# Global Regulation of a $\sigma^{54}$ -Dependent Flagellar Gene Family in Caulobacter crescentus by the Transcriptional Activator FlbD

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Biosynthesis of the Caulobacter crescentus polar flagellum requires the expression of a large number of flagellar (fla) genes that are organized in a regulatory hierarchy of four classes (I to IV). The timing of fla gene expression in the cell cycle is determined by specialized forms of RNA polymerase and the appearance and/or activation of regulatory proteins. Here we report an investigation of the role of the C. crescentus transcriptional regulatory protein FlbD in the activation of  $\sigma^{54}$ -dependent class III and class IV fla genes of the hierarchy by reconstituting transcription from these promoters in vitro. Our results demonstrate that transcription from promoters of the class III genes flbG, flgF, and flgI and the class IV gene fljK by  $Escherichia\ coli\ E\sigma^{54}$  is activated by FlbD or the mutant protein FlbD<sup>S140F</sup> (where S140F denotes an S-to-F mutation at position 140), which we show here has a higher potential for transcriptional activation. In vitro studies of the flbG promoter have shown previously that transcriptional activation by the FlbD protein requires ftr (ftr for flagellar transcription regulation) sequence elements. We have now identified multiple ftr sequences that are conserved in both sequence and spatial architecture in all known class III and class IV promoters. These newly identified ftr elements are positioned ca. 100 bp from the transcription start sites of each  $\sigma^{54}$ -dependent fla gene promoter, and our studies indicate that they play an important role in controlling the levels of transcription from different class III and class IV promoters. We have also used mutational analysis to show that the ftr sequences are required for full activation by the FlbD protein both in vitro and in vivo. Thus, our results suggest that FlbD, which is encoded by the class II flbD gene, is a global regulator that activates the cell cycle-regulated transcription from all identified  $\sigma^{54}$ -dependent promoters in the *C. crescentus fla* gene hierarchy.

The dimorphic bacterium Caulobacter crescentus generates two functionally and structurally distinct progeny cells after each cell division: a motile swarmer cell with a single polar flagellum and a sessile stalked cell. The new swarmer cell is formed during division of the stalked cell and requires the sequential assembly of surface structures during the cell cycle at the stalk-distal pole, including a single flagellum. The C. crescentus flagellum, like other bacterial flagella, can be divided into three basic parts: the basal body, a short curved structure called the hook, and the external flagellar filament (22). Flagellum biosynthesis requires the activity of ca. 50 flagellar (fla) genes that have been assigned to one of four classes (I to IV) arranged in a regulatory hierarchy on the basis of their time of expression in the cell cycle, promoter architecture, and results of epistasis experiments. The flagellum is assembled from the inside of the cell to the outside in the order basal body-hook-flagellar filament, and the sequence of fla gene expression in the C. crescentus cell cycle reflects this order. Thus, the class II MS-ring and switch protein genes are expressed early, and their expression is required in turn for expression of the class III hook protein gene and of the class IV flagellin genes later in the cell cycle (for reviews, see references 6 and 33).

Flagellum biosynthesis and flagellar gene expression in *C. crescentus* are coupled in some manner to DNA synthesis (41, 50), and more recent results indicate that chromosome replication is required for transcription of the class II genes, which are near the top of the hierarchy (13, 52). The class I genes, although not yet identified, are presumed to encode proteins that respond directly to cell division cycle signals and to be

required in turn for expression of class II genes. Class II genes encode the first components of the flagellar basal body, including proteins for flagellar protein-specific export (13, 44, 45, 62) and regulatory proteins required for the expression of class III and class IV genes (34, 43, 61). Class III genes, which encode the components of the outer basal body and hook, are required for expression of the class IV flagellin genes (11, 12, 18, 29, 40).

Extensive analyses of promoter sequences and genetic and biochemical studies of *fla* gene regulation have shown that *fla* genes at different classes in the genetic hierarchy are transcribed by specialized forms of RNA polymerase. Class II genes contain at least two promoter types, those of class IIA and class IIB. Class IIB genes, including *fliF* (54), *fliQ* (13), *fliL* (52, 62), and *rpoN* (2), share a common promoter consensus that is unlike that of other bacterial promoters and may be recognized by a specialized activator or form of RNA polymerase (3, 45, 52). The sequence of the class IIA promoter of the *flhA* is not similar to the class IIB promoter consensus (44, 48), and it is presumably recognized by another specialized form of RNA polymerase (3, 45).

The class III and class IV fla gene promoters at the bottom of the hierarchy have been the most extensively studied. DNA sequence analysis originally indicated that they are  $\sigma^{54}$ -dependent promoters (see Table 1) (9, 26, 28). This conclusion was supported by extensive site-directed mutagenesis of the class III flbG and flgK promoters, which demonstrated that the conserved sequences and spacing characteristic of this promoter class are absolutely required for their transcription both in vivo (30) and in vitro (36). More recently, it has been demonstrated that the rpoN gene, which encodes  $\sigma^{54}$ , is required for class III (2, 7) and class IV (59) fla gene expression.

The enteric  $E\sigma^{54}$  ( $\sigma^{54}$ -containing RNA polymerase) is

The enteric  $E\sigma^{54}$  ( $\sigma^{54}$ -containing RNA polymerase) is unique among prokaryotic RNA polymerase holoenzymes, in that formation of transcription-competent open complexes de-

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pends on the function of activation proteins that typically bind to specific DNA sequence elements located upstream of the transcriptional start site (for reviews, see references 20 and 23). The *cis*-acting *ftr* sequences (*ftr* for flagellar transcription regulation) with an apparently similar function were originally identified ca. 100 bp from the transcription start sites of the class III *ftbG* and *ftgK* promoters and of the class IV *ftjK* and *ftjL* flagellin promoters (26, 28). The requirement of these sequence elements for transcription from the class III *ftbG* and *ftgK* genes was subsequently demonstrated both in vivo (15, 30, 31) and in vitro (3).

Genetic evidence has shown that the flbD gene, identified by Ramakrishnan and Newton (43), is essential for transcription of the fla genes from  $\sigma^{54}$ -dependent promoters (34, 43). Its predicted product, FlbD, is homologous to NtrC and a family of response regulators known to activate transcription by  $E\sigma^{54}$ in Escherichia coli, Salmonella typhimurium, Rhizobium melliloti, and Pseudomonas putida (for reviews, see references 20, 23, and 57). Recent studies of the flbG promoter have demonstrated that FlbD is an ftr-specific DNA-binding protein that contacts symmetrical guanine residues in the ftr1 and ftr1\* sequence elements (3, 4). The FlbD protein was also shown to make similar contacts with ftr4 (54), a DNA sequence which overlaps the fliF promoter (3). Comparable observations of FlbD binding at ftr sequences have been made with C-terminal fragments of FlbD containing the helix-turn-helix domain (32) and a poly-His-tagged FlbD derivative (58). By reconstituting transcription in vitro with highly purified proteins, work from this laboratory has demonstrated directly that the FlbD protein activates transcription from the class III flbG promoter and represses transcription from the class II fliF promoter by its interactions with ftr sequence elements that have previously been shown genetically to be essential for the regulation of flbG (ftr1) and fliF (ftr4) expression (3).

Other conserved sequences, designated RF-2 and RF-3, upstream from class III genes flgH, flgI, and flgF have also been described (6, 11). Although the impact of these putative cisacting sequences on transcription has not been studied in detail, their identification raises the question of whether activation by FlbD at ftr sequence elements, as described above for flbG (3, 4), is necessary and sufficient for regulation of all class III and class IV genes. To address this question, we have extended our studies of flbG to other  $\sigma^{54}$ -dependent class III and class IV promoters to determine the requirement of FlbD and ftr sequences for transcriptional activation. We have found that all class III transcription units described to date and the class IV genes, which encode the 25- and 27-kDa flagellins, contain multiple ftr sequences that are positioned ca. 100 bp from the transcription start sites. We have also used a reconstituted in vitro transcription system containing E. coli  $E\sigma^{54}$  to examine in detail the class III flgI, flgF, and flbG promoters and the class IV fljK promoter. Our results demonstrate that FlbD activates transcription from each of these promoters and that full activation by FlbD both in vitro and in vivo requires an intact pair of ftr sequences. These results lead us to propose that FlbD globally regulates transcription from all  $\sigma^{54}$ -dependent class III and class IV fla gene promoters and that its function is mediated by interaction with the cis-acting ftr sequence elements.

### MATERIALS AND METHODS

**Site-directed mutagenesis of flbD.** The flbD mutation encoding FlbD<sup>S140F</sup> was constructed with the Sall-SacII fragment from pGIR132, which encodes the central domain of FlbD from C. crescentus. The fragment was ligated into the mutagenesis vector pALTER-1 (Promega) to generate pJW102. It was then mutated by site-directed mutagenesis with the oligonucleotide MJW-1: CCAG

GTCGCCCCTTCGAAGCCTCG. This changed the codon for Ser-140 to a codon for phenylalanine and introduced a *BstBI* site to the resultant plasmid pMJW1.

Construction of vector for protein expression. The wild-type FlbD protein was overexpressed in *E. coli* by using plasmid pGIR168 as described previously (3, 4). pGIR168 is a derivative of pAR3039/pET3-b in which a 2-kb *Bam*HI-*Eco*RV fragment containing the entire *flbD* gene is fused transcriptionally to the gene 10 promoter of bacteriophage T7.

The plasmid used for overproduction of the mutant FlbD, FlbD<sup>S140F</sup>, was constructed as follows. The *NcoI* fragment of pGIR168 containing the wild-type *flbD* gene was replaced by the *NcoI* fragment from pMJW1 containing the S140F mutation. The resultant plasmid, pJW201, like pGIR168, contains the entire *flbD* gene on a 2-kb *Bam*HI-*EcoR*V fragment that was fused transcriptionally to the gene 10 promoter of bacteriophage T7.

Protein overexpression and purification. Plasmids pGIR168 and pJW201 were transformed separately into *E. coli* BL21 (DE3) to overproduce the FlbD<sup>WT</sup> (WT, wild type) and FlbD<sup>S140F</sup> (S140F denotes an S-to-F mutation at position 140) proteins, respectively. Cells were grown in ML-Amp100 liquid medium at 37°C overnight. Four liters of fresh medium was inoculated with 40 ml of the overnight cultures and then incubated at 37°C. When the optical density at 600 nm reached ca. 1.0, the cultures were induced to overproduce proteins with PTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 1 mM. The cells were harvested and washed with TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA). The proteins were purified as described previously (3). This procedure yielded FlbD protein that was greater than 95% pure, as judged by Coomassie blue staining of sodium dodecyl sulfate-polyacrylamide gels. The purified protein was dialyzed against TGED (20 mM Tris [pH 7.9], 5% glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol) in 50% glycerol and stored at −80°C.

Mutation of ftr5, ftr5\*, and ftr5\*\*. A 280-bp EcoRI fragment which contains the ftgF promoter region was generated by PCR and introduced into the EcoRI site of pBluescript II to yield pAKC26. The identification and orientation of the insert were verified by dideoxy sequencing. The 280-bp ftgF from pAKC26 was cloned as a BamHI-HindIII fragment into the BamHI-HindIII sites of the mutagenesis vector pALTER-1 to yield pAKC27. It was then mutated by site-directed mutagenesis with the following three oligonucleotides: (i) FTR5-A, AATCTGCCGTCAGAGTCCG, which makes a transversion from G to T at position –92 of ftr5 to yield pJWMF1; (ii) FTR5-B, TGGTCGCAGTCTGAATCGCC, introducing the transversion from G to T at position –106 of ftr5\* to yield pJWMF2; and (iii) FTR5-C, TTTTGCCGGTCAATTGGTCG, introducing the transversion from G to T at position –120 of ftr5\* to yield pJWMF3. The presence of mutations was confirmed by dideoxy sequencing.

ftr7 and ftr7\* mutations. The fljK promoter was isolated from a 1.1-kb EcoRI fragment derived from pCN200 (27) and was inserted into the EcoRI site of pUC18, to generate pAKC7. Then the 520-bp PsII fragment of pAKC7 was subcloned into the PsII site of the mutagenesis vector pALTER-1, to yield pAKC12. The orientation of the insert was confirmed by restriction enzyme digestion. It was then mutated by site-directed mutagenesis to make single nucleotide changes with the following oligonucleotides: FTR7-A, AGGCTC TCGTCAGAAATTGC, which introduces a transversion from G to T at position –101 of ftr7 to yield pJWMK1; FTR7-B, AGAAATTGCTGGGAGCCTTC, which makes a transition from G to A at position –112 of ftr7 to generate pJWMK2; and FTR7-A\*, CAGATAGTGCCGTCAACAATTTCCCA, which introduces a transversion from G to T at position –140 of ftr7\* to produce pJWMK3. The mutations were verified by dideoxy sequencing.

Construction of templates for transcription assays. All plasmids used for in vitro transcription assays in this study were derived from plasmid pTE103, which contains the pUC8 multiple cloning sites that are positioned 215 bp upstream of a strong *rho*-independent transcription terminator of bacteriophage T7 (14).

(i) flbG promoter. The plasmid pNJ5 (3, 4, 36) carries a 650-bp HindIII-EcoRI fragment containing the flbG promoter cloned into the HindIII and EcoRI sites of pTE103. The transcriptional start site for the flbG operon is positioned 365 bp upstream from the T7 terminator (36) (see Fig. 3).

(ii) flgF promoter and mutant fir elements. A 280-bp BamHI-HindIII fragment containing the flgF promoter and its wild-type ftr elements from pAKC27 was subcloned into the BamHI and HindIII sites of pTE103, to generate pAKC28. In pAKC28, the transcription initiation site of flgF is located 336 bp upstream of the T7 termination site. The BamHI-HindIII fragments containing mutant ftr5, ftr5\*, or ftr5\*\* sequences were constructed from plasmids pJWMF1, pJWMF2, and pJWMF3 by subcloning 280-bp BamHI-HindIII fragments into the BamHI-HindIII sites of pTE103, to generate plasmids pJWF1, pJWF2, and pJWF3, respectively. In these three plasmids, as in pAKC28, the flgF transcriptional initiation sites are positioned 336 bp upstream of the T7 terminator (see Fig. 3 and 4).

(iii) fljK promoter and mutant ftr elements. Plasmid pAKC8 was constructed by insertion of the 520-bp PstI fragment of pAKC7 containing the fljK promoter into the PstI site of pTE103. The orientation of the insert was confirmed by digestion with the restriction enzyme EcoRI. Plasmids containing the mutant ftr7 or ftr7\* site were constructed by insertion of the PstI fragments from pJWMK1, pJWMK2, and pJWMK3 into pTE103, to generate pJWK1, pJWK2, and pJWK3, respectively. In pAKC8, as well as in pJWK1, pJWK2, and pJWK3, the fljK transcriptional start site lies 451 bp upstream of the T7 termination site (see Fig. 3 and 5).

(iv) flgI promoter. pAKC30 was constructed as follows. A 310-bp EcoRI fragment containing the flgI promoter region was generated by PCR and cloned into the EcoRI site of pBluescript II, to yield the plasmid pAKC29. The identification and orientation of the insert were confirmed by dideoxy sequencing. The BamHI-HindIII fragment from pAKC29 was subcloned into the BamHI and HindIII sites of pTE103, to generate pAKC30. In pAKC30, the flgI transcriptional initiation site was placed 368 bp upstream of the T7 terminator (see Fig. 3).

Transcription assays. The formation of heparin-resistant open complexes at the  $\sigma^{54}$ -dependent promoters was measured in single-cycle transcription assays as described previously (3, 4, 37). The transcription assay was performed at 34°C in a total volume of 50 μl in transcription buffer containing 50 mM Tris-HCl (pH 7.8), 100 mM NaCl, 1 mM dithiothreitol, 200 μg of bovine serum albumin per ml, 0.1 mM EDTA, and 10 mM MgCl<sub>2</sub>. Purified reagents were added to the final concentrations indicated as follows: *E. coli* core RNA polymerase (100 nM), *E. coli*  $\sigma^{54}$  (200 nM), NtrC (400 nM), NtrB (160 nM), and supercoiled plasmid template (5 nM). The concentrations of the FlbD<sup>WT</sup> and FlbD<sup>S140F</sup> proteins were varied as indicated in individual experiments. End-labeled *Sau*3A fragments of pUC18 were used as DNA size markers.

Construction of fla promoter-lacZ fusions. The lacZ reporter gene fusions were constructed by subcloning fla gene promoters into pANZ5 (38), which contains a promoterless lacZ reporter gene in a plasmid capable of autonomous replication in C. crescentus. DNA fragments containing the flgF promoter region with wild-type ftr elements or with mutant ftr5, ftr5\*, or ftr5\*\* were subcloned into the Kpn1 and Xba1 sites of pANZ5, to generate transcriptional fusion vectors pJWFZ, pJWF1Z, pJWF2Z, and pJWF3Z, respectively. The fljK promoter was subcloned as a PsrI fragment from pAKC8 into the PsrI site of pANZ3, to yield the fljKp-lacZ fusion plasmid pJWKZ. The promoter fragment and construction of the flbGp-lacZ fusion plasmid pANZ405 have been described previously (34). A Kpn1-Xba1 fragment containing the flgI gene promoter from plasmid pAKC30 was subcloned into the KpnI and XbaI sites of pANZ5, to yield the flgIp-lacZ fusion plasmid pJWIZ.

**β-Galactosidase activity assays.** *C. crescentus* strains containing *fla* promoter*lacZ* fusion plasmids were grown to mid-logarithmic phase (optical density at 600 nm, 0.3 to 0.7) in PYE medium (40). Assays of  $\beta$ -galactosidase activity were performed according to the method of Miller (24).

Construction of fla promoter-xylE fusions. Wild-type and ftr-mutant fljK promoter fragments were fused transcriptionally to a promoterless xylE reporter gene by cloning the PstI-SalI fragments from the wild-type pAKC8 and from mutants pJWK1, pJWK2, and pJWK3 into the PstI-XhoI sites of plasmid pRKX-1. This created plasmids pAKC81 (wild type), pAKC84 (ftr7 [C101T]), pAKC85 (ftr7 [G112A]), and pAKC86 (ftr7\* [G140T]), respectively. The parental pRKX-1 plasmid carries a 1.5-kb DNA fragment containing the xylE gene (19) that was inserted into the unique BamHI site of pRK2L10.

**XylE activity assays.** XylE activity was measured in cell extracts prepared from logarithmically growing cells. Cells were grown in M2 minimal medium (40), harvested by centrifugation, resuspended in 50 mM potassium phosphate buffer, pH 7.5, containing 100 mM KCl, and lysed by passage through a French pressure cell. Cellular debris was removed by centrifugation, and the extracts were assayed for protein concentration with the Bio-Rad protein assay kit. Assays were performed on equal amounts of protein at 30°C as described by Konyecsni and Deretic (19).

# **RESULTS**

Organization of ftr sequences in class III and class IV fla genes. Recent in vitro transcription results have shown that the C. crescentus FlbD protein, which is homologous to the NtrC family of bacterial response regulators (43), can activate the transcription of the  $\sigma^{54}$ -dependent class III flbG promoter in vitro and that this activity depends on intact sequence elements ftr1 (3, 4) and ftr1\* (3a). To determine if other  $\sigma^{54}$ -dependent fla genes contain a similar regulatory architecture, we examined the  $\sigma^{54}$ -dependent promoters of the seven class III and class IV fla genes which have been described in C. crescentus and found that each of them contains a pair of 19-bp ftr sequence elements that align with the FlbD binding sites ftr1 and ftr1\* of the flbG operon and ftr4 of the fliF operon (Table 1). The only exception to this observation is flgF, which contains three ftr sequence elements. All of the sequences contain the symmetrically placed guanine residues (Table 1, underlined bases) that in ftr1, ftr1\*, and ftr4 are specifically contacted by the full-length FlbD protein (3, 4). The consensus sequence of the symmetrical 19-bp ftr, which is CCCGGCARNRNYTGC CGGG, agrees well with that, published previously, based on fewer sequences (3, 54).

It is interesting to note that the *ftr* elements are almost always present in pairs approximately 100 bp upstream of transcriptional start sites, except in *flgK*, where the elements are ca. 100 bp downstream of the start site (15, 30, 31). The exact spacing between centers of adjacent *ftr* sites varies from promoter to promoter and ranges from 33 to 34 bp for the *flbG* and *flgI* promoters to 12 to 15 bp for the *fljL* and *flgH* promoters (Table 1). The possible significance of these differences in spacing is considered in the Discussion.

Activation of transcription in vitro by FlbDWT and FlbD<sup>S140F</sup>. Purified wild-type FlbD activates transcription in vitro from the flbG promoter at relatively high concentrations without obvious phosphorylation of the purified protein (3). This is in contrast to the well-characterized NtrC protein, whose activity absolutely requires phosphorylation of a conserved aspartate residue near its amino terminus (55). Although there is evidence that FlbD can be phosphorylated (59), a cognate histidine protein kinase has not been identified. Therefore, to facilitate our in vitro studies we examined a mutant FlbD (FlbD<sup>S140F</sup>) to determine if it is a more efficient transcription activator than the wild-type protein. FlbD<sup>S140F</sup>, which has been used previously in in vivo studies of transcription (59), corresponds to one of the well-characterized NtrC-(Con) mutant proteins, NtrCS160F, that is known to partially bypass the requirement for phosphorylation in the E. coli system (Fig. 1) (55).

We first compared the capacities of FlbDWT and FlbDS140F to activate transcription from the class III flgF operon (rod and L-ring protein genes) and the class IV fljK gene (25-kDa flagellin protein gene) promoters which contain the ftr sequences shown in Table 1. The flgF gene transcription start site in the supercoiled plasmid template pAKC28 lies 336 bp upstream of the T7 rho-independent terminator of the parental plasmid pTE103. The addition of FlbD (Fig. 2A) produced a transcript that migrated very close to the expected position of a 336nucleotide transcript predicted from the in vivo mapped start site (51). When FlbDS140F was substituted for FlbDWT, the same-size transcript was observed, but with a much stronger signal, and transcription was detected at FlbD<sup>S140F</sup> concentration. tions as low as 40 nM. In contrast, concentrations of FlbD WT in excess of 240 nM were required for an equivalent signal. To quantify these results, we compared the band intensities of FlbD<sup>WT</sup>- and FlbD<sup>S140F</sup>-dependent transcripts for each of the different concentrations tested. Results of PhophorImage analysis showed that the level of flgF-specific transcript activated by FlbDS140F is 14- to 16-fold higher than that by its wild-type counterpart, FlbD<sup>WT</sup>, at each concentration examined. Thus, as predicted from the homology to NtrC<sup>S160F</sup>, the FlbD<sup>S140F</sup> protein has a greater capacity to catalyze open complex formation by  $E\sigma^{54}$  than wild-type FlbD in the absence of any modification.

We also examined the class IV *fljK* promoter for activation by FlbD<sup>WT</sup> and FlbD<sup>S140F</sup> (Fig. 2B). When FlbD was added to the reaction mixtures containing the *fljK* promoter template, an RNA of the size expected from the previously mapped in vivo start site (451 nucleotides) (26) was observed in all the reaction mixtures, even at an FlbD<sup>WT</sup> or FlbD<sup>S140F</sup> concentration as low as 40 nM. The levels of transcript increased as the concentrations of the FlbD<sup>WT</sup> or FlbD<sup>S140F</sup> protein were increased, and as observed above for the *flgF* promoter, FlbD<sup>S140F</sup> was a more effective transcription activator than FlbD<sup>WT</sup>. Quantification of these results by PhosphorImage analysis as described above showed that FlbD<sup>S140F</sup> generated about fivefold-more transcript than FlbD<sup>WT</sup> at each protein concentration. Thus, the S140F substitution in FlbD increased the capacity of the protein to activate transcription from both

TABLE 1. Alignment of ftr sequence elements and $\sigma^{54}$ -dependent promoters of C. crescer
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Class	Gene	ftr	Spacing <sup>a</sup>	Position <sup>b</sup>	ftr sequence <sup>c</sup>	$\sigma^{54}$ -dependent promoter $^d$	Reference(s)
IIB	fliF	ftr4	7	-16	GTG <u>G</u> CTAAATCCTGCCTAC	$NA^e$	22, 52
III	$\mathit{flbG}$	ftr1 ftr1*	3	$-117 \\ -150$	CTCGCCAAAAAGCGCCCCA CGCGGCAACTCCCGTCCCT	T <u>GG</u> CCCGACCGTT <u>GC</u>	23
III	flgK	ftr2 ftr3	4.5	+59 +100	CCC <u>GG</u> CAAGTTTCG <u>CC</u> GGG CGC <u>GG</u> TTTGCCGAG <u>CC</u> GAA	T <u>GG</u> CGCCGACCTT <u>GC</u>	25
III	flgF	ftr5 ftr5* ftr5**	2.5	-96 -110 -124	CCC <u>GG</u> CAGAGTCCGA <u>C</u> CAG GCA <u>GG</u> CTGAATCTG <u>CC</u> CGG CCG <u>GG</u> CAATTGGTCG <u>C</u> AGG	T <u>GG</u> CACGAAGGTT <u>GC</u>	9
III	flgI	ftr6 ftr6*	3	-81 -115	CCC <u>GG</u> CCTTGTCCGGAATC CCG <u>GG</u> CAGAATCTG <u>CC</u> GTG	TGGCCCGCTTCTGGC	15
III	flgH	ftr9 ftr9*	1.5	$-135 \\ -147$	CAT <u>GG</u> CCGCCGCCGCC CAA <u>GG</u> CCATGGCCG <u>CC</u> GCC	A <u>GG</u> CCCAGAGCTT <u>GC</u>	9
IV	fljK	ftr7 ftr7*	2.5	$-116 \\ -145$	CTC <u>GG</u> CAGAAATTG <u>CC</u> GGG GCC <u>GG</u> CAACAATTT <u>CC</u> CAT	T <u>GG</u> CCCGGCAATT <u>GC</u>	20
IV	fljL	ftr8 ftr8*	1.5	-130 -147	CCCGGCGTAAGTTGCATTC TCTGGAAAAAGCTGCCGCC	T <u>GG</u> CCCGGCGCTT <u>GC</u>	20, 45
Consensus <sup>f</sup>					CCC <u>GG</u> CARNRNYT <u>G</u> CCGGG GGGCC <u>G</u> TYNRNRAC <u>GG</u> CCC	T <u>GG</u> CCCNNNNNTT <u>GC</u>	

<sup>&</sup>lt;sup>a</sup> Number of helical turns from centers of a pair of ftr sequences.

the flgF and the fljK promoters and permitted the use of much lower protein concentrations to generate strong transcription signals in our assays. Consequently, we used the mutant FlbD<sup>S140F</sup> protein in all the in vitro transcription assays described below.

Comparison of the activity of FlbD on  $\sigma^{54}$ -dependent fla gene promoters. To investigate the role of FlbD-ftr interactions in the regulation of the class III and class IV fla genes analyzed in Table 1, we compared transcription in vitro from the  $\sigma^{54}$ -dependent promoters of fljK (25-kDa flagellin), flgF (rod and L-ring proteins), flbG (hook components), and flgI (P-ring and basal body components). Of these, only the flbG promoter has been examined in vitro previously. The structures of the promoters and regulatory elements are diagrammed in Fig. 3A.

Transcripts were not detected from reaction mixtures containing any of the four templates when the FlbD<sup>S140F</sup> protein was absent (Fig. 3B; lanes 1, 5, 9, and 13). The addition of 100 nM FlbD<sup>S140F</sup> activator protein in the reaction mixtures containing plasmids pAKC8 (fljK; Fig. 3B, lane 2), pAKC28 (flgF; Fig. 3B, lane 6), pNJ5 (flbG; Fig. 3B, lane 10), and pAKC30 (flgI; Fig. 3B, lane 14) resulted in the formation of transcripts with expected sizes of 451, 336, 365, and 368 nucleotides, respectively, which are based either on in vivo transcription start site mapping (11, 26) or, in the case of flgI, on the location of the  $\sigma^{54}$ -dependent promoter (18). Although the abundance of the transcripts generated from these promoters was low at 100 nM FlbD<sup>S140F</sup>, the levels were substantially increased with the addition of more activator protein (Fig. 3B). However, the

efficiency of transcription from the promoters examined differed widely, with the class IV fljK promoter being the most efficient and the class III flgI promoter being the least efficient under our assay conditions, as judged by visual inspection of the autoradiograms in Fig. 3. We consider possible mechanisms that affect levels of transcription in the experiments described below.

We have extended this in vitro analysis to examine the requirement of flbD in vivo. Each of the four promoters was fused to a promoterless β-galactosidase gene present in the plasmid pANZ5 (see Materials and Methods). The expression of  $\beta$ -galactosidase from these fusions was then determined in a wild-type strain (CB15) and an flbD::Tn5 mutant strain (PC5510). In each case, transcription was abolished or severely depressed in the flbD mutant strain (Table 2). Previous studies of flbG (39), flgF (11), and fljK (58) transcription in vivo have yielded similar results and support the in vitro results presented in Fig. 3, which indicate that FlbD directly mediates transcriptional activation of these promoters. We also noted the same rank order in levels of FlbD-mediated expression from this set of  $\sigma^{54}$ -dependent promoters assayed in vitro and in vivo, but the relative difference between levels of transcription from the promoters was less when assayed in vivo.

Requirement of ftr sequences for flgF and fljK transcription. The ftr sequence elements identified in Table 1 are located upstream of the  $\sigma^{54}$ -dependent fla promoters in the same relative position as are ftr1 and ftr1\* in the flbG promoters (3, 32). To determine the requirement of these sequences for pro-

<sup>&</sup>lt;sup>b</sup> Measured from +1 to edge of ftr sequence.

<sup>&</sup>lt;sup>c</sup> Nucleotides which match those in the consensus sequence are shown in boldface type; underlined nucleotides are potential FlbD contact sites, homologous to those found in ftr1, ftr1\*, and ftr4 (3).

<sup>&</sup>lt;sup>d</sup> Nucleotides which match those in the  $\sigma^{54}$ -dependent promoter consensus are shown in boldface type; underlined nucleotides indicate the -12 and -24 sites of  $\sigma^{54}$ -dependent promoter.

<sup>&</sup>lt;sup>e</sup> NA, not applicable.

R, purines; Y, pyrimidines; N, any bases. Contact sites between flbD and guanine residues of ftr1, ftr1\*, and ftr4 are underlined in the consensus sequence (3, 4).

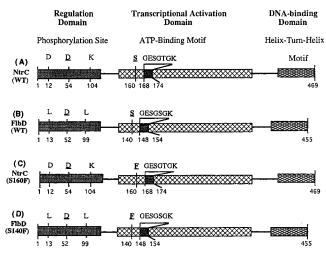
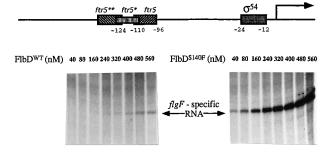


FIG. 1. Comparison of potential function domains of FlbD with NtrC. (A) The S. typhimurium wild-type NtrC contains 469 amino acids and consists of three functional domains. The regulatory domain (about 120 amino acids), which is located at the amino terminus, contains three amino acids (indicated by the one-letter amino acid code) that are signatures of the response regulator class of proteins and typically forms an acid pocket around the site of phosphorylation in the CheY crystal structure (53). The transcriptional activation domain (about 240 amino acids), which is located in the central portion of the protein, contains an ATP-binding motif. (The sequence shown for the NtrC protein is taken from the corrected sequence reported by Weiss et al. [56].) The DNA-binding domain (about 60 amino acids), which is located at the carboxyl terminus of the protein, contains a putative helix-turn-helix motif. Phosphorylation of NtrC is absolutely required for the activity of this protein. (B) The C. crescentus wild-type FlbD contains 455 amino acids and has a structure homologous to that of NtrC, with the exception that only one of the three most highly conserved amino acids is present in the regulatory domain of FlbD. (C) The structure of a mutant NtrC protein, NtrC<sup>S160F</sup>. In this protein, the serine residue at position 160 has been replaced by a phenylalanine residue. This protein, unlike its wild-type counterpart, has a weak capacity for transcriptional activation without phosphorylation. (D) The structure of the mutant FlbD protein, FlbD<sup>S140F</sup>, which has been used in this study. In FlbD<sup>S140F</sup>, the serine residue at position 140 has been replaced by a phenylalanine residue, corresponding to the substitution made in NtrCS160F

moter activation, we constructed a series of plasmids containing mutations at highly conserved positions within the *ftr* sequences. Dimethyl sulfate footprinting assays have shown that FlbD makes base-specific contacts at highly conserved guanine residues in each half site of the *ftr* sequences (Table 1) (3). Consequently, five of the mutations in *ftr* elements made in this study were G-to-T transversions and one was a G-to-A transition at these conserved positions.

The levels of transcripts from supercoiled templates containing the flgF promoter with the G-to-T transversion at position -92 of ftr5 (pJWF1), position -106 of ftr5\* (pJWF2), or position -120 of ftr5\*\* (pJWF3) were significantly reduced compared with those from the wild-type flgF promoter (pAKC28) (Fig. 4). Although transcription was activated from all four templates by FlbD<sup>S140F</sup> at concentrations of 100 nM or higher, visual inspection of the band intensities revealed that the levels of flgF-specific transcripts derived from the mutant templates were lower than those derived from the wild-type template at any given FlbD concentration. In the mutant templates carrying only a single point mutation, we observed residual levels of transcription, e.g., pJWF1 (ftr5), pJWF2 (ftr5\*), and pJWF3 (ftr5\*\*). These activities could be due to the interaction of FlbD<sup>S140F</sup> with the two remaining intact ftr elements. Alternatively, the FlbD<sup>S140F</sup> protein may still recognize and bind to the mutant ftr, but with lowered affinity. Irrespective of the explanation, these results clearly indicate that full activation by FlbD

# A Class III flgF promoter (Ring and Rod Proteins)



### B Class IV fljK promoter (25-kD Flagellin Protein)



FlbD<sup>WT</sup> (nM) 40 80 160 240 320 400 480 560 FlbD<sup>S140F</sup> (nM) 40 80 160 240 320 400 480 560

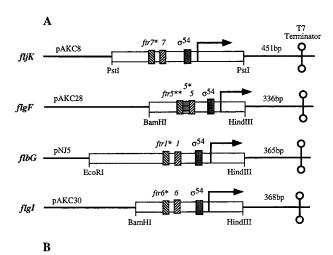
| fljK - specific | RNA

FIG. 2. Analysis of the activities of FlbD<sup>WT</sup> and FlbD<sup>S140F</sup>. Drawings of the templates used for this study are shown. Plasmid pAKC28 contains the *flgF* gene promoter in which the start site was placed 366 bp upstream of the *rho*-independent terminator site. Plasmid pAKC8 contains the *fljK* gene promoter in which the transcriptional initiation site was inserted 451 bp upstream of the termination site. The arrows indicate the positions of transcriptional initiation sites and the orientations of the transcripts. The  $\sigma^{54}$ -dependent promoters and *ftr* elements are indicated by boxes. Transcription from the class III *flgF* gene promoter (A) and from the class IV *fljK* gene promoter (B) was activated by FlbD proteins. All transcription reaction mixtures contained 5 nM supercoiled template (pAKC28 [A] or pAKC8 [B]), 100 nM *E. coli* RNA polymerase core enzyme, and 200 nM *E. coli*  $\sigma^{54}$ . The concentrations of the FlbD<sup>WT</sup> and *fljbS*-ida<sup>F</sup> proteins are indicated above the lanes. The positions of *flgF*- and *fljK*-specific transcripts on the gels are indicated by arrows.

requires intact  $\mathit{ftr}$  elements upstream of the  $\sigma^{54}$ -dependent  $\mathit{ftgF}$  promoter.

We also examined the effect of mutations in the ftr7 and ftr7\* sequence elements on transcription from the class IV fljK promoter in plasmids pJWK1 (C to T at position –101 of ftr7), pJWK2 (G to A at position –112 of ftr7), and pJWK3 (G to T at position –140 of ftr7\*) (see Materials and Methods). Each of these ftr mutations (Fig. 5) dramatically reduced the levels of transcription from the fljK promoter compared with the template containing wild-type ftr elements (Fig. 5). On the basis of the intensity of the labeled bands, the levels of transcripts generated from the mutant ftr7 and ftr7\* templates were severalfold lower than those generated from the wild-type template. This contrasts with the more modest effect of ftr mutations in the flgF promoter discussed above (Fig. 4), but it confirms that result by demonstrating that intact, multiple ftr sequence elements are also required for full activation of the fljK promoter.

We have noted that the level of transcript made from the wild-type *fljK* template pAKC8 was strongly concentration dependent at FlbD<sup>S140F</sup> concentrations of from 100 to 400 nM. The concentration dependency was, however, much less marked in transcription from mutant templates with altered *ftr* elements (pJWK1, pJWK2, and pJWK3) over the same range



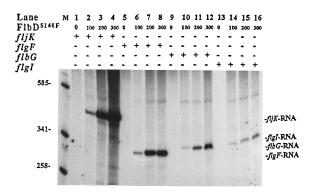


FIG. 3. Transcriptional activation of *fla* gene promoters by FlbD<sup>S140F</sup> protein. (A) Drawings of the structures of the transcription templates containing the *fljK* (pAKC8), *flgF* (pAKC28), *flbG* (pNJ5), and *flgI* (pAKC30) gene promoters used in this study are shown. Horizontal lines represent the sequences of the vectors. Open boxes indicate the sequences of *fla* genes. Filled boxes indicate the arrangement of the *flr* sequence elements and  $\sigma^{54}$ -dependent promoter sequences. The *rho*-independent terminator is also indicated. The number of base pairs indicates the distance between transcriptional start site and terminator is. The orientations of the transcripts are indicated by arrows. (B) Single-cycle transcription assays were performed with 5 nM supercoiled plasmids pAKC8 (lanes 1 to 4), pAKC28 (lanes 5 to 8), pNJ5 (lanes 9 to 12), and pAKC30 (lanes 13 to 16). Each reaction mixture contained 100 nM *E. coli* RNA polymerase core enzyme and 200 nM *E. coli*  $\sigma^{54}$ . The concentrations (nanomolar) of FlbD<sup>S140F</sup> are indicated above the lanes. Labeled size standards (lane M; in base pairs [shown on the left]) were derived from *Sau*3A-digested pUC18.

of  ${
m FlbD^{S140F}}$  concentrations. As considered in the Discussion, the mutant *ftr* sequences may impair cooperative binding by the  ${
m FlbD^{S140F}}$  protein.

The results obtained from the in vitro experiments described above were confirmed by examining the effects of these *ftr* element mutations on activation of transcription from *flgFp-lacZ* fusions (Table 3) and *fljKp-xylE* fusions (Table 4) in vivo. Transcription from the *flgF* promoter was substantially reduced in all *flgFp-lacZ* fusions containing *ftr* mutations, and the levels of *lacZ* expression were further decreased in the *flbD*::Tn5 genetic background (Table 3). We observed similar effects of *ftr* mutations on transcription from the *fljK* promoter in the *fljKp-xylE* fusions: levels of *xlyE* expression were significantly reduced in the three constructs containing mutated *ftr* sequences (Table 4). These results further indicate that intact pairs of *ftr* sequence elements are required for the full activation of transcription from both class III and class IV gene promoters by FlbD.

TABLE 2. Requirement of *flbD* and *ftr* sequences for expression from class III and class IV promoters

Strain	Genotype	Promoter fusion	lacZ expression <sup>a</sup>
PC9971	Wild type	fljKp-lacZ	368
PC9972	flbD::Tn5	fljKp-lacZ	12
PC9906	Wild type	flgFp-lacZ	173
PC9910	flbD::Tn5	flgFp-lacZ	8
PC9918	Wild type	flbGp-lac $Z$	177
PC9923	flbD::Tn5	flbGp-lac $Z$	5
PC9915	Wild type	flgIp-lacZ	77
PC9920	flbD::Tn5	flgIp-lacZ	7

<sup>&</sup>lt;sup>a</sup> Data are Miller units, corrected for vector (pANZ5) activity of 34 Miller units.

Role of ftr elements in the control of transcriptional efficiency. The efficiency of transcription initiation from the four promoters examined in Fig. 3 varied in the order fljK > flgF > flbG > flgI under the conditions we tested. Possible explanations for these differences include the effectiveness of the RNA polymerase-promoter recognition and FlbD-ftr binding. To evaluate the contribution of these interactions, we first compared the capacities of NtrC (in the presence of the histidine kinase NtrB), whose activity should be independent of specific DNA binding, and of FlbD<sup>S140F</sup> to catalyze open complex formation at the fljK and flgF promoters. As shown in Fig. 6 (lanes 5 and 6), we identified conditions in which NtrC and FlbD<sup>S140F</sup> were approximately equivalent in activating transcription from the fljK promoter. The fact that FlbD<sup>S140F</sup> is much less efficient at stimulating open complex formation from the flgF promoter than was NtrC under the same reaction

# Class III flgF promoter (Ring and Rod Proteins)

ftr5\*\*ftr5\*ftr5

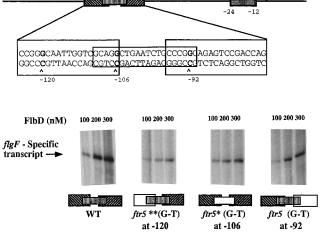
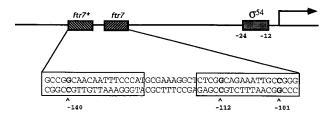


FIG. 4. Effects of *ftr* mutations upon activation of transcription from class III *ftgK* promoter by FlbD<sup>S140F</sup> protein. Shown are the structures of the class III *ftgF* gene promoter and the *ftr5*, *ftr5*\* and *ftr5*\*\* sequence elements. All symbols are as described in the legend to Fig. 3. The positions of the nucleotides (in boldface type) that are mutated are indicated. Single-cycle transcription assays were carried out by using 5 nM supercoiled templates, including pAKC28 containing the wild-type *ftr* sequences, pJWF1 containing a mutant *ftr5* (G to T at position −92), pJWF2 containing a mutant *ftr5*\*\* (G to T at position −106), and pJWF3 containing a mutant *ftr5*\*\* (G to T at position −120). The *ftgF* promoter in all four constructs was fused 336 by upstream of the *rho*-independent terminator. Each reaction mixture also contained 100 nM *E. coli* RNA polymerase core enzyme and 200 nM purified σ<sup>54</sup>-dependent factor. The concentrations (nanomolar) of FlbD<sup>S140F</sup> protein are indicated above the lanes.

### Class IV fljK promoter (25-kD Flagellin Protein)



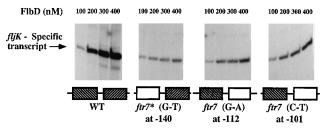


FIG. 5. Effects of mutations in ftr7 and ftr7\* on the transcriptional activation from class IV fljK gene promoter by FlbDS<sup>140F</sup>. Shown is the structure of the class IV fljK gene promoter and its ftr elements ftr7 and ftr7\*. All symbols are as described in the legend to Fig. 3. The mutated nucleotides are indicated by boldface type. The positions of these nucleotides are indicated. Single-cycle transcription assays were performed by using 5 nM supercoiled templates, including pAKC8 containing the fljK gene promoter with wild-type ftr7 and ftr7\*, pJWK1 containing the fljK promoter with a mutant ftr7 (C to T at position –101), pJWK2 containing fljK with another mutant ftr7 (G to A at position –112), and pJWK3 containing fljK with a mutant ftr7\* (G to T at position –140). The fljK promoter in all four plasmids was placed 451 bp upstream of the termination site. Each reaction mixture contained 100 nM E. coli RNA core polymerase and 200 nM E. coli  $\sigma$ 54-dependent factor. FlbDS<sup>140F</sup> concentrations (nanomolar) are indicated over the lanes.

conditions (Fig. 6; lanes 8 and 9) suggests that FlbD<sup>S140F</sup> is more efficient in activating transcription from the fljK promoter than from the flgF promoter. These results indicate that the rate of open complex formation at these promoters is limited not by  $E\sigma^{54}$ -promoter binding but rather by FlbD-ftr interaction. The fact that NtrC stimulates nearly equal levels of transcript from both promoters implies that the FlbD<sup>S140F</sup> concentration is limiting in the reactions with the flgF template.

To confirm this conclusion, we carried out identical assays using an equal molar mixture of both the *flgF* and *fljK* promoter templates. The result shows that NtrC generated approximately equal levels of transcripts from each promoter as expected (Fig. 6, lane 2), but when FlbD<sup>S140F</sup> was present (Fig. 6, lane 3), only the *fljK* transcript was detected. The addition of

TABLE 3. Effect of *ftr* mutations on expression of *flgFp-lacZ* promoter fusions

Strain	Genotype	ftr <sup>a</sup>	lacZ expression <sup>b</sup>
PC9906	Wild type	Wild type	173
PC9907	Wild type	ftr5 (G92T)	21
PC9908	Wild type	ftr5* (G106T)	32
PC9909	Wild type	ftr5** (G120T)	69
PC9910	flbD::Ťn5	Wild type	8
PC9911	flbD::Tn5	ftr5 (G92T)	12
PC9912	flbD::Tn5	ftr5* (G106T)	10
PC9913	flbD::Tn5	ftr5** (G120T)	9

<sup>&</sup>lt;sup>a</sup> See the legend to Fig. 4.

TABLE 4. Effect of *ftr* mutations on expression of *fljKp-xylE* promoter fusions

Strain	Genotype	ftr <sup>a</sup>	xylE expression <sup>b</sup>
PC1101	Wild type	Wild type	13.68
PC1102	Wild type	ftr7 (C101T)	0.35
PC1103	Wild type	ftr7 (G112T)	0.88
PC1104	Wild type	frt7* (G140T)	1.43

<sup>&</sup>lt;sup>a</sup> See the legend to Fig. 5.

both FlbD<sup>S140F</sup> and NtrC to the reaction mixtures (Fig. 6, lane 4) restored nearly equal levels of transcription from both promoters. If the difference in the levels of transcription between flgK and fljF (Fig. 6, lanes 6 and 9) were a consequence of interactions of  $E\sigma^{54}$  with the respective promoter sequences, we would have predicted that NtrC, which presumably is functioning nonspecifically with the *C. crescentus* promoters, would generate more transcripts from the fljK template than from the flgF template, as was the case with FlbD<sup>S140F</sup>. Thus, in our in vitro transcription system, the efficiency of transcription is unlikely to be a consequence of  $E\sigma^{54}$  interaction with the respective promoters, and we speculate that the efficiency of FlbD interactions with the respective ftr binding sites may be a factor governing the efficiency of transcription from the different  $\sigma^{54}$ -dependent fla gene promoters.

### DISCUSSION

The sequential order of fla gene expression in the C. crescentus cell cycle is governed, at least in part, by the appearance and/or activation of RNA polymerases containing specialized  $\sigma$  factors, as well as by specific regulatory proteins. In this report, we have examined regulation of the  $\sigma^{54}$ -dependent promoters of class III and class IV fla genes. We have found that all of these genes contain multiple ftr sequence elements

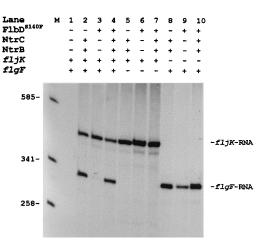


FIG. 6. The role of ftr sequence elements in determination of the transcriptional efficiency of fta gene promoters. Single-cycle transcription assays were performed by using either a mixture of two supercoiled plasmids, pAKC8 containing the class IV ftjK gene promoter and pAKC28 containing the class III ftgF gene promoter (lanes 1 to 4), or each of these plasmids alone (pAKC8, lanes 5 to 7, and pAKC28, lanes 8 to 10) (5 nM for each). E. coli RNA polymerase core enzyme and  $\sigma^{54}$ -dependent factor were present at final concentrations of 100 and 200 nM, respectively. NtrC, NtrB, and FlbDs140F were added to final concentrations of 400, 160, and 400 nM, respectively. Labeled size standards (lane M; in base pairs [shown on the left]) were derived from Sau3A-digested pUC18.

<sup>&</sup>lt;sup>b</sup> Data are Miller units, corrected for vector (pANZ5) activity of 34 Miller units

<sup>&</sup>lt;sup>b</sup> Specific activities of catechol oxidase in units described previously (19) and corrected for vector activity of 0.31.

ca. 100 bp from the transcriptional start sites (Table 1). Our results also demonstrate that transcription from class III flbG, flgF, and flgI promoters and the class IV fljK promoter is activated by the FlbD protein and that FlbD-dependent transcription requires pairs of intact ftr sequence elements in vitro (Fig. 3, 4, and 5) as well as in vivo (Tables 3 and 4). On the basis of these observations, we propose that FlbD functions at ftr sequence elements as a global activator of class III and class IV fla gene transcription in the C. crescentus cell cycle.

**FlbD** is a transcriptional activator of class III and class IV genes. We have extended our initial studies of FlbD activity on the class III flbG promoter (3, 4, 32, 59) by examining the function of FlbD on a wide range of  $\sigma^{54}$ -dependent promoters, including the promoters of the flgF, flgI, and flbG operons at class III and the fljK gene at class IV. Our results show (Fig. 3) that purified FlbD is sufficient for the transcriptional activation of each of these promoters. The only identified  $\sigma^{54}$ -dependent class III fla gene promoters we have not examined are flgH and flgK. These promoters also contain pairs of ftr sequence elements at ca. 100 bp from the transcription start sites (Table 1), and the ftr2 and ftr3 sequence elements of flgK are known from previous work to be required in vivo for normal regulation of this fla gene (15, 31). We predict that both the flgK and flgH promoters would be recognized by purified FlbD in our reconstituted  $E\sigma^{54}$  transcription system.

Regulation of FlbD activity. The FlbD protein is structurally similar to the NtrC protein (43), whose activity is modulated as a result of phosphorylation by the histidine kinase NtrB in response to nitrogen limitation (17, 35). The C. crescentus activator contains three functional domains: the regulatory domain at the amino terminus, the activation domain at the central part, and the DNA-binding domain at the carboxyl terminus (Fig. 1). In NtrC, the phosphorylation site is within an amino-terminal domain of ca. 120 residues (Fig. 1) (17). A homolog of this domain has been found on more than 20 bacterial regulatory proteins whose activities are known or thought to be modulated by phosphorylation (reviewed in references 1 and 55). The role of phosphorylation in governing the function of NtrC is believed to be in modulation of the cooperativity of NtrC dimers to form a tetrameric or oligomeric activation complex that possesses the ATPase activity necessary for driving  $E\sigma^{54}$  into the open complex (42, 57).

FlbD is unusual among the transcription activators of the NtrC family, because its amino-terminal domain contains only one (D-52) of the three residues (D-12, D-54, and K-104) present in most other members of this response regulator family (Fig. 1) (53). Our results clearly indicate that, unlike NtrC, wild-type FlbD proteins activate transcriptional initiation from flbG (2, 4, 5), flgF (Fig. 2A), and fljK (Fig. 2B) promoters without any apparent phosphorylation. These observations suggest that unmodified FlbD contains a basal level of activity or that it uses a unique mechanism to activate the initiation of transcription from  $\sigma^{54}$ -dependent fla genes in C. crescentus. Other lines of evidence indicate, however, that FlbD can be phosphorylated in vivo (59) and that the high-energy-phosphate-containing molecule phosphoramidate stimulates FlbD activity in vitro (4). These results suggest a role for phosphorylation in governing FlbD function. It may be that the biologically relevant activity of FlbD requires phosphorylation or some other form of modification. Establishing a direct role for phosphorylation in the transcription regulation will rely on identification of a FlbD kinase and demonstration of its effects on FlbD activity.

**Organization of** *cis***-acting sequences.** The organization of the -100 ftr sequence elements and the -24, and -12 promoter sequences in the C. crescentus fla genes is highly remi-

niscent of the structure of many globally regulated *ntr* and *nif* promoters of the enteric bacteria and *Rhizobium* spp. that promote transcription of genes involved in nitrogen assimilation. The *cis*-acting elements upstream of the *nif* and the *ntr* promoters have been shown to be functionally equivalent to enhancers and upstream activation sequences (8, 46).

We and others have previously demonstrated that FlbD, which contains a putative helix-turn-helix motif at its carboxyl terminus (Fig. 1), binds in close proximity to symmetrical guanine residues on both strands of the ftr1, ftr1\* (3, 32), and ftr4 (3, 32, 58) sequences. Mutation of the ftr1 sequence at one of these contact sites (G to T at position -113) severely inhibits transcription both in vivo (30, 31) and in vitro (3). These results, in conjunction with the results of transcription assays on templates with mutated ftr sequences presented here, demonstrate that the conserved guanine residues are critical for FlbD function at these promoters. Our results also suggest that pairs of ftr sequence elements may maximize oligomerization of the FlbD protein near the  $\sigma^{54}$ -dependent promoters. This conclusion is supported by the symmetry of FlbD binding to the arms of the ftr dyad (3), the organization of many class III gene ftr sequence elements in pairs (Table 1), and the observation that ftr mutations significantly reduce the levels of transcription from cognate promoters both in vitro (Fig. 4 and 5) and in vivo (Tables 3 and 4). Thus, FlbD may function as an oligomer. The requirement of oligomerization for transcriptional activation has been demonstrated with NtrC (42, 57) and FNR (21). It has been proposed that binding pairs of dimers facilitates the formation of a tetrameric NtrC complex at low NtrC concentrations that can effectively catalyze isomerization of the closed  $E\sigma^{54}$  complex into the open complex (42, 57). Further biochemical experiments will be necessary to demonstrate that FlbD activates transcription by a similar mechanism.

In addition to the *ftr* elements studied in this report, two other sequence elements have been reported upstream from several class III promoters (reviewed in reference 6). A sequence designated RF-2 has been identified upstream of the *flgH* gene promoter (12), and a second conserved sequence element termed RF-3 has been reported upstream of the *flgI* (18) and the *flgF* (51) promoters. The RF-3 sequences have not been examined genetically, but alignment of these sequences with the *ftr* sequences in Table 1 suggests that they are in fact *ftr* elements: the RF-3 in *flgF* corresponds to *ftr5\**, and the RF-3 in *flgI* corresponds to *ftr6\** (Table 1).

The similarity of the RF-2 site to the consensus NifA binding site has been noted previously by Dingwall et al. (12), and these authors suggested that it behaves as a functional homolog of the NifA binding site in flgH regulation. The flgH gene has been shown previously, however, to be transcribed from both its own promoter and by read-through transcription from the flgF operon (11), whose promoter we have examined here. Thus, the flgH promoter may represent a secondary means for expression of flgH that is independent of FlbD control. Although our transcription studies suggest that FlbD is sufficient to modulate  $E\sigma^{54}$ -dependent transcription from the class III and class IV fla genes, other proteins may also contribute to their control. These include 70- and 90-kDa proteins in C. crescentus extracts that bind to ftr oligonucleotides (16).

**Promoter efficiency.** The number of protein molecules which oligomerize to form the different flagellar substructures varies widely. If the cellular concentration of each subunit reflects its abundance in the flagellum, one might speculate that finetuning of transcriptional efficiency would be one way to govern the relative levels of expression. The center-to-center spacing between adjacent *ftr* sites is different in individual promoters (Table 1): (i) three turns between *ftr1* and *ftr1\** (*flbG*) and *ftr6* 

and ftr6\* (ftgI), which is strikingly similar to the arrangement of adjacent high-affinity binding sites for NtrC at the glnAp2 promoter (37, 46, 49); (ii) two and one-half turns between ftr5 and ftr5\* (ftgF) and ftr7 and ftr7\* (ftjK), which is similar to that of upstream NtrC binding sites found in the ntfLA promoter (25, 60); and (iii) one and one-half turns between ftr9 and ftr9\* (ftgH) and ftr8 and ftr8\* (ftjL), which is similar to that of two of the NtrC binding sites upstream of the glnH promoter (10).

We speculate that these differences in organization of ftr sequence elements, as well as differences in the individual ftr sequences, could determine the capacity of FlbD to bind and thus provide a mechanism for controlling the efficiency at which the respective promoters are transcribed. The results shown in Fig. 3 clearly indicate that much more transcription can be generated from the flgF and fljK promoters (two and one-half turns, ftr separation) than from the flbG and flgI promoters (three turns, ftr separation) under the same conditions. Moreover, our results (Fig. 6) suggest that the transcriptional efficiency from the flgF and fljK promoters in vitro is determined by the properties of ftr elements and not by the  $\sigma^{54}$ -dependent promoter itself. We base this idea on the observation that NtrC, which is likely to be functioning via nonspecific binding or from solution, can stimulate open complex formation from these two promoters with equal efficiency, whereas FlbD, which presumably acts by binding ftr elements, shows a greater capacity to activate fljK transcription than flgF

One interpretation of our results is that binding of FlbD to two ftr elements with a spacing of two and one-half turns permits activation at a lower FlbD concentration than binding to sequence elements with a spacing of three turns (47). In the case of NtrC, transcription activation is most efficient when the binding sites are three helical turns apart, presumably as a consequence of more efficient oligomerization. Therefore, the difference in optimal organization of FlbD and NtrC binding sites may reflect different interactions between the FlbD and NtrC monomers in the dimeric proteins or a difference in the way that the dimers recognize their respective binding sites. It will be important to test this idea, because one alternative possibility is that the ftr sequence itself is responsible for the transcriptional efficiency. In this case, the ftr elements of fljK, which contains the most efficient promoter, would have higheraffinity binding sites. We note in this context that ftr elements ftr7 and ftr7\* of fljK most closely match the ftr consensus shown in Table 1.

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