenesis. Oligonucleotide primer mutagenesis of M13mp19-LS1 was performed as described previously (25). Primers were 5′ phosphorylated with ATP and T4 polynucleotide kinase and annealed to single-stranded viral M13mp19-LS1 DNA isolated from the phage particles grown on *E. coli* RZ1032. After polymerization with T7 DNA polymerase and ligation with T4 DNA ligase, the resulting DNA molecules were used to transfect *E. coli* DH5α. Clones with the desired mutation were identified by nucleotide sequence analysis.

Nucleotide sequence accession number. The nucleotide sequence shown in Fig. 2 is part of a larger sequence that has been filed with GenBank, Los Alamos National Laboratory, under accession number M69228.

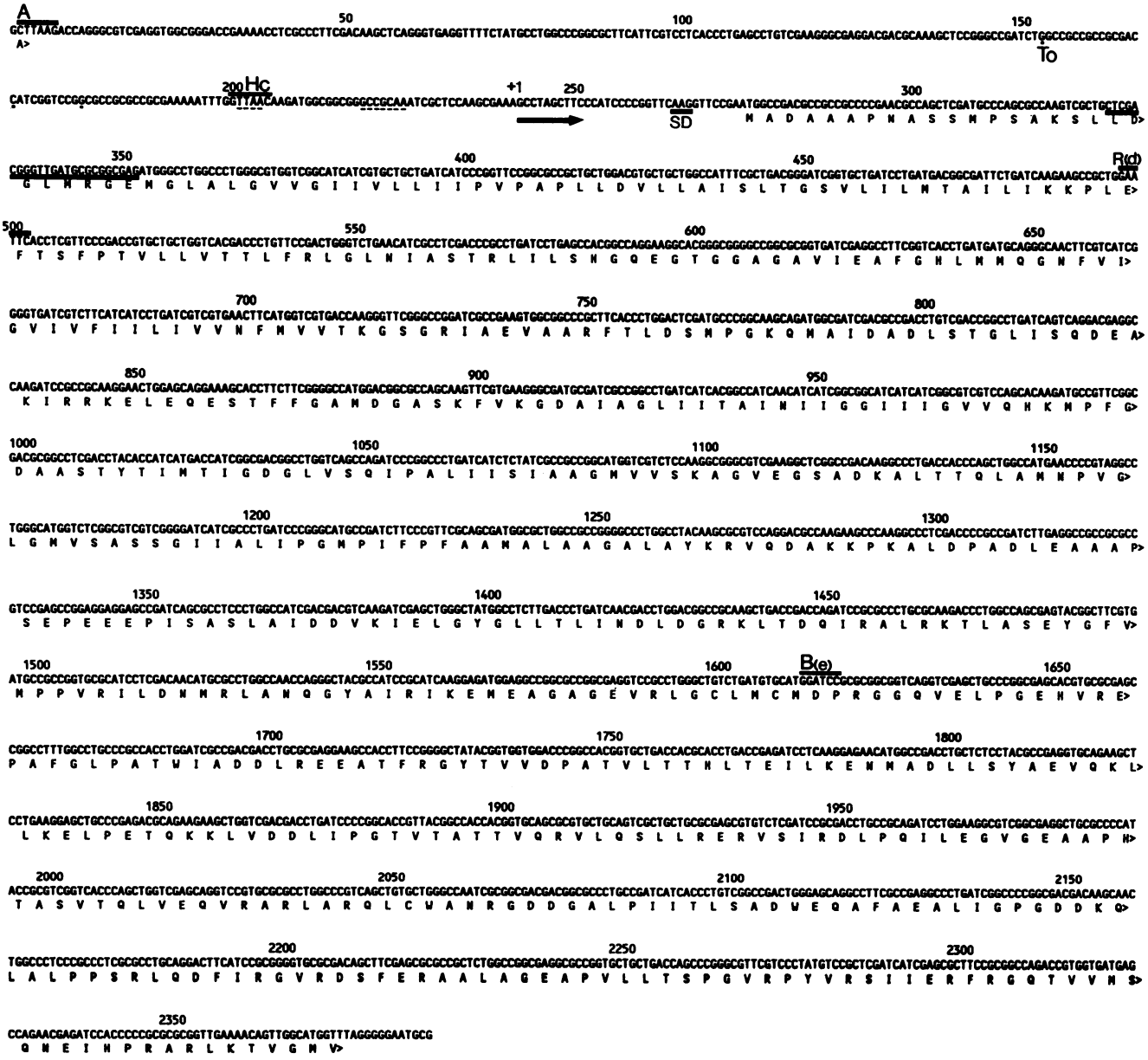

FIG. 2. Nucleotide sequence of the 3′-*flaO*-5′-*flbF* region. The translated sequence of ORF700 (*flbF*) is shown in single-letter amino acid code. A possible ribosome-binding site for FlbF protein is underlined and designated SD. Dots under the sequence mark 3′ ends of the *flaO* operon transcript, and To marks the position of the major 3′ end. The *flbF* transcription initiation point (+1) is indicated by an arrow below the sequence. The oligonucleotide primer used for mapping the +1 in *flbF* is underlined. Conserved −10 and −35 elements of the *flbF* promoter are underscored with dashes. Selected restriction sites are indicated. Hc, *Hin*cII; other restriction sites are the same as in the legend to Fig. 1.

RESULTS

Subcloning and nucleotide sequence of the *flbF* region. The organization of genes and transcription units in the *flaO-flbF* region of the *C. crescentus* flagellar hook gene cluster has been reported previously (39) and is shown in Fig. 1A. Bacteriophage λ Hk1 is a recombinant λ EMBL3 derivative that carries the *flaO-flbF* region of the hook gene cluster (57), and it was used as a source of DNA for subcloning *flbF* (Fig. 1A). Genetic complementation studies and nucleotide sequence analysis have previously identified the *flbD* protein-coding region (39, 43, 51), and these results suggested that *flbF* is located between the *Afl*II site in the last codon of *flbD* and the *Hin*dIII(b) site (Fig. 1B). pRK2L1-3.2 contains the 3.2-kb fragment extending from a filled-in *Afl*II site in the last codon of *flbD* to the *Hin*dIII(b) site (Fig. 1A), and, as predicted, it restored wild-type motility to *flbF* mutant strains SC1061(*flbF177*::Tn*5*) and SC1132(*flbF608*::Tn*5*), as measured by using a swarm plate assay (data not shown).

To help identify the *flbF* promoter and structural gene, we determined the nucleotide sequence (46) of a 2.3-kb fragment beginning at the *Afl*II site and extending to the right (Fig. 1B). The sequence is shown in Fig. 2, and the sequencing strategy is summarized graphically in Fig. 1D. The first 139 nt of the sequence shown in Fig. 2 were reported previously

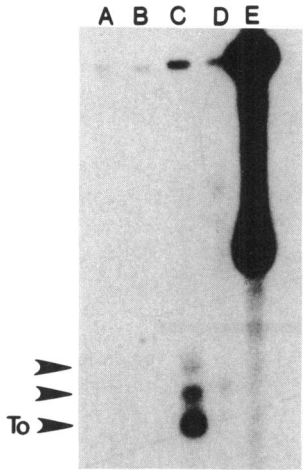

FIG. 3. Mapping the 3′ ends of the *flaO* transcript. Total RNA was prepared from *C. crescentus* strains and used in nuclease S1 assays as described in Materials and Methods. (A) The 474-nt *Afl*II-*Hin*fI fragment, 3′ ^{32}P labeled at the *Afl*II end (Fig. 1C), was used as a probe. Nuclease S1 was added to the reactions in lanes A to D. Arrows indicate protected fragments of 174, 164, and 149 nt (To). The source of RNA in each reaction is as follows: A, SC1052(*flaO172*::Tn*5*); B, SC1032(*flbD198*::Tn*5*); C, CB15 (wild type); D and E, yeast tRNA.

by Ramakrishnan and Newton (43) and are included here to illustrate the relationship between *flbD* and *flbF*.

Mapping the 3′ ends of the *flaO* operon transcript. The transcription initiation site for the *flaO* operon has been determined previously (32), and we were interested in defining the 3′ end of this transcription unit to investigate its relationship to *flbF*. When RNA from strain CB15 was probed with the 474-nt *Afl*II-*Hin*fI fragment 3′ ^{32}P end labeled at the *Afl*II end (Fig. 1C) in a nuclease S1 protection assay, partially protected fragments of 149, 164, and 174 nt were detected (Fig. 3, lane C). The major 3′ end of the *flaO* transcript thus mapped about 180 nt 3′ from the TAA translation termination codon of *flbD* (Fig. 2). Genetic evidence that the protected fragments detected with this assay correspond to 3′ ends of the *flaO* transcript comes from examination of 3′ *flaO* transcript levels in strains with Tn*5* inserted in the *flaO* operon (Fig. 1B). As expected, strains

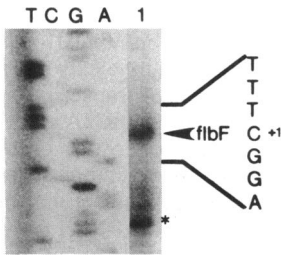

FIG. 4. Mapping the 5′ end of the *flbF* transcript. RNA isolated from strain CB15 was reverse transcribed with the 5′-end-labeled synthetic oligonucleotide complementary to nt 328 to 351 (Fig. 2). The products (lane 1) were denatured, electrophoresed, and located by autoradiography. Size markers were provided by chain terminating sequencing reactions with the same labeled primer and by using single-stranded M13mp19-LS1 DNA as the template. +1 indicates the 5′ nucleotide of the *flbF* transcript, and * marks a band corresponding to a shorter apparent 5′ end.

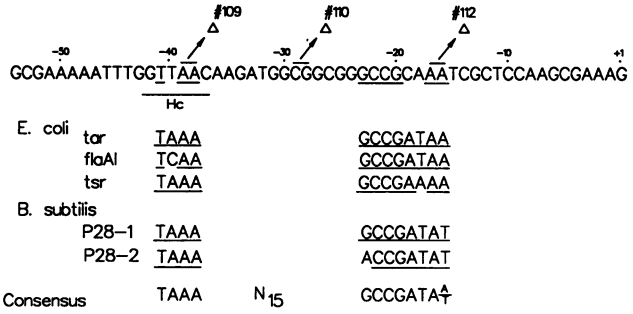

FIG. 5. Conserved sequences in the *flbF* promoter region. Data on σ^{28} promoters were summarized from references 1 and 18. Nucleotides matching the consensus for σ^{28} promoters are underlined, and arrows above the sequence indicate the position and nature of mutations created and analyzed in this work. Hc, cleavage site for *Hin*cII. Numbering indicates the distance in base pairs from the *flbF* transcription start site.

SC1052(*flaO172*::Tn*5*) and SC1032(*flbD198*::Tn*5*) had barely detectable levels of 3′ *flaO* mRNA compared with wild-type strain CB15 (Fig. 3, compare lanes A and B to C). These data suggest that *flbD* is the last gene in the *flaO* operon, because the largest open reading frame with a start codon (ATG or GTG) between the predicted *flbD* translation termination point and the 3′ end of the transcript could only code for a 28-amino-acid polypeptide.

Mapping the 5′ end of the *flbF* transcript. The 5′ end of the *flbF* transcript maps near the *Eco*RI(d) end of the 2.2-kb *Eco*RI(c)-*Eco*RI(d) fragment (36). We confirmed this result by using the 354-nt *Bgl*I-*Eco*RI(d) fragment (Fig. 1C) as a probe in nuclease S1 assays (data not shown), and primer extension mapping was used to accurately locate the 5′ end of the in vivo *flbF* transcript (Fig. 4). The longest RNA detected (Fig. 4, lane 1) corresponds to a 5′ end at the G residue at bp 241, which is approximately 89 bp downstream from the major 3′ end of the *flaO* transcript (Fig. 2).

A shorter transcript with the 5′ end apparently at the G residue at bp 251 (Fig. 4, lane 1) was also detected each time that the primer extension reaction was done. It is possible that two promoters initiate transcription at positions 241 and 251 or that the transcript initiated at bp 241 is processed between bp 250 and 251. However, we favor the interpretation that the reverse transcriptase terminated primer extension synthesis prematurely at position 251, because nuclease S1 protection analysis did not detect a second transcript and no transcript corresponding to the 5′ end at position 241 was detected for *flbF* promoter mutants that failed to synthesize the transcript with 5′ end at nucleotide 251, as discussed below (see Fig. 7a). These results placed the *flbF* transcript 5′ end at the G residue at bp 241, which is approximately 89 bp downstream from the major 3′ end of the *flaO* transcript (Fig. 2).

Comparison of the nucleotide sequence 5′ from the *flbF* transcription start site to conserved sequence motifs of well-characterized promoters revealed a sequence that matched at 6 of 8 nt to the −10 box and at 3 of 4 nt to the −35 box of promoters from other organisms that are transcribed by σ^{28} RNA polymerase (Fig. 5). The conserved −10 box in *flbF* is located 16 bp 5′ from the proposed transcription start site instead of the usual 8 to 10 bp for σ^{28} promoters, and the −10 and −35 boxes are separated by 14 bp instead of the 15 bp observed for the consensus sequence (1, 3, 18).

Cloning the *flbF* promoter. The *flbF* promoter was sub-

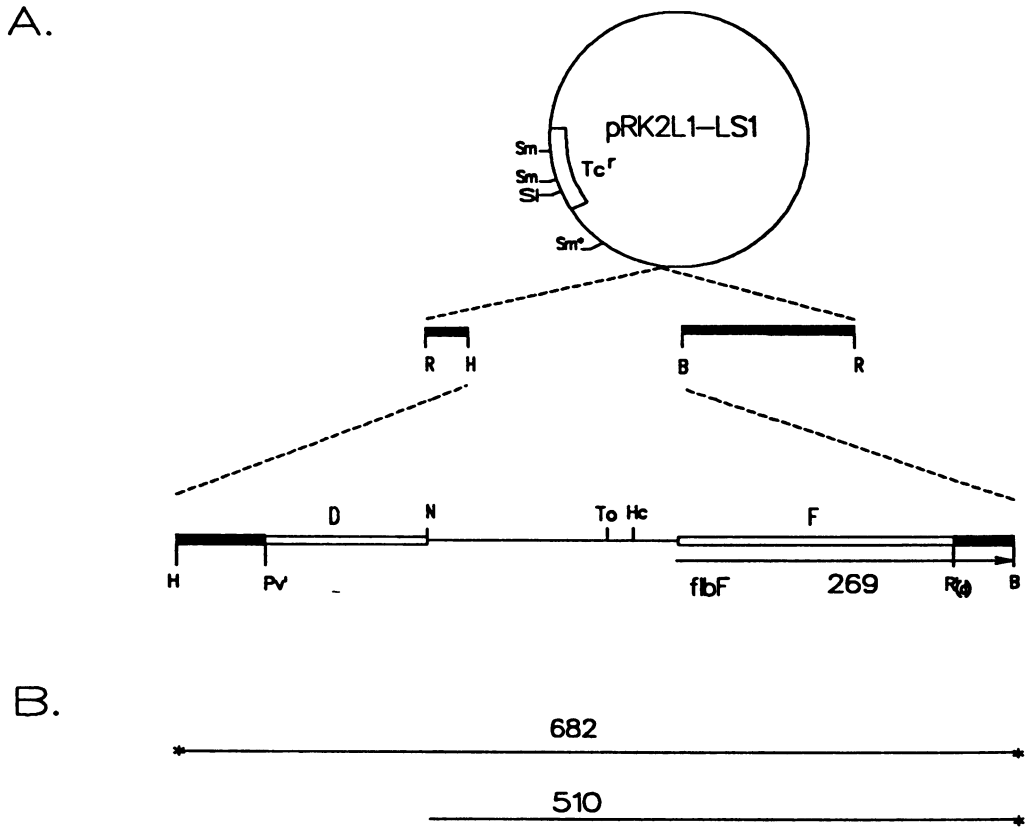

FIG. 6. Cloning the *flbF* promoter. (A) Genetic and physical map of pRK2L1-LS1. Hc marks the *Hin*cII site; other restriction sites are the same as in the legend to Fig. 1. Pv′ and R′ indicate that the ends were made flush ended in a polymerization reaction. The arrow below the sequence indicates the origin and direction of transcription, and To marks the major 3′ end of the *flaO* transcript. Open bars marked D and F indicate FlbD and FlbF protein-coding sequences, respectively. (B) Probes for nuclease S1 assays of the level of plasmid-encoded *flbF* mRNA in pRK2L1-LS. Asterisks indicate 5′-^{32}P-labeled ends.

cloned to investigate the transcriptional requirement for nucleotides in the conserved −10 and −35 boxes by using site-directed mutagenesis. The 652-bp *Pvu*II-*Eco*RI(d) fragment (Fig. 1B) was made flush ended by a DNA synthesis reaction with the Klenow fragment of DNA polymerase I and ligated to the *Hin*cII site of M13mp19 to yield M13mp19-LS1. The *flbF* promoter fragment in M13mp19-LS1 flanked by M13 polylinker DNA was then recloned in pRK2L1 as a *Hin*dIII-*Bam*HI fragment to yield pRK2L1-LS1 (Fig. 6A). pRK2L1 is a low-copy-number plasmid that is able to replicate in *C. crescentus*, and it has been used previously for investigating the activity of *C. crescentus fla* gene promoters (33, 52).

When RNA isolated from strain CB15(pRK2L1-LS1) was probed with the 682-nt *Hin*dIII-*Bam*HI fragment labeled at both 5′ ends (Fig. 6B) in nuclease S1 assays, a protected fragment with an estimated size of 269 nt was detected (Fig. 7a, lane C), and this is the size expected for the *flbF* transcript expressed from the plasmid. Protected fragments were not detected when RNA from strain CB15 or strain CB15(pRK2L1) was assayed (Fig. 7a, lanes A and B). Only transcripts made from the *flbF* promoter on pRK2L1-LS1 can protect the 5′ ^{32}P-labeled *Bam*HI end of the 682-bp *Bam*HI-*Hin*dIII probe from nuclease S1 hydrolysis, because the labeled 5′ phosphates in this probe are on nucleotides derived from the plasmid polylinker sequence. These results show that the nuclease S1 assay is specific for the *flbF* promoter cloned in pRK2L1-LS1 and that transcription initiated at the expected position. The double-end-labeled 682-nt probe was routinely used in the nuclease S1 assays because it yielded the same-sized protected fragment as the 510-nt *Not*I-*Bam*HI fragment 5′ end labeled at the *Bam*HI end (Fig. 6B) and was easier to prepare.

Mutagenesis of the *flbF* promoter. Two-base-pair deletions were introduced into the conserved σ^{28}-like −10 and −35 boxes and nonconserved spacer region in M13mp19-LS1 by using oligonucleotide mutagenesis, and these mutations are summarized in Fig. 5. After confirming the mutations by nucleotide sequence analysis, each mutant promoter was cloned in pRK2L1 on a 680-bp *Hin*dIII-*Bam*HI fragment and transferred to *C. crescentus* by electroporation. The relative level of plasmid-encoded *flbF* transcripts was measured by a nuclease S1 assay using in vivo RNA and the 682-nt *Hin*dIII-*Bam*HI fragment as a probe. All three of the deletion mutations resulted in undetectable levels of plasmid-encoded *flbF* mRNA (Fig. 7a, lanes D, E, and F). As a control for the level and intactness of RNA, the relative level of 5′ *flaO* mRNA was also determined by using a nuclease S1 assay with the 285-nt *Bam*HI(d)-*Hin*dIII(a) fragment as a probe (Fig. 1C). Figure 7b shows that the level of *flaO* transcript was similar in each preparation. Similarly, nuclease S1 analysis detected the genomic *flbF* transcript in each of these preparations (data not shown). These results demonstrate that mutations disruptive of the conserved −10 or −35 sequences or the spacing between them interfered with *flbF* promoter function.

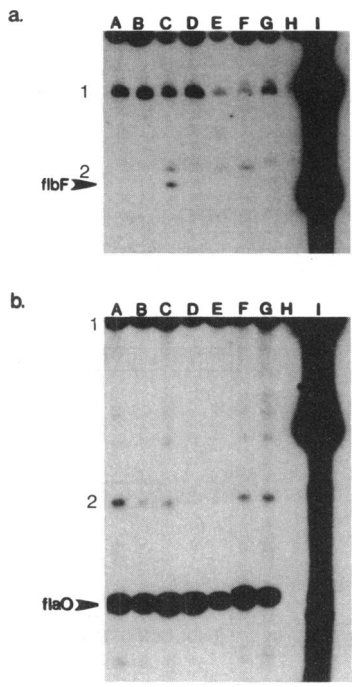

FIG. 7. Nuclease S1 analysis of the level of plasmid-encoded *flbF* mRNA in pRK2L1-LS1. Nuclease S1 assays were performed as described in the legend to Fig. 3, except that the 682-nt *Hin*dIII-*Bam*HI fragment (Fig. 6B) or the 285-nt *Hin*dIII-*Bam*HI fragment (Fig. 1C) was used as a probe. Arrowheads mark the *flbF* and *flaO* protected fragments, 1 denotes full-length probe, and 2 denotes a nuclease S1 degradation product of the probe. The source of RNA in each assay is indicated. (a) Probe is the 682-nt *Hin*dIII-*Bam*HI fragment. Lanes: A, CB15; B, CB15(pRK2L1); C, CB15(pRK2L1-LS1); D, CB15(pRK2L1-LS1#109); E, CB15(pRK2L1-LS1#110); F, CB15(pRK2L1-LS1#112); G, CB15(pRK2L1-LS2); H and I, yeast tRNA. (b) Probe is the 285-nt *Hin*dIII-*Bam*HI fragment; RNA preparations are the same as in panel a.

We also determined the effect of deleting the nucleotides 5′ from the −35 sequence element. pRK2L1-LS2 contains the 309-bp fragment extending from the *Hin*cII site to the polylinker *Bam*HI site cloned in pRK2L1 (Fig. 6A), and the relative level of *flbF* transcripts expressed from this plasmid in vivo was measured by a nuclease S1 protection assay. pRK2L1-LS2 lacks the sequences 5′ from the conserved −35 sequence element and also has a T-to-C point mutation at −34 because of sequence differences in the *Hin*cII sites in *flbF* and the pUC18 polylinker. The level of *flbF* transcripts expressed from pRK2L1-LS2 was greatly reduced relative to that of the parent plasmid (Fig. 7a, lane G).

Nucleotide sequence of *flbF*. To identify the *flbF* structural gene, we searched the DNA sequence 3′ from the *flbF* promoter region for long open reading frames (longer than 100 codons) with potential start codons and ribosome-binding sites. Only one open reading frame was identified, and its length is 700 codons (ORF700). ORF700 begins at the ATG at +33 in *flbF* and extends rightward to bp 2373, where it ends at a TAG translation terminator (Fig. 2). Eight base pairs upstream from the start codon of ORF700 is a sequence (5′-AAG) that is complementary to the 3′ end of the *C. crescentus* 16S rRNA (12) and that might serve as a ribosome-binding site (49). The next in-frame ATG is located 31 bp downstream, and it has a potential ribosome-binding site (5′-GAA) that is located 11 bp away. ORF700 has a codon usage bias that is very similar to that found in other *C. crescentus* flagellar genes (reference 47 and data not shown).

Comparison of the deduced amino acid sequence of ORF700 with the amino acid sequences translated from the GenBank data base (version 67) revealed a sequence that has significant similarity to ORF5 in the low-calcium-response region (*lcr*) of virulence plasmid pYVO3 from *Y. enterocolitica* (Fig. 8) and that corresponds to the N-terminal 159 amino acid residues of the inferred LcrD protein (53). Except for a small localized gap, these sequences are similar over the entire length of LcrD (46% identity), but the similarity is even stronger when residues with similar chemical properties are considered (Fig. 8). In addition to amino acid sequence similarity, ORF700 and LcrD also share a pattern of four conserved hydrophobic regions that are similar to membrane-spanning elements of integral membrane proteins (10, 44) (Fig. 8). The striking sequence similarity and predicted structural homology between ORF700 and LcrD suggest that the former is a protein-coding sequence. We propose that ORF700 or a reading frame beginning at a nearby ATG residue in the same reading frame corresponds to the *flbF* structural gene, as determined by its physical location in the *flbF* transcription unit. Consistent with this gene assignment, pRK2L1-3.2, which contains ORF700, corrected the motility defect in *flbF* mutant strains SC1061 and SC1132 (Fig. 1A). In contrast, pRK2L1-1.6 and pRK2L1-3.0, which lack the carboxy- and amino-terminal portions of ORF700, respectively, failed to restore motility to strains SC1061 and SC1132 (Fig. 1A).

DISCUSSION

The *C. crescentus* flagellar genes are under strict temporal regulation in the cell division cycle, and they are organized into a regulatory hierarchy. *flbD* and *flbF* are of particular interest in relation to the regulatory hierarchy because they are required in *trans* for expression of several flagellar gene transcription units, including *flaN*, *flbG*, *flgL*, and *flgK* (30, 32, 38). Analysis of the *flaO* operon DNA sequence revealed that *flbD* codes for a 52-kDa protein, and its inferred amino acid sequence and domain organization is very similar to that of the NtrC(NRI) protein (43, 51). The homology to NtrC suggested the possibility that FlbD might be the transcriptional regulator of a two-component regulatory system and that its activity might be regulated through phosphorylation by a histidine protein kinase (43, 50).

In this report, we have investigated the organization and expression of the transcription unit encoding *flbF* to identify its gene product and to locate and analyze the *flbF* promoter. We defined the 3′ boundary of the *flaO* operon transcript by nuclease S1 mapping, and our data support the prediction by Ramakrishnan and Newton (43) that *flbD* is the last gene in the *flaO* operon, because the largest open reading frame with a start codon (ATG or GTG) between the predicted *flbD* translation termination point and the 3′ end of the transcript could only code for a 28-amino-acid polypeptide. To begin characterizing the *flbF* promoter and other *cis*-acting sequences, we precisely mapped the 5′ end of the *flbF* transcript by using primer extension analysis and found that the neighboring DNA sequence contains −10 and −35 elements that are similar to the consensus sequence for promoters in other organisms that are transcribed by σ^{28} RNA polymerase (1, 3, 18). It would not be surprising if the σ^{28}-like −10 and −35 sequences identified in *flbF* constitute the promoter, because flagellar and chemotaxis genes from organisms as diverse as *E. coli*, *S. typhimurium*, and *Bacillus subtilis*

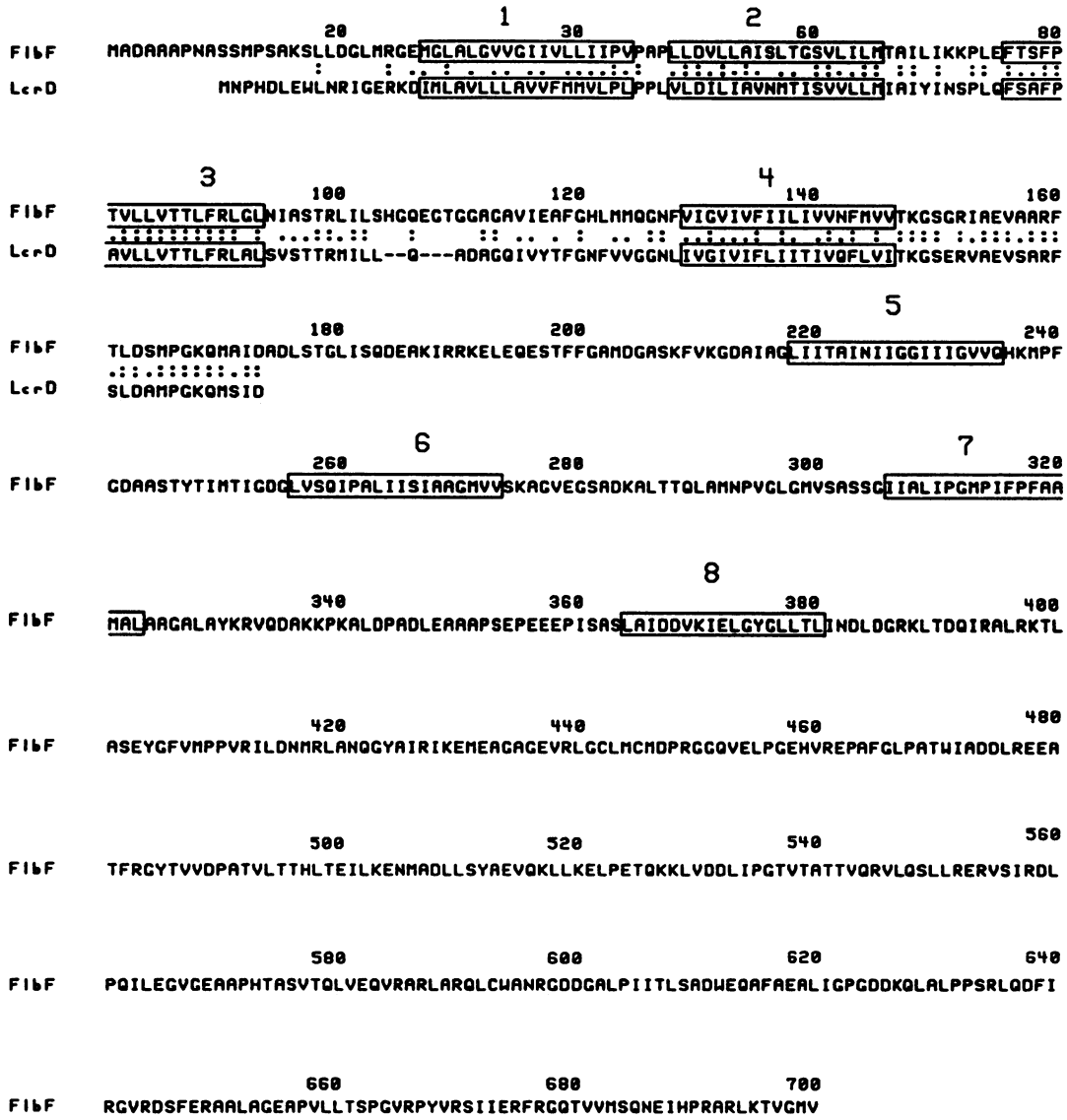

FIG. 8. Homology of FlbF to LcrD. The LcrD amino acid sequence corresponds to the first 159 residues translated from *lcrD* of *Y. enterocolitica* (53). Sequences are shown in single-letter amino acid code. Double dots indicate amino acid identity, and single dots indicate chemically similar residues. Hydrophobic regions predicted to form parts of membrane-spanning segments are boxed.

utilize σ^{28}-type promoters (1, 18, 26). Evidence for the presence of a σ^{28} RNA polymerase analog in *C. crescentus* comes from a report by Frederikse and Shapiro that the *S. typhimurium* chemoreceptor gene *tsr* was transcribed in *C. crescentus* from its native σ^{28} promoter (14). In addition, the *C. crescentus flaE* gene contains a sequence near the transcription start site that matches the −10 element of σ^{28} promoters at four of eight positions (22). *flaE* lacks the conserved 5′-TAAA sequence at −35, and in this respect it resembles the class I and II flagellar genes of *S. typhimurium*, which also lack the −35 box (25). The functions of the conserved −10- and −35-like sequences in *flbF* were investigated by cloning the *flbF* promoter and using site-directed mutagenesis. Two-base-pair deletions that disrupted the −10 or −35 region or changed the spacing between them resulted in no detectable *flbF* transcript, suggesting that these sequences are required for promoter function. The −10 element is located 16 bp upstream of the proposed 5′ end of the *flbF* transcript rather than the usual 8 to 10 bp for σ^{28} promoters (1). It is possible that the *flbF* transcript is rapidly and specifically processed at its 5′ end and that the actual start site is located closer to the −10 element. Apparent processing has previously been reported in the case of the *flaN* (33) and *flbG* (8) operon transcripts. We propose that the σ^{28}-like consensus elements identified for *flbF* are used in vivo as a promoter.

Genes at the lowest level of the regulatory hierarchy include *flgK* and *flgL*, which encode the 25- and 27-kDa flagellins, respectively, and these genes have sequences near the transcription start site that match the consensus sequence for promoters transcribed by σ^{54} RNA polymerase (30, 32). The *flaN* and *flbG* operon genes occupy a position above *flgK* and *flgL* and also utilize σ^{54}-type promoters (32, 33, 37). The *flaO* operon genes occupy a level in the hierarchy above the *flaN* and *flbG* operon genes, and the 5′ region of the operon lacks a σ^{54}-like promoter. Instead, *flaO*

contains a sequence, essential for transcription, that matches the consensus −10 box for *E. coli* promoters transcribed by σ^{32} RNA polymerase (34, 52). It has become evident that genes at different levels of the *C. crescentus* flagellar gene regulatory hierarchy have different promoter sequences, suggesting the possibility that forms of RNA polymerase utilizing alternate σ factors may be required for transcription of these genes. Although the requirement for a cascade of alternate σ factors for sequential activation of *B. subtilis* sporulation genes has been clearly demonstrated (9, 28), the role of alternate σ factors in the temporal regulation of *C. crescentus* flagellar genes remains to be determined.

We determined the nucleotide sequence of *flbF* to study its role in flagellar gene transcription. The predicted *flbF* product is a slightly acidic protein (pI 5.2) of 700 amino acid residues and with a calculated molecular weight of 74,523.7. The amino acid sequence appears to be divided into two almost equal domains, with the N-terminal portion consisting of eight strongly hydrophobic regions that are predicted to be membrane-spanning segments. FlbF is thus predicted to be an integral membrane protein. FlbF does not contain a helix-turn-helix DNA-binding motif characteristic of some DNA regulatory proteins (16). In light of its requirement in *trans* for *flaN* and *flbG* transcription, *flbF* appeared to be a candidate for a histidine protein kinase that might regulate FlbD activity or for a σ^{54} analog. However, it does not contain the conserved C-terminal amino acid residues characteristic of NtrB and other histidine protein kinases (for a recent review, see reference 50), and it is not similar in sequence to the σ^{54}-like proteins in GenBank (version 67).

The FlbF amino acid sequence is very similar to the inferred sequence of the LcrD protein encoded in the low-calcium-response (*lcr*) region of plasmid pYVO3 of *Y. enterocolitica* (4) (Fig. 8). The *lcr* region of *Yersinia* virulence plasmids is highly conserved between several species examined, and it encodes genes involved in Ca^{2+} signal transduction (13). Consistent with the structural predictions made for FlbF protein, LcrD encoded by the virulence plasmid of *Yersinia pestis* is also predicted to be an integral membrane protein with eight membrane-spanning segments (40).

Mapping of the in vivo 5′ ends of transcripts in the *lcr* region of pYVO3 has revealed several possible promoter sequences, and Northern (RNA) blot analysis using a probe containing *lcrD* sequences identified transcripts of 2.8 and >3.0 kb that might encode LcrD (53). Several theoretical promoters were identified on the basis of their similarity to the consensus sequence for *E. coli* σ^{70} promoters (17), although the similarity was not strong (53). We identified a sequence (TAAT-N_{15}-GCCGAAAA) 138 bp upstream from the ATG for LcrD that matches the consensus σ^{28} promoter at 7 of 8 residues at −10 and 3 of 4 residues at −35 (shared residues are underlined). It is possible that the homology between FlbF and LcrD extends to their expressions and that both may be transcribed by a similar form of RNA polymerase.

We are extending the analysis of the *cis* requirements for *flbF* promoter function and investigating the biochemical mechanism by which FlbF functions in flagellar gene transcription.

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