JOURNAL OF BACTERIOLOGY, Jan. 1993, p. 367–376
0021-9193/93/020367-10$02.00/0

Vol. 175, No. 2

Identification of the Promoter and a Negative Regulatory Element, *ftr4*, That Is Needed for Cell Cycle Timing of *fliF* Operon Expression in *Caulobacter crescentus*

SUSAN M. VAN WAY,[1] AUSTIN NEWTON,[2] ANN H. MULLIN,[1] AND DAVID A. MULLIN[1]*

Department of Cell and Molecular Biology, Molecular and Cellular Biology Program, Tulane University, New Orleans, Louisiana 70118-5698,[1] and Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544-1014[2]

Received 23 April 1992/Accepted 12 November 1992

The *fliF* operon of *Caulobacter crescentus*, which was previously designated the *flaO* locus, is near the top of the flagellar-gene regulatory hierarchy, and it is one of the earliest transcription units to be expressed in the cell cycle. In this report, we have identified two *cis*-acting sequences that are required for cell cycle regulation of *fliF* transcription. The first sequence was defined by the effects of three 2-bp deletions and five point mutations, each of which greatly reduced the level of *fliF* operon transcript in vivo. These eight mutations lie between −37 and −22 within an 18-bp sequence that matches, at 11 nucleotides, sequences in the 5′ regions of the *flaQR* (*flaS* locus) and *fliLM* operons, which are also expressed early and occupy a high level in the regulatory hierarchy (A. Dingwall, A. Zhuang, K. Quon, and L. Shapiro, J. Bacteriol. 174:1760–1768, 1992). We propose that this 18-bp sequence contains all or part of the *fliF* promoter. We have also identified a second sequence, 17 bp long and centered at −8, which we have provisionally designated *ftr4* because of its similarity to the enhancer-like *ftr* sequences required for regulation of σ^{54} promoters *flaN* and *flbG* (D. A. Mullin and A. Newton, J. Bacteriol. 171:3218–3227, 1989). Six of the seven mutations in *ftr4* examined resulted in a large increase in *fliF* operon transcript levels, suggesting a role for *ftr4* in negative regulation. A 2-bp deletion at −12 and −13 in *ftr4* altered the cell cycle pattern of *fliF* operon transcription; the transcript was still expressed periodically, but the period of its synthesis was extended significantly. We suggest that the *ftr4* sequence may form part of a developmental switch which is required to turn off *fliF* operon transcription at the correct time in the cell cycle.

Flagellum biosynthesis in *Caulobacter crescentus* is under strict spatial and temporal regulation, and it has been used as a model for studying the control of developmentally regulated genes (for reviews, see references 29 and 42). Genetic requirements for transcription of these flagellar (*fla*) genes have been identified by comparing the relative levels of individual *fla* gene transcripts in wild-type cells with those in various nonmotile mutants. These studies have demonstrated that, as in *Escherichia coli* (18, 19) and *Salmonella typhimurium* (22), the *C. crescentus fla* genes are organized into a regulatory hierarchy in which genes at one level of the hierarchy are required for expression of genes lower in the hierarchy (4, 26, 30, 33, 44).

The *C. crescentus fla* gene hierarchy, as currently understood, contains four levels or classes of genes (Fig. 1) (11). Flagellin genes *flgK* and *flgL* occupy level IV, the lowest level of the hierarchy, and their expression depends on genes in the *flaN* and *flbG* operons, which occupy level III. The level III genes depend, in turn, on level II genes like the *flbF* and the *fliF* operon genes for their expression, and the gene or genes at the top of the hierarchy that are presumably required for expression of level II genes have been placed at level I. No level I genes have been identified yet.

A unique feature of *fla* gene expression in *C. crescentus* is that all of the genes in the regulatory hierarchy that have been examined are under strict temporal regulation, because their expression is confined to a discrete period in the cell cycle that corresponds to the time of flagellum assembly. The *fliF* operon is particularly interesting in relation to understanding the timing of *fla* gene expression because it is one of the earliest transcription units to be expressed and because delaying its expression by bringing it under the control of a promoter that is periodically transcribed at a later time in the cell cycle results in a similar delay in expression of transcription units *flbG*, *flgK*, and *flgL* (32), which are below it in the hierarchy.

The 5.1-kb *fliF* operon, which was previously called the *flaO* locus, contains five open reading frames, the first of which (*fliF*) is homologous to that of the *S. typhimurium fliF* gene (38) that encodes the M-ring protein, which is the first ring protein assembled in the basal body of the flagellum (17). The last gene in the operon, *flbD*, codes for a protein that belongs to a family of response regulators that includes the *E. coli* NtrC protein, which is required for transcription initiation by σ^{54} RNA polymerase (36). Immediately downstream from the *fliF* operon is the *flbF* transcription unit, which codes for a 74-kDa integral membrane protein (39) that is a homolog of the *Yersinia pestis* LcrD, *Shigella flexneri* VirH, and *Salmonella* InvA proteins (13, 37, 40). *flbD* and *flbF* are both required for transcription from the σ^{54} promoters of *flaN*, *flbG*, *flgK*, and *flgL* (4, 24, 26), and both are also negative regulators of *fliF* operon transcription (30).

The genes at level IV and some of those at level III share a common set of *cis*-acting sequences that includes a σ^{54} promoter, an enhancer-like sequence called *ftr* (24, 26, 27), and a sequence that matches the consensus binding site for *E. coli* integration host factor (14, 28). In contrast, the *fliF* operon 5′ region does not contain a sequence that conforms to the σ^{54} RNA polymerase consensus sequence (21, 26), and we report here the results of site-directed mutagenesis experiments that identify two important *cis*-acting elements

* Corresponding author.

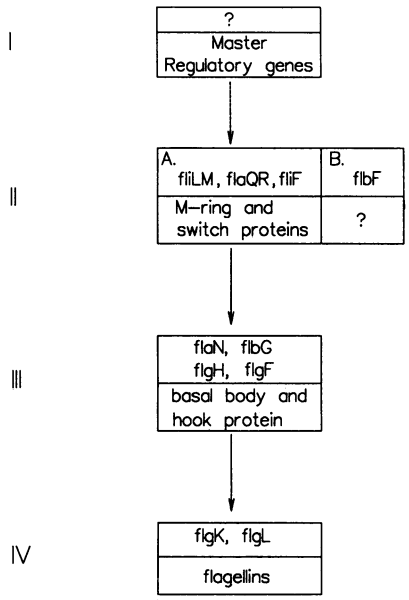

FIG. 1. Proposed hierarchy for *C. crescentus fla* genes. Class I, II, and III genes referred to by Shapiro and coworkers (7, 9, 46) are designated here level II, III, and IV genes, respectively (11). Genes at level II are classed A or B on the basis of differences in promoter sequences.

required for regulated expression of the *fliF* operon. The first sequence identified by mutagenesis is essential for *fliF* expression, and it lies within an 18-bp sequence that is strongly conserved in the 5′ region of the *fliF*, *flaQR* (*flaS* locus), and *fliLM* transcription units, all of which are expressed early in the cell cycle and occupy level II in the *fla* gene regulatory hierarchy (9, 32, 46). We propose that this 18-bp sequence contains all or part of the *fliF* promoter. The second sequence identified by mutagenesis is provisionally designated *ftr4* because of its similarity to the enhancer-like *ftr* elements identified previously (26, 27). *ftr4* appears to function as a site of negative transcription regulation, and we present evidence that this sequence may be part of a developmental switch which is required to turn off *fliF* operon transcription at the correct time in the cell division cycle.

MATERIALS AND METHODS

Strains and culture conditions. The bacterial strains, plasmids, and phages used in this work are listed in Table 1. *C. crescentus* wild-type strain CB15 and mutant derivatives were grown in a peptone-yeast extract medium (PYE) (35), and plasmid-containing strains were grown in PYE broth or M2 glucose minimal-salt broth (16) supplemented with 0.02% (wt/vol) Casamino Acids and 2 μg of tetracycline per ml (M2tet). *E. coli* strains were grown in a yeast extract-tryptone (YT) medium (15), and strains containing plasmids were grown in YT medium supplemented with appropriate antibiotics: ampicillin, 50 μg/ml; chloramphenicol, 10 μg/ml; kanamycin, 50 μg/ml; or tetracycline, 10 μg/ml.

Plasmid and phage construction. To construct M13mp 19SV4, the 287-bp *Bam*HI-*Hin*dIII fragment (Fig. 2B) was made fully double stranded by filling in the single-stranded ends with the Klenow fragment of DNA polymerase I and all four deoxyribonucleoside triphosphates (dNTPs), and the resulting fragment was ligated to the *Hin*cII site of M13mp19. pUC18SV4 was constructed by ligating the 320-bp *Bam*HI-*Hin*dIII fragment from M13mp19SV4 to *Bam*HI- and *Hin*dIII-digested pUC18. The *fliF* operon promoter in this construct is proximal to the *Hin*dIII site in the multiple-cloning-site polylinker. pRK2L1-SV4 (Fig. 3A) was constructed by ligating the 320-bp *Bam*HI-*Hin*dIII fragment from M13mp19SV4 to pRK2L1 that had been cleaved with *Bam*HI and *Hin*dIII. pRK2L1 is a derivative of pRK290 that contains the multiple-cloning-site polylinker from pUC18. M13mp19532 was constructed by ligating the 532-bp *Bam*HI-*Sac*I fragment to M13mp19 double-stranded DNA digested with *Bam*HI and *Sac*I.

pAM1 was constructed by ligating the 177-bp *Hin*dIII-*Bst*UI fragment from pUC18SV4 to pUC18 digested with *Hin*dIII and *Hin*cII. pAM2 was constructed by ligating the 143-bp *Hin*dIII-*Sau*3AI fragment from pUC18SV4 to pUC18 digested with *Hin*dIII and *Bam*HI. pAM3 was constructed by ligating the 114-bp *Hin*dIII-*Rsa*I fragment to pUC18 digested with *Hin*dIII and *Hin*cII. pRK2L1-AM1 (Fig. 3B) was constructed by ligating the 187-bp *Bam*HI-*Hin*dIII fragment from pAM1 to pRK2L1 that had been digested with *Hin*dIII and *Bam*HI. pRK2L1-AM2 (Fig. 3B) was constructed by ligating the 152-bp *Hin*dIII-*Kpn*I fragment from pAM2 to pRK2L1 that had been digested with *Hin*dIII and *Kpn*I. pRK2L1-AM3 (Fig. 3B) was constructed by ligating the 124-bp *Bam*HI-*Hin*dIII fragment from pAM3 to pRK2L1 that had been digested with *Bam*HI and *Hin*dIII. Plasmids were introduced into *C. crescentus* strains by electroporation using a Bio-Rad Gene Pulser Electroporator (27). Plasmid and phage DNAs were introduced into *E. coli* strains by $CaCl_2$ transformation (23).

Isolation of RNA and nuclease S1 protection assays. RNA was purified from *C. crescentus* cells as described previously (33), and its concentration was estimated by measuring its A_{260}. Nuclease S1 protection reactions for analysis of RNA purified from log-phase cells and synchronized cells contained 100 and 10 μg of RNA, respectively.

Restriction endonuclease-generated DNA fragments were 5′ end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and used as probes in nuclease S1 assays (2). The 287-

TABLE 1. Bacterial strains, plasmids, and phages

Strain, plasmid, or phage	Relevant features	Source or reference
Strains		
C. crescentus		
CB15	Wild type	35
CB15F	Density variant of CB15	12
SC511	*flaK155*::IS*511*	34
SC1032	*flbD198*::Tn*5*	34
SC1132	*flbF177*::Tn*5*	34
E. coli		
HB101	Host for plasmids	3
DH5 α	Male, Km^r	BRL[a]
CJ236	*dut ung*, male, Cm^r	20
Plasmids		
pRK2L1	Cloning vector, Tc^r	27
pUC18	Cloning vector, Ap^r	45
pRK2LM10	Promoterless CAT gene, Tc^r	39
Bacteriophages M13mp18 and -19	Cloning vectors	45

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

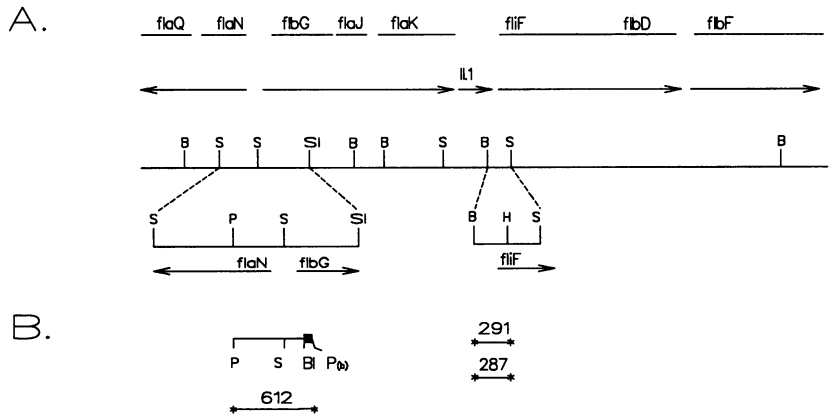

FIG. 2. Physical and genetic map of the hook gene cluster. (A) The organization of transcription units, flagellar genes, and restriction map of the hook gene cluster. The location of transcription start sites for *flbF* and the *flaN*, *flbG*, and *fliF* operons have been described previously (4, 26, 37, 40). Transcription unit II.1 is located between the *flbG* and *fliF* operons (4). Relevant restriction sites are indicated as follows: B, *Bam*HI; S, *Sst*I; Sl, *Sal*I; P, *Pst*I; H, *Hin*dIII. (B) Probes for nuclease S1 mapping. The location of a *Bal* 31 deletion endpoint (Bl) and ^{32}P-labeled ends (*) are indicated. All of the probes except the 291-nt probe (which is 3′ end labeled) are 5′ end labeled. Genomic sequences are indicated by thin solid lines. ■, polylinker DNA derived from pUC18.

nucleotide (287-nt) *Bam*HI-*Hin*dIII fragment labeled at both 5′ ends (Fig. 2B) was used as a probe for the chromosomal *fliF* operon transcript, and the 320-nt *Bam*HI-*Hin*dIII fragment labeled at both 5′ ends (Fig. 3C) was used as a probe for *fliF* operon mRNA expressed from pRK2L1-SV4. The 291-nt *Bam*HI-*Hin*dIII fragment, ^{32}P labeled at both 3′ ends (Fig. 2B), was generated by filling in the single-stranded ends of the 287-bp *Bam*HI-*Hin*dIII fragment by using the Klenow fragment of DNA polymerase I and dCTP, dGTP, dTTP, and [α-^{32}P]dATP, and it was used in nuclease S1 protection assays as a probe for the II.1 gene transcript. The 612-nt *Pst*I fragment labeled at both 5′ ends (Fig. 2B) was used as a probe to detect genomic *flaN* and *flbG* transcripts. The *Pst*I(b) end of the 612-nt probe was generated by ligating a *Hin*cII-*Pst*I fragment from the pUC18 polylinker to the flush end of a genomic DNA fragment generated by the action of *Bal* 31 nuclease (27). The 5′ ^{32}P label at the *Pst*I(b) end is protected by the *flbG* transcript in nuclease S1 assays presumably because the polylinker-derived sequence at this end (5′-GGTCCATG) is complementary to the *flbG* mRNA sequence (5′-CAUGACC) except for a single nucleotide mismatch 5 bp in from the 5′ end in the former sequence. An excess amount of ^{32}P-labeled probe DNA was added to each nuclease S1 assay to ensure that the intensity of the protected fragments reflected the level of the 5′ transcript. The hybridization temperature for the DNA probes and RNA was 55°C. The products of the nuclease S1 protection assays were denatured by heating to 95°C for 5 min in a formamide-containing marker dye solution and fractionated by electrophoresis in 6% polyacrylamide gels that contained 8 M urea.

As a control, we compared the levels of the *fliF* operon transcripts expressed from pRK2L1-SV4 (Fig. 3A) in four independently isolated RNA preparations and found the relative levels to be very similar (data not shown). This finding suggests that the level of the *fliF* operon transcript detected in CB15(pRK2L1-SV4) does not vary widely from preparation to preparation.

Preparation of synchronous cultures of strain CB15F. *C. crescentus* CB15F is a density variant of strain CB15, in which the swarmer cells can be separated from stalked and predivisional cells by centrifugation (12). Strains CB15F (pRK2L1-SV4) and CB15F(pRK2L1-SV4#2) were grown in

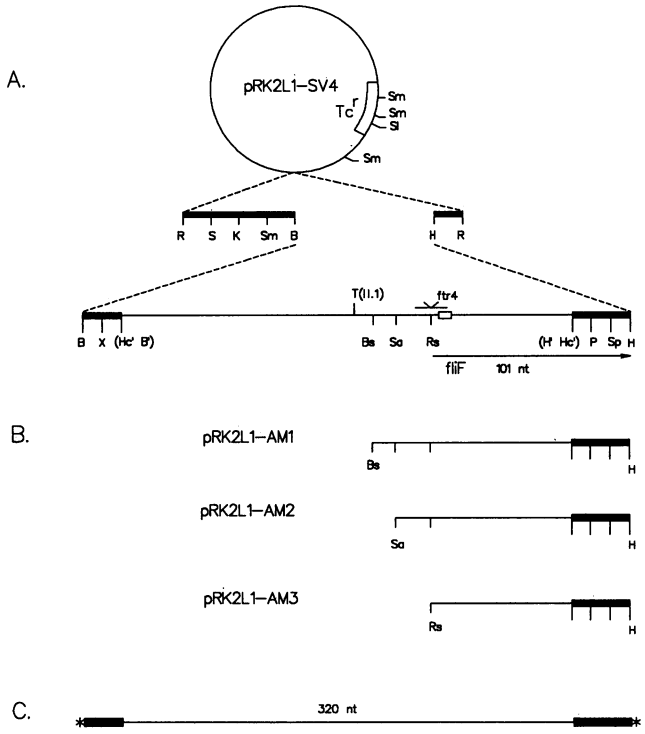

FIG. 3. Physical map of pRK2L1-SV4 and deletion derivatives. (A) Physical map of pRK2L1-SV4. The thin-line circle represents sequences derived from pRK290 (10); the thick lines represent DNA derived from the pUC18 multiple-cloning-site polylinker (45). Restriction sites: Bs, *Bst*UI; Hc, *Hin*cII; K, *Kpn*I; R, *Eco*RI; Rs, *Rsa*I; Sa, *Sau*3AI; Sm, *Sma*I; Sp, *Sph*I. H′ Hc′, a *Hin*cII end ligated to a filled-in *Hin*dIII end; Hc′ B′, a filled-in *Bam*HI end ligated to a *Hin*cII end. Other restriction sites are as indicated in the legend to Fig. 2. The arrow below the sequence indicates the origin and direction of *fliF* transcription, and it corresponds to the 101-nt protected fragment detected in nuclease S1 assays. *ftr4* (□) and the arms of a sequence with dyad symmetry (⌣) are indicated. T(II.1), the approximate location of the 3′ end of the II.1 gene transcript. (B) Restriction fragments from the *fliF* promoter region cloned in pRK2L1. (C) *Bam*HI-*Hin*dIII probe (320 nt) for nuclease S1 assays of pRK2L1-SV4-encoded *fliF* mRNA. *, 5′ ^{32}P-labeled ends.

TABLE 2. Transcriptional activities of 5′ *fliF* mutants determined by CAT assay

fliF promoter	Base change (position)	Activity[a]
Wild type		1.00 ± 0.10
Mutants		
2333	A→C (−37)	0.31 ± 0.07
2362	C→G (−33)	0.22 ± 0.04
59	C→A (−32)	0.72 ± 0.10
33	C→A (−22)	0.50 ± 0.16
2961	T→G (−7)	1.86 ± 0.11
2941	C→T (−6)	1.59 ± 0.15
2930	T→G (−4)	3.80 ± 0.48
2948	C→T (−2)	4.27 ± 0.25
2919	T→G (+1)	1.23 ± 0.11

[a] Relative to wild-type activity.

M2tet broth. Early stationary-phase cultures were diluted 1:100 into 1 liter of M2tet broth and grown to stationary phase (final optical density at 650 nm = 1.2). Cells were centrifuged at 5,000 rpm for 30 min in a Sorvall H-6000A rotor, resuspended in 80 ml of ice-cold M2 broth, mixed with ice-cold ludox (6 ml of ludox [pH 7.5]–10 ml of M2 broth supplemented with 40 μg of dextran T40 per ml plus 10 ml of cells), and spun at 8,000 rpm for 40 min at 4°C in a Sorvall SS34 rotor. Swarmer cells were collected from the gradient, diluted with 15 ml of ice-cold M2 broth, centrifuged at 8,000 rpm for 10 min at 4°C, and then washed twice in 100 ml of ice-cold M2 broth. The cells were diluted into a total volume of 260 ml of M2 broth at 30°C; 25 ml of cells was removed at 30-min intervals and added to a tube containing 200 μg of chloramphenicol per ml, 4 mM sodium azide, and crushed ice; and RNA was extracted immediately. This synchronization technique resulted in greater than 95% swarmer cells, as determined by light microscopy. The quality of the synchrony and progression through the cell cycle was monitored by observing the morphological development of the cells in the culture by light microscopy at 30-min time points, and as an additional control for the quality of the synchrony, we measured the cell cycle patterns of *flaN* and *flbG* transcripts, which are detected only during a short interval after the *fliF* transcript is detected (32).

Primer extension mapping. Primer extension was performed as described previously (40). Five picomoles of the 5′ ^{32}P-labeled synthetic oligodeoxyribonucleotide primer RT1 (5′-GACGCCCGACGCCGAACTGCC) was mixed with 10 μg of *C. crescentus* RNA and heated in a 95°C water bath for 2 min and then at 42°C for 1 h. The primer extension reaction was allowed to proceed for 1 h at 37°C with 20 U of avian myeloblastosis virus reverse transcriptase and 0.5 mM dNTPs. Nucleic acids were phenol extracted, ethanol precipitated, and fractionated by electrophoresis in a 6% polyacrylamide gel next to a dideoxynucleotide sequencing ladder generated by using M13mp19532 single-stranded DNA as a template and the 5′-^{32}P-end-labeled RT1 oligonucleotide as a primer. Primer RT1 anneals at +97 to +117 from the *fliF* transcription start site.

Chloramphenicol acetyltransferase assays. The effects of *fliF* promoter mutations (Table 2) on gene expression were quantified by using promoter-testing plasmid pRK2LM10 (39), which contains a promoterless chloramphenicol acetyltransferase (CAT) gene. Mutations of interest carried on the 320-bp *Bam*HI-*Hin*dIII fragment (Fig. 3A) were ligated between the *Hin*dIII and *Bam*HI sites in pRK2LM10. The *fliF* promoter in these plasmid constructs is oriented so that it may direct transcription of the CAT gene. pRK2LM10 carrying a wild-type or mutant *fliF* promoter was introduced into *C. crescentus* CB15, and the level of CAT protein was measured with a commercially available enzyme-linked immunosorbent assay (ELISA) from Five Prime to Three Prime, Inc., by using a protocol suggested by the manufacturer. All assays were done in duplicate on at least two independent cell extracts for each mutant analyzed. Promoter activity reported in Table 2 was expressed as a fraction of wild-type activity.

Oligonucleotide mutagenesis. Oligonucleotide mutagenesis of the *fliF* operon promoter in M13mp19SV4 was performed essentially as described by Kunkel et al. (20). Mutagenic oligonucleotides were phosphorylated by using ATP and T4 polynucleotide kinase, and the phosphorylated primers were annealed to single-stranded viral M13mp19SV4 DNA isolated from the phage particles after growth on *E. coli* CJ236. After polymerization using T4 DNA polymerase and all four dNTPs and ligation with T4 DNA ligase, the resulting DNA molecules were used to transfect *E. coli* DH5 α. After identifying mutants by nucleotide sequence analysis, double-stranded phage DNAs with mutations were cleaved with *Bam*HI and *Hin*dIII and the promoter-containing fragments were ligated to pRK2L1 or pRK2LM10 cleaved with *Bam*HI and *Hin*dIII. These plasmid constructs were introduced into *C. crescentus* cells by electroporation, and the level of plasmid-encoded *fliF* operon expression was determined.

Nucleotide sequencing. DNA fragments of interest were cloned into M13mp19 double-stranded DNA (45) for dideoxy nucleotide sequencing (41), which was performed by using [α-^{32}P]dATP as the radiolabel, and 7-deazaguanosine 5′-triphosphate was substituted for GTP in the sequencing reactions to reduce compression artifacts (1, 25).

Nucleotide sequence accession number. The nucleotide sequence shown in Fig. 4 is part of a larger sequence that has been filed with GenBank, Los Alamos National Laboratory, under accession number M84717.

RESULTS

Cloning and expression of the *fliF* operon promoter. The chromosomal *fliF* operon transcription start site was previously mapped within the 291-bp *Bam*HI-*Hin*dIII fragment, about 80 bp upstream from the *Hin*dIII restriction site, by using nuclease S1 protection analysis (Fig. 2B) (26), and here we used primer extension analysis to locate the precise 5′ end of the *fliF* operon transcript made in vivo. The primer extension reaction (see Materials and Methods) yielded a single labeled product detected in autoradiograms (Fig. 5A, lane 1), which corresponds to a 5′ end at the T marked +1 in Fig. 4. This result is consistent with the previous nuclease S1 mapping result, and it places the 5′ end of the *fliF* operon transcript 81 bp upstream from the *Hin*dIII site and approximately 82 bp downstream from the major 3′ end of the II.1 gene transcript (28). In subsequent discussion, the T at +1 will be referred to as the transcription start site.

fliF operon transcription in *C. crescentus* was studied by using the 291-bp *Bam*HI-*Hin*dIII DNA fragment (Fig. 2B) cloned in pRK2L1 (27), a derivative of low-copy-number plasmid pRK290 (10). The resulting plasmid, pRK2L1-SV4 (Fig. 3A) was transferred to *C. crescentus* wild-type strain CB15 by electroporation, and the relative level of the plasmid-encoded *fliF* operon transcript was determined by using a nuclease S1 protection assay with the 320-nt *Bam*HI-*Hin*dIII fragment (Fig. 3C) as a probe. As expected, tran-

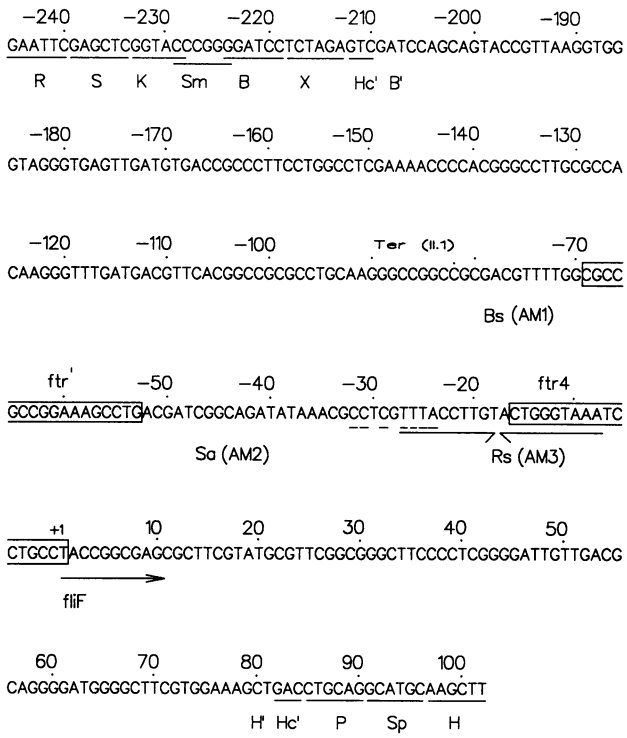

FIG. 4. Nucleotide sequence of the *fliF* operon promoter region in pRK2L1-SV4. The nucleotide sequence shown includes part of the flanking pUC18-derived multiple-cloning-site polylinker (45). Restriction sites are as indicated in the legend to Fig. 3. *ftr*-like sequences are boxed; single-barbed arrows indicate the location of a sequence with dyad symmetry. The double-barbed arrow below the sequence indicates the initiation point and direction of *fliF* transcription. Nucleotides between −24 and −32 that match the conserved −10 sequence element of *E. coli* σ^{32} promoters (6) are underlined. Ter, end of II.1 gene transcript (28).

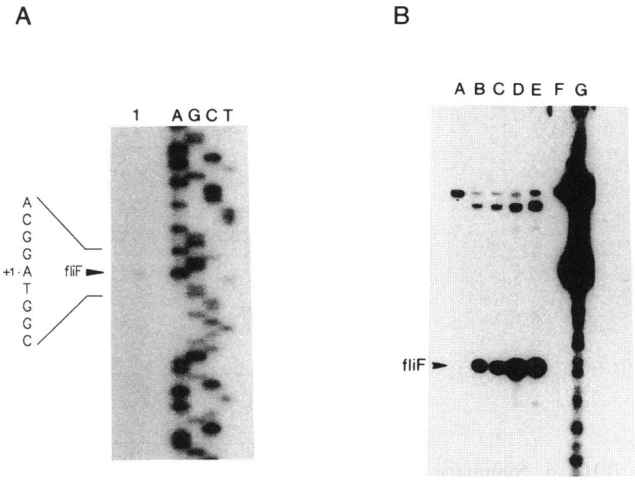

FIG. 5. Transcription start site and genetic requirements for *fliF* expression in pRK2L1-SV4. Total RNA was prepared from a log-phase *C. crescentus* culture, and it was used in nuclease S1 protection assays or primer extension reactions as described in Materials and Methods. (A) The 5′ end of the genomic *fliF* transcript determined by primer extension. +1, the 5′ nucleotide of the *flbF* transcript. (B) Nuclease S1 protection assays. Reaction products in lanes A through G were probed with the 320-nt *Hin*dIII-*Bam*HI DNA fragment (Fig. 3C). The source of RNA in each reaction product is indicated as follows: lane A, CB15(pRK2L1); lane B, CB15(pRK2L1-SV4); lane C, SC511 (*flaK155*::IS*511*)/pRK2L1-SV4; lane D, SC1032 (*flbD198*::Tn*5*)/pRK2L1-SV4; lane E, SC1132 (*flbF177*::Tn*5*)/pRK2L1-SV4; lanes F and G, *Saccharomyces cerevisiae* tRNAs. Nuclease S1 was included in lanes A through F. The 101-nt *fliF* protected fragment is indicated.

scripts from the chromosomal *fliF* operon promoter were not detected in strain CB15 carrying the parental vector pRK2L1 (Fig. 5B, lane A). This assay measures only transcription from the plasmid-borne *fliF* operon promoter as a partially protected 101-nt fragment when RNA from strain CB15(pRK2L1-SV4) is analyzed (Fig. 5B, lane B), because the 5′ ends of the probe were derived from the multiple-cloning-site polylinker in pRK2L1-SV4 (Fig. 3C). The site of transcription initiation determined by this assay is consistent with the location of the initiation nucleotide for the genomic *fliF* operon transcript determined by primer extension. As a control for the amount and integrity of the transcript in each RNA preparation, the level of transcription unit II.1 RNA (Fig. 2A) (28) was routinely measured with a nuclease S1 assay using the 291-nt DNA fragment (Fig. 2B) as a probe. The relative levels of the II.1 transcript were found to be similar in all of the RNA preparations that were compared in this report (data not shown).

Previous studies have shown that the *fliF* operon is negatively regulated by *flbD* and *flbF* (30), and we demonstrated that the cloned *fliF* operon promoter is regulated like the genomic promoter by comparing the relative levels of the plasmid-encoded *fliF* transcript expressed from pRK2L1-SV4 in nonmotile mutants. The level of the plasmid-encoded *fliF* transcript in a *flbD198*::Tn*5* mutant strain was higher than that in wild-type strain CB15 (Fig. 5B; compare lanes B and D), and a *flbF177*::Tn*5* mutant strain also contained an elevated level of the plasmid-encoded *fliF* transcript (Fig. 5B, lane E). The relative level of the plasmid-encoded *fliF* transcript in the *flbF* mutant was reproducibly less than that in the *flbD* mutant strain. The level of the *fliF* transcript from pRK2L1-SV4 in a strain with a *flaK155*::IS*511* mutation (Fig. 5B, lane C) was about the same as that in strain CB15, as expected because *flaK* occupies a level in the *fla* gene regulatory hierarchy below the level occupied by *fliF*. These results suggest that the cloned *fliF* operon promoter is under the same negative regulation as the genomic promoter. The 320-bp *Bam*HI-*Hin*dIII fragment also contains the *cis*-acting sequences necessary for cell cycle regulation of *fliF* operon transcription, as discussed below (see Fig. 8A).

Mutagenesis of the *fliF* operon promoter. The nucleotide sequence near the *fliF* transcription start site has been reported previously (26), and Fig. 4 shows an extended sequence that includes the 287-bp *Bam*HI-*Hin*dIII fragment and flanking polylinker sequence in pRK2L1-SV4. Comparison of the nucleotide sequence near the *fliF* transcription start site to known promoter motifs revealed a sequence centered at −28 (5′-CCTCGTTTA) (Fig. 4) that matches at seven of nine nucleotides the −10 element consensus sequence (CCCCATtTA) for *E. coli* promoters that are transcribed by σ^{32} RNA polymerase (6), although the distance to the 5′ end of the *fliF* transcript detected in vivo is 24 bp rather than the 7 to 8 bp usually observed for *E. coli* σ^{32} promoters. In our initial experiments, mutations were introduced into this sequence, each DNA fragment was cloned into pRK2L1 (see Materials and Methods) and transferred to *C. crescentus* by electroporation, and the relative level of

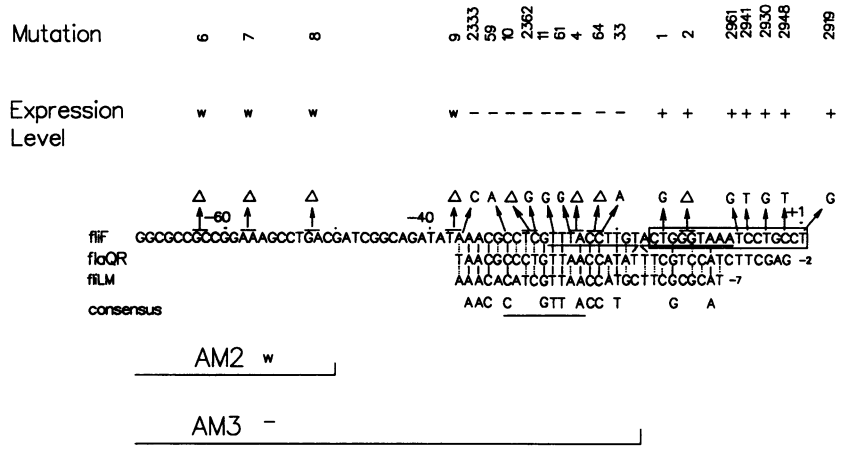

FIG. 6. Summary of primer mutagenesis of the *fliF* promoter region. The positions and nature of mutations created and analyzed in this work are indicated. +1, the *fliF* transcription start site. *ftr4* is boxed. Effects of mutations on the relative level of the *fliF* transcript (summarized from the data in Fig. 7 and Table 2) are indicated as follows: w, wild-type level; −, reduced level; +, increased level. The similarity in nucleotide sequence between the 5′ sequences of *fliF* (*flaO*), *flaQR* (*flaS*), and *fliLM* was shown previously (9, 46) and is shown here in relation to the *fliF* mutations analyzed. The consensus sequence indicates the positions of identical nucleotides found in all three promoter regions, and the region that is similar to the *E. coli* σ^{32}-like −10 sequence in *fliF* is underlined. Lines under AM2 and AM3 indicate the extents of these deletions.

the plasmid-encoded *fliF* transcript was measured by nuclease S1 protection assay.

All of the mutations examined (mutations 10, 11, 61, 4, and 64) (Fig. 6 and Fig. 7a) led to a significant decrease in the level of the *fliF* transcript, and mutations 10 and 11 resulted in almost undetectable levels of expression (Fig. 7a, lanes D and E). Thus, the 9-bp sequence examined here contains nucleotides required for transcription of the *fliF* operon.

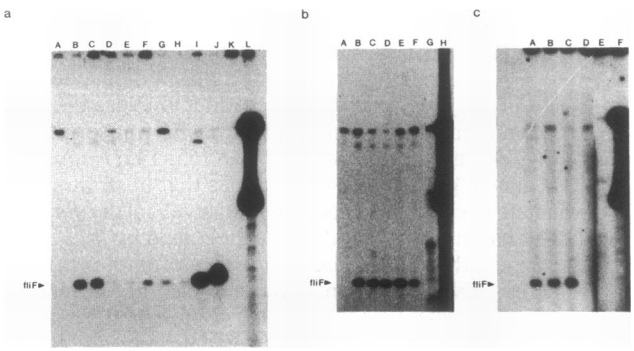

FIG. 7. Nuclease S1 analysis of point mutations and deletions in the *fliF* promoter region. Nuclease S1 protection assays were carried out as described in Materials and Methods. (a) Mutations in the −10 to −28 region. The position of the 101-nt *fliF* protected fragment is indicated. Nuclease S1 was present in lanes A through K. The DNA probe was the 320-nt *Hin*dIII-*Bam*HI fragment labeled at both 5′ ends (Fig. 3C). Lane A, CB15(pRK2L1); lane B, CB15 (pRK2L1-SV4); lane C, CB15(pRK2L1-SV4#9); lane D, CB15 (pRK2L1-SV4#10); lane E, CB15(pRK2L1-SV4#11); lane F, CB15 (pRK2L1-SV4#61); lane G, CB15(pRK2L1-SV4#4); lane H, CB15 (pRK2L1-SV4#64); lane I, CB15(pRK2L1-SV4#1); lane J, CB15 (pRK2L1-SV4#2); lanes K and L, yeast tRNAs. (b) Mutations 5′ from −28. Nuclease S1 was present in lanes A through G. Lane A, CB15(pRK2L1); lane B, CB15(pRK2L1-SV4); lane C, CB15 (pRK2L1-SV4#6); lane D, CB15(pRK2L1-SV4#7); lane E, CB15 (pRK2L1-SV4#8); lane F, CB15(pRK2L1-SV4#9); lanes G and H, yeast tRNAs. (c) Deletion mutations. Nuclease S1 was present in lanes A through E. Lane A, CB15(pRK2L1-SV4); lane B, CB15 (pRK2L1-AM1); lane C, CB15(pRK2L1-AM2); lane D, CB15 (pRK2L1-AM3); lanes E and F, yeast tRNAs.

We also examined the effects of four additional mutations in this region (mutations 2333, 2362, 59, and 33) (Fig. 6) by cloning DNA fragments with mutations in pRK2LM10 (see Materials and Methods), which has a promoterless CAT gene. After transfer to *C. crescentus* by electroporation, *fliF* promoter activity was quantified by measuring the level of CAT protein in log-phase cells by using an ELISA. The data in Table 2 show that all four of the mutations in this region resulted in a decrease in the level of *fliF* expression relative to the level for the wild-type promoter. These results are consistent with the findings described above that were obtained by using nuclease S1 protection assays, and they suggest that nucleotides outside the 9-bp σ^{32}-like −10 sequence are also required for expression of *fliF*.

To determine whether there is a requirement for sequences upstream of −37 for *fliF* expression, 2-bp deletions (mutations 6 to 9) were introduced between −37 and −63 and their effects on the level of the plasmid-encoded *fliF* transcript were determined. When assayed by nuclease S1 analysis, none of these 5′ mutations had an effect on the level of the *fliF* transcript (Fig. 7b; compare lane B with lanes C to F).

The requirement of 5′ sequences was tested further by analyzing the expression of DNA fragments extending from the *Hin*dIII site to the *Bst*UI (AM1), *Sau*3AI (AM2), or *Rsa*I (AM3) site (Fig. 4). pRK2L1-AM2 contains a *fliF* promoter fragment extending from *Hin*dIII to *Sau*3AI (Fig. 3B and 4), and RNA isolated from a strain carrying pRK2L1-AM2 contained a wild-type level of the plasmid-encoded *fliF* transcript (Fig. 7c; compare lanes A and C), suggesting that sequences 5′ to the *Sau*3AI site at −50 are dispensable for *fliF* expression. pRK2L1-AM3 contains a fragment extending from *Hin*dIII to *Rsa*I at −17 (Fig. 3B), and thus, it lacks the essential 9-bp sequence centered at −28 and upstream sequences. As expected, no plasmid-encoded *fliF* transcript was detected in strain CB15(pRK2L1-AM3) (Fig. 7c, lane D).

We previously reported a sequence at −53, indicated as *ftr'* in Fig. 4, that matches at 10 of 17 nucleotides the *ftr* (*fla* gene transcription regulator) consensus sequence, 5'-CNCG GCRAAAMBBGCCG (26, 28). *ftr* elements are *cis*-acting enhancer-like sequences that have been found 3' or 5' from *C. crescentus fla* genes with σ^{54} promoters, and they are required for transcription regulation (24, 26–28). Our results presented here show that *ftr'* is not essential for *fliF* transcription because pRK2L1-AM2 (Fig. 3B) lacks *ftr'* but expresses wild-type levels of plasmid-encoded *fliF* mRNA. A second *ftr*-like sequence that matches at 13 of 17 bases the *ftr* consensus sequence is designated *ftr4* (Fig. 4), and its function is considered in the next section.

***ftr4* is required for negative regulation of *fliF*.** Previous studies have shown that strains with mutations in *flbD* and *flbF* contain elevated levels of the *fliF* transcript, suggesting that the products of these two genes act as negative regulators of transcription (30). We tested the role of nucleotides in *ftr4* (Fig. 6) in negative regulation of *fliF* by introducing mutations and measuring the effects on *fliF* expression. Deletion of the nucleotides at −12 and −13 (mutation 2) and a T-to-G mutation at −15 (mutation 1) both resulted in greatly elevated levels of the *fliF* transcript, as measured with nuclease S1 protection assays (Fig. 7a; compare lane B with lanes I and J). We also introduced five additional mutations in *ftr4* (mutations 2961, 2941, 2930, 2948, and 2919) (Fig. 6), and after cloning the mutated DNA fragments in pRK2LM10, the effects on *fliF* expression were determined by using an ELISA for CAT protein. All five of these mutations resulted in increased levels of *fliF* expression (Table 2), suggesting that nucleotides between +1 and −15, which lie within *ftr4*, are involved in negative regulation of *fliF* transcription.

***ftr4* is required for normal temporal expression of *fliF*.** The temporal order in which *fliF*, *flaN*, *flbG*, and flagellin genes *flgK* and *flgL* are expressed in the cell cycle corresponds to their relative positions in the *fla* gene regulatory hierarchy (Fig. 1) (24, 32). Expression of these *fla* genes is under strict temporal regulation, as indicated by the discrete interval of *fliF* expression that begins shortly before the onset of *flaN* and *flbG* transcription (32). This observed order of gene expression is consistent with the finding that FlbD protein, the product of the last gene in the *fliF* operon, is an activator of *flaN* and *flbG* transcription (4, 26, 33, 36).

The temporal pattern of plasmid-encoded *fliF* mRNA was determined by preparing a synchronized culture of CB15F(pRK2L1-SV4) swarmer cells by density gradient centrifugation (12), and at 30-min intervals throughout the cell cycle, RNA was isolated and the relative levels of plasmid-borne *fliF* transcripts were assayed by nuclease S1 protection. We also measured genomic *flaN* and *flbG* expression, because their expression patterns provide well-characterized markers for relative time in the cell cycle. The cell cycle patterns of *flaN* and *flbG* operon transcription in CB15F(pRK2L1-SV4) are shown in Fig. 8C and D, respectively, and they match the previously published temporal patterns, because both transcripts were periodically detected beginning at about 0.62 division units of the cell cycle (4, 32). Fig. 8A shows that the plasmid-encoded *fliF* transcript from pRK2L1-SV4 was expressed during a discrete interval that began at about 0.43 division units of the cell cycle, shortly before *flaN* and *flbG* mRNAs were detected (Fig. 8C and D). This demonstrates that the plasmid-encoded *fliF* transcript made by pRK2L1-SV4 is expressed with the same cell cycle pattern as the genomic *fliF* transcript examined previously (32).

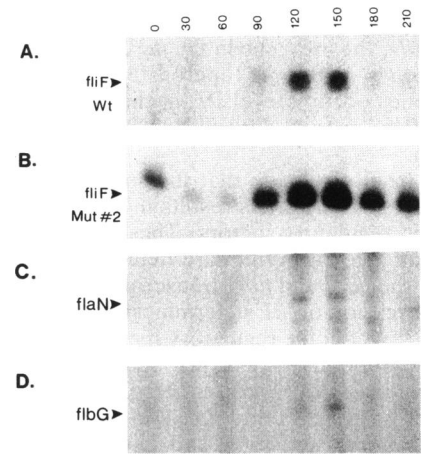

FIG. 8. Temporal pattern of plasmid-encoded *fliF* operon transcription. Nuclease S1 protection assays were carried out as described in Materials and Methods. The probe for genomic *flaN* and *flbG* was the 612-nt *Pst*I fragment (Fig. 2B). The probe for plasmid-encoded *fliF* was the 320-nt *Hin*dIII-*Bam*HI fragment (Fig. 3C). Time (in minutes) in the cell cycle is indicated at the top. Cell division was monitored by microscopic observation, and it occurred between 180 and 210 min. (A) Temporal pattern of plasmid-encoded *fliF* expression in CB15(pRK2L1-SV4). Wt, wild type. (B) Temporal pattern of plasmid-encoded *fliF* transcription in CB15(pRK2L1-SV4#2). Mut #2, mutation 2. (C) Temporal pattern of genomic *flaN* transcription in CB15(pRK2L1-SV4). (D) Temporal pattern of genomic *flbG* transcription in CB15(pRK2L1-SV4).

We examined the effect of mutation 2, which resulted in an elevated level of the *fliF* transcript (Fig. 7a, lane J), on the temporal pattern of the plasmid-encoded *fliF* transcript in synchronized *C. crescentus* cells to determine whether the nucleotides at −12 and −13 in *ftr4* (Fig. 6) play a role in timing of *fliF* expression. The temporal pattern of genomic *flaN* and *flbG* transcription was also measured for CB15F(pRK2L1-SV4#2), and it was identical to that shown in Fig. 8C and D (data not shown). Fig. 8A and B show the cell cycle regulation of plasmid-encoded *fliF* transcripts expressed from pRK2L1-SV4 and pRK2L1-SV4#2, respectively. Although the levels of the *fliF* transcripts expressed from both of these plasmids varied periodically and peak transcript levels were detected at approximately the same time in the cell cycle, the plasmid-encoded *fliF* transcript expressed from pRK2L1-SV4#2 was made in increased amounts and the interval during which it was detected was extended beyond the relatively restricted interval of expression from the wild-type *fliF* promoter on plasmid pRK2L1-SV4. Most notably, *fliF* expression apparently continues after cell division, because a significant amount of the transcript was present in the new swarmer cells (0 min) while it is never detected in cells with a wild-type *fliF* promoter. This result suggests that the nucleotides at −12 and −13 in *ftr4* may constitute part of a molecular switch that is required to turn off transcription of *fliF* at the correct time in the cell cycle.

DISCUSSION

The *C. crescentus fla* genes are organized into a regulatory hierarchy, and *flbF* and the *fliF* operon genes are near the top of the *fla* gene regulatory hierarchy (level II) along with *flaQR* and *fliLM* (4, 30, 46). *flaN* and *flbG* are classified with

the level III genes because their transcription depends on *flbF* and *flbD*, the last gene in the *fliF* operon (26, 30) as well as other genes in the *fliF* operon (38). The *flgK* and *flgL* flagellin genes occupy level IV, at the bottom of the hierarchy, and they in turn depend on level III genes for their expression.

Several results indicate that the sequential expression of the *C. crescentus fla* genes results in part from a cascade of *trans*-acting regulatory factors. These include findings that the *flbF* and *fliF* transcripts are detected earlier in the cell cycle than the *flaN* and *flbG* transcripts (32, 37) and that the time of expression of *fliF* determines the time of expression of genes below it in the regulatory hierarchy, as indicated by the observation that delaying *fliF* operon expression caused a corresponding delay in expression of the *flbG* operon genes and flagellin genes *flgK* and *flgL* (32). *fliF* is also negatively regulated by *flbD* and *flbF*, and like the other known *C. crescentus fla* genes, it is periodically expressed in the cell cycle (30, 32). Elucidating the molecular mechanism of *fliF* operon regulation is, thus, an important step toward understanding the temporal regulation of *fla* gene expression in *C. crescentus*.

Characterization of the *fliF* promoter. We pointed out previously that the nucleotide sequence 5′ of the *fliF* operon was different from that of the σ^{54} promoters that are required for expression of genes at levels II and III of the regulatory hierarchy (26). It is also different from the promoter sequence of *flbF* (37, 40). In this study, we have presented new results mapping the precise 5′ end of the *fliF* operon transcript made in vivo and we reported the results of initial experiments to investigate the *cis*-acting sequences needed for expression of *fliF* and its temporal regulation in the cell cycle. We identified two sequences that are required for the periodic expression, and one of these is also required for the negative autoregulation of this transcription unit. Together, these regulatory sequences appear to be essential for the normal cell cycle-regulated expression of *fliF*.

Our results indicate that one element required for *fliF* expression is included in a sequence extending from −37 to approximately −17, and we propose that this sequence contains all or part of the *fliF* promoter. Results supporting this proposition are as follows: (i) sequences upstream of the *Sau*3AI site at −50 (Fig. 4) are not required, because the *Sau*3AI-*Hin*dIII fragment in pRK2L1-AM2 expressed normal levels of the *fliF* transcript and contains all of the elements needed for the normal expression of the transcription unit (Fig. 7c); (ii) neither the 2-bp deletion at −37 and −38 (mutation 9) nor those upstream (mutations 6 to 8) alter the level of the *fliF* transcript (Fig. 7a); and (iii) single base changes and 2-bp deletions in the −34 to −22 sequence abolish or severely decrease expression. Thus, the −37 to −17 sequence, which contains nucleotides required for *fliF* expression, is defined at the 5′ end by a sequence in which 2-bp deletions did not alter expression and downstream by the *ftr4* sequence discussed below, which appears to be required for negative regulation of *fliF*.

In Fig. 6, we have aligned the 5′ sequence of *fliF* with the corresponding sequences of *flaQR* and *fliLM*, which are also level II genes (Fig. 1) (9, 32, 46). In addition to being expressed early, these three transcription units are also expressed during the same interval in the cell cycle, between about 0.43 and 0.90 division units (9, 32, 46). The similarity of these sequences, which has been pointed out by Dingwall et al. (9), suggests the possibility that these genes contain similar promoters, and the results presented here are consistent with this conclusion. Several of the residues shown to be required for transcription from the *fliF* promoter (Fig. 6) are conserved in the sequences 5′ of *fliLM* and *flaQR*, suggesting that the corresponding sequences may also be important for expression of *fliLM* and *flaQR*.

Although a sequence within the *fliF* operon 5′ region is similar to the consensus −10 element of σ^{32} promoters of enteric bacteria (6) and residues within this sequence are required for *fliF* expression, several observations suggest that it is unlikely that this transcription unit is expressed from a σ^{32} promoter: (i) the 5′ sequence of *fliF* lacks a properly spaced σ^{32}-like −35 sequence element, and deletion of the region that would spatially correspond to the −35 element had no effect on transcript levels; (ii) *fliF* expression was not induced by a heat shock in *E. coli* or *C. crescentus* (data not shown); (iii) purified σ^{32} RNA polymerase from *E. coli* did not recognize the *fliF* promoter in vitro (data not shown); and (iv) two mutations 3′ of the σ^{32}-like −10 sequence (mutations 64 and 33) also result in reduced levels of the *fliF* transcript, as did one mutation 5′ from the σ^{32}-like −10 element (mutation 2333) (Fig. 7a and Table 2). Thus, the *fliF* operon contains a new class of promoter different from the σ^{54} promoter, as suggested previously by Mullin et al. (26), and this promoter appears to be shared by at least two other *C. crescentus* transcription units, *fliLM* and *flaQR* (9, 46). Further mutagenesis experiments and in vitro transcription studies will be required to define more precisely the exact nature of the *fliF* promoter.

Although *fliF*, *flaQR*, and *fliLM* may share a common promoter sequence, this sequence is not shared by all genes near the top of the *C. crescentus fla* gene regulatory hierarchy. Previous studies have shown that *flbF*, which is expressed before *fliF* in the cell cycle (36) but is not required for *fliF* expression, has a different promoter sequence (37, 40). Thus, this and previous work have defined at least three different classes of promoters that are responsible for expression of genes at different levels of the *C. crescentus fla* gene regulatory hierarchy. Flagellin genes *flgK* and *flgL* (level IV) and genes *flaN*, *flbG*, and *flbN* (level III) have σ^{54}-like promoters (8, 24, 26, 27), while *flbF* and *fliF* (level II) have essential 5′ sequences that differ from each other and from the σ^{54} promoters (37, 40). The *flbF* 5′ region contains sequences that are similar to the conserved −10 and −35 sequence elements found in *E. coli* and *Bacillus subtilis* σ^{28} promoters, and mutations in these sequences reduce or abolish *flbF* expression (39, 40).

It is possible that the *fliF* promoter sequence is centered at about −25, as indicated by our mutagenesis results. Another possibility is that the *fliF* transcript is processed and that the transcription start site is 5′ of the site mapped in this work by primer extension. In the case of at least two *Caulobacter* operons, for which the transcription start sites can be convincingly inferred from genetic data and in vitro transcription results, processed transcripts with discrete single (*flaN*) or multiple (*flbG*) 5′ mRNA ends have been mapped by nuclease S1 assays (4, 31).

Characterization of negative regulation by *ftr4*. The second sequence identified by these studies, *ftr4*, appears to be required for the previously reported negative autoregulation of *fliF* (30), because seven mutations in *ftr4* resulted in elevated levels of *fliF* expression (Fig. 6). The *ftr4* sequence extends from +1 to −16, and if significant processing of the *fliF* transcript occurred and the start site were 5′ to the one proposed, then it could be argued that the effect of *ftr4* mutations on levels of *fliF* mRNA might have resulted from enhanced stability of the transcript and not regulation of expression. This seems unlikely for two reasons. First, if

mutations 1 and 2 result in elevated production of *fliF* due to enhanced transcript stability, then the mutant transcripts should be longer at their 5′ ends; however, primer extension analysis has demonstrated that the mutant and wild-type transcripts have the same length. Second, the levels of transcript from the *fliF* operon carrying mutations 1 and 2 were not increased in a *flbD* mutant strain (data not shown), as would be expected if mutations 1 and 2 enhanced transcript stability. This second result is also consistent with the conclusion that *ftr4* is, in fact, the site of negative autoregulation exerted by *flbD* (30).

The FlbD amino acid sequence and organization of predicted functional motifs are very similar to those of the transcriptional regulatory protein, NtrC (36). Both proteins have a C-terminal helix-turn-helix DNA-binding motif, and NtrC protein depends upon this sequence for its DNA-binding activity (5). Thus, FlbD could act directly as a transcriptional repressor of *fliF*, but there is no direct evidence for this activity.

We previously identified a sequence at −53 in *fliF* that was designated *ftr′* because of its similarity to the 17-bp *ftr* consensus sequence, 5′-CNCGGCRAAAMBBGCCG. The enhancer-like *ftr* elements were first discovered in a variety of locations upstream or downstream of *fla* genes with σ^{54} promoters (26). For example, *ftr1* at −100 from the *flbG* transcription start is required for *flbG* expression and for the negative regulation of *flaN* (27), while *ftr2* at +86 from the *flaN* transcription start site is required for *flaN* transcription and *ftr3* at +120 in the same transcription unit appears to be a negative regulator of both *flaN* and *flbG* expression (28). The results presented here, however, show that *ftr′* at −53 is not required for *fliF* expression, because pRK2L1-AM2 (Fig. 3) lacks this sequence but expresses wild-type levels of *fliF* transcript. Inspection of the *fliF* promoter region revealed another *ftr*-like element, designated here *ftr4* (Fig. 6), that matches at 13 of 17 bp the *ftr* consensus sequence, and two different 2-bp deletions and six point mutations in *ftr4* resulted in elevated levels of *fliF* expression. Given the functions of *ftr1* and *ftr3* in negative regulation of transcription (27, 28) and the effects of *ftr4* mutations on *fliF* expression, it seems likely that *ftr4* also functions as a negative transcription regulator.

Role of *ftr4* in temporal expression of the *fliF* operon. The pattern of *fliF* transcription in synchronously dividing cells carrying pRK2L1-SV4 with *ftr4* mutation 2 suggests that this negative control element may be involved in the temporal regulation of *fliF* expression. Normally *fliF* mRNA is detected only during a brief interval in the cell division cycle, beginning just before *flaN* and *flbG* expression (32); however, mutation 2 resulted in a significant extension of the period of *fliF* transcription, since expression that is normally terminated before cell separation continued after division in the new swarmer cell. This finding suggests to us that deletion of the nucleotides at −12 and −13 in *ftr4* may have damaged a sequence that forms part of a developmental switch which is required to turn off *fliF* transcription at the correct time in the cell cycle. This may represent a general mechanism of shutting off *fla* gene expression in *C. crescentus*. Previous work has shown that some mutations in the polyhook gene, *flaJ*, result in increased expression of the hook protein and also extend the period of *flbG* (hook) operon expression because of a failure to turn off gene expression at the normal time in the cell cycle (43).

ACKNOWLEDGMENTS

We thank L. Shapiro and B. Ely for discussions about the organization and assignment of gene class in the *Caulobacter* hierarchy, and we thank C. Gross for generously providing *E. coli* σ^{32} RNA polymerase for in vitro transcription experiments. Ludox used in the cell synchrony experiments was provided by the DuPont Chemical company. This work was supported by grants to D.A.M. from the Board of Regents of the State of Louisiana LEQSF(87-90)-RD-A-19 and the Newcomb College Foundation and PHS grant GM22299 to A.N. from the National Institutes of Health.

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