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

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Received 17 January 1995/Accepted 31 March 1995

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* Er$^{54}$ is activated by FlbD or the mutant protein FlbD$^{S140F}$ (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 *flF* (54), *flJ* (13), *flL* (52, 62), and *ropN* (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 *flJ* 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 *ropN* gene, which encodes $\sigma^{54}$, is required for class III (2, 7) and class IV (59) *fla* gene expression.

The enteric Er$^{54}$ ($\sigma^{54}$-containing RNA polymerase) is unique among prokaryotic RNA polymerase holoenzymes, in that formation of transcription-competent open complexes de-
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 fr sequences (fr for flagellar transcription regulation) with an apparently similar function were originally identified ca. 100 bp from the transcription start sites of the class III flgB and flgK promoters and of the class IV flgK and flgL flagellin promoters (26, 28). The requirement of these sequence elements for transcription from the class III flgB and flgK 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 σ70-dependent promoters (34, 43). Its predicted product, FlbD, is homologous to NtrC and a family of response regulators known to activate transcription by Eκα in Escherichia coli, Salmonella typhimurium, Rhizobium meliloti, and Pseudomonas putida (for reviews, see references 20, 23, and 57). Recent studies of the flgB promoter have demonstrated that FlbD is an fr-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 flf4 (54), a DNA sequence which overlaps the flf4 promoter (3). Comparable observations of FlbD binding at flf4 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 flgB promoter and represses transcription from the class II flf4 promoter by its interactions with fr sequence elements that have previously been shown genetically to be essential for the regulation of flgB (ftr1) and flf4 (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 cis-acting sequences on transcription has not been studied in detail, their identification raises the question of whether activation by FlbD at fr sequence elements, as described above for flgB (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 FlbD to other σ70-dependent class III and class IV promoters to determine the requirement of FlbD and fr 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 fr 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 RNA polymerase to examine in detail the class III flg, flf4, and flgB promoters and the class IV flgK 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 fr sequences. These results lead us to propose that FlbD globally regulates transcription from all σ70-dependent class III and class IV fla gene promoters and that its function is mediated by interaction with the cis-acting fr sequence elements.

MATERIALS AND METHODS

Site-directed mutagenesis of flbD. The flbD mutation encoding FlbDΔRF-3 was constructed with the Sall-SacI 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 pW102. It was then mutated by site-directed mutagenesis with the oligonucleotide MJW-1: CCAG GTGCCCTCTTCGAAAGCTC. This changed the codon for Ser-140 to a codon for phenylalanine and introduced a BsrBI site to the resultant plasmid pMW1.

Construction of vector for protein expression. The wild-type FlbD protein was overexpressed in E. coli by using plasmid pGIR168 as described previously (3, 34). pGIR168 is a derivative of a 2340 bp BamHI fragment in which a 520-bp HI-EcoRI 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, FDBSAPC, was constructed as follows. The NcoI fragment of pGIR168 containing the wild-type flbD gene was replaced by the NcoI fragment from pMW1 containing the SI40F promoter. The resultant plasmid, pJW201, like pGIR168, contains the entire flbD gene on a 2-kb BamHI-EcoRV 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 FlbDWT (WT, wild type) and FlbDΔRF-3 (SI40F) proteins. The resultant plasmids, pJW1, like pGIR168, contains the entire flbD gene on a 2-kb BamHI-EcoRV fragment that was fused transcriptionally to the gene 10 promoter of bacteriophage T7.

Construction of vector for protein expression. Plasmids pGIR168 and pJW201 were transformed separately into E. coli BL21 (DE3) to overproduce the FlbDWT (WT, wild type) and FlbDΔRF-3 (SI40F) proteins. The resultant plasmids, pJW1, like pGIR168, contains the entire flbD gene on a 2-kb BamHI-EcoRV fragment that was fused transcriptionally to the gene 10 promoter of bacteriophage T7.

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) FlbD promoter. The plasmid pNJS (3, 34, 36) carries a 650-bp HindIII-EcoRI fragment containing the flbD promoter cloned into the HindIII and EcoRI sites of pTE103. The transcriptional start site for the flbD operon is positioned 365 bp upstream of the T7 terminator (36) (see Fig. 3).

(ii) Flf4 promoter and mutant fr elements. A 280-bp BamHI-HindIII fragment containing the flf4 promoter and its wild-type fr elements from pAKC27 was subcloned into the BamHI and HindIII sites of pTE103, to generate pAC28. In pAC28, the transcription initiation site of flf4 is located 336 bp upstream of the T7 terminator site. The BamHI-HindIII fragments containing mutant fr5, fr*3, or fr** sequences were constructed from plasmids pWMF1, pWMF3, and pWMF3 by subcloning 280-bp BamHI-HindIII fragments into the BamHI-HindIII sites of pTE103, to generate plasmids pWJF1, pWJF2, and pWJF3, respectively. In these three plasmids, as in pAC28, the flf4 transcriptional initiation sites are positioned 336 bp upstream of the T7 terminator (see Fig. 3).

(iii) Flk promoter and mutant fr elements. Plasmid pAC28 was constructed by insertion of the 520-bp PstI fragment of pAC27 containing the flkK 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 fr7 or fr*7 sites were constructed by insertion of the PstI fragments from pJWMK1, pJWMK2, and pJWMK3 into pTE103, to generate pJWK1, pJWK2, and pJWK3, respectively. In pACK8, as well as in pWJF1, pWJF2, and pWJF3, the flk transcriptional start site lies 451 bp upstream of the T7 terminator site (see Fig. 3 and 5).
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 fkr, 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 fblg and fblg promoters to 12 to 15 bp for the fjlL and fjlH promoters (Table 1). The possible significance of these differences in spacing is considered in the Discussion.

Activation of transcription in vitro by FlbD<sup>WT</sup> and FlbD<sup>S140F</sup>. Purified wild-type FlbD activates transcription in vitro from the fblg 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-<br/>Con mutant proteins, NtrC<sup>S140F</sup>, that is known to partially bypass the requirement for phosphorylation in the E. coli system (Fig. 1) (55).

We first compared the capacities of FlbD<sup>WT</sup> and FlbD<sup>S140F</sup> to activate transcription from the class III fblg operon (rod and L-ring protein genes) and the class IV fjlK gene (25-kDa flagellin protein gene) promoters which contain the ftr sequences shown in Table 1. The fblg 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 336-nucleotide transcript predicted from the in vivo mapped start site (51). When FlbD<sup>S140F</sup> was substituted for FlbD<sup>WT</sup>, the same-size transcript was observed, but with a much stronger signal, and transcription was detected at FlbD<sup>S140F</sup> concentrations as low as 40 nM. In contrast, concentrations of FlbD<sup>WT</sup> 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 PhosphorImage analysis showed that the level of fblg-specific transcript activated by FlbD<sup>S140F</sup> 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 Er<sup>54</sup> than wild-type FlbD in the absence of any modification.

We also examined the class IV fjlK 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 fjlK 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 fblg 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 five-fold-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...
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$^{S140F}$ protein in all the in vitro transcription assays described below.

Comparison of the activity of FlbD on $\alpha^{54}$-dependent fla gene promoters. To investigate the role of FlbD-flr interactions in the regulation of the class III and class IV fla genes analyzed in Table 1, we compared transcription in vitro from the $\alpha^{54}$-dependent promoters of fljK (25-kDa flagellin), flgF (rod and L-ring proteins), flbG (hook components), and fljL (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$^{S140F}$ protein was absent (Fig. 3B; lanes 1, 5, 9, and 13). The addition of 100 nM FlbD$^{S140F}$-activator protein in the reaction mixtures containing plasmids pAKC8 (flk; Fig. 3B, lane 2), pAKC28 (flgF; Fig. 3B, lane 6), pNJ5 (flbG; Fig. 3B, lane 10), and pAKC30 (fljL; 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 fljL, on the location of the $\alpha^{54}$-dependent promoter (18). Although the abundance of the transcripts generated from these promoters was low at 100 nM FlbD$^{S140F}$, 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 fljL promoter being the most efficient and the class III flgF 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 $\beta$-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 $\alpha^{54}$-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 flr sequence elements for flgF and fljK transcription. The flr sequence elements identified in Table 1 are located upstream of the $\alpha^{54}$-dependent fla promoters in the same relative position as are flr1 and flr1+ in the flbG promoters (3, 32). To determine the requirement of these sequences for pro-
In this study, transcription, e.g., pJWF1 (*pJWF1), positions

flgF

promoter (A) and from the class IV

fljK

promoter (B) was activated by

rho

promoter in which the startsite was placed 366 bp upstream of the rho-inde-
dependent 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 σ54-dependent promoters and
fr elements are indicated by boxes. Transcription from the class III fljK 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 σ54. The concentrations of the FlbDWT and
FlbDS140F proteins are indicated above the lanes. The positions of fljK- and
fljK-specific transcripts on the gels are indicated by arrows.

We also examined the effect of mutations in the flr7 and flr7* sequence elements on transcription from the class IV fljK promoter in plasmids pJKW1 (C to T at position -101 of flr7), pJKW2 (G to A at position -112 of flr7), and pJKW3 (G to T at position -140 of flr7*) (see Materials and Methods). Each of these flr mutations (Fig. 5) dramatically reduced the levels of transcription from the fljK promoter compared with the template containing wild-type flr elements (Fig. 5). On the basis of the intensity of the labeled bands, the levels of transcripts generated from the mutant flr7 and flr7* templates were severalfold lower than those generated from the wild-type template. This contrasts with the more modest effect of flr mutations in the flgF promoter discussed above (Fig. 4), but it confirms that result by demonstrating that intact, multiple flr 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 FlbDS140F concentrations of from 100 to 400 nM. The concentration dependency was, however, much less marked in transcription from mutant templates with altered flr elements (pJWK1, pJKW2, and pJKW3) over the same range.

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 Weis 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 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, NtrC569F. 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, FlbDS140F, which has been used in this study. In FlbDS140F, the serine residue at position 140 has been replaced by a phenylalanine residue, corresponding to the substitution made in NtrC569F.
of FlbD<sub>S140F</sub> concentrations. As considered in the Discussion, the mutant <i>ftr</i> sequences may impair cooperative binding by the FlbD<sub>S140F</sub> protein.

The results obtained from the in vitro experiments described above were confirmed by examining the effects of these <i>ftr</i> element mutations on activation of transcription from <i>ftrGp-lacZ</i> fusions (Table 3) and <i>fljKp-xylE</i> fusions (Table 4) in vivo. Transcription from the <i>ftrG</i> promoter was substantially reduced in all <i>ftrGp-lacZ</i> fusions carrying <i>ftr</i> mutations, and the levels of <i>lacZ</i> expression were further decreased in the <i>ftrGp-lacZ</i> promoter in all three constructs containing mutated <i>ftr</i> sequences (Table 4). These results further indicate that intact pairs of <i>ftr</i> 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 <i>flbD</i> and <i>ftr</i> sequences for expression from class III and class IV promoters

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<th>Strain</th>
<th>Genotype</th>
<th>Promoter fusion</th>
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* Data are Miller units, corrected for vector (pANZ5) activity of 34 Miller units.

### Role of <i>ftr</i> elements in the control of transcriptional efficiency

The efficiency of transcription initiation from the four promoters examined in Fig. 3 varied in the order: <i>fljK</i> > <i>flgF</i> > <i>flbG</i> > <i>fljG</i> 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<sub>S140F</sub> to catalyze open complex formation at the <i>fljK</i> and <i>flgF</i> promoters. As shown in Fig. 4 (lanes 5 and 6), we identified conditions in which NtrC and FlbD<sub>S140F</sub> were approximately equivalent in activating transcription from the <i>fljK</i> promoter. The fact that FlbD<sub>S140F</sub> is much less efficient at stimulating open complex formation from the <i>flgF</i> promoter than was NtrC under the same reaction conditions.

#### Class III <i>flgF</i> promoter (Ring and Rod Proteins)

![Class III flgF promoter diagram](image)

**FIG. 4. Effects of <i>ftr</i> mutations upon activation of transcription from class III <i>flgK</i> promoter by FlbD<sub>S140F</sub> protein.** Shown are the structures of the class III <i>flgK</i> gene promoter and the <i>ftr5</i>, <i>ftr5</i>*, and <i>ftr5</i>** 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 mM supercoiled templates, including pAKC28 containing the wild-type <i>ftr</i> sequences, pJWF1 containing a mutant <i>ftr</i> (G to T at position -120), pJWF2 containing a mutant <i>ftr5</i> (G to T at position -106), and pJWF3 containing a mutant <i>ftr5</i>* (G to T at position -106). The <i>flgF</i> promoter in all four constructs was fused 336 bp upstream of the <i>rho</i>-independent terminator. Each reaction mixture also contained 100 mM E. coli RNA polymerase core enzyme and 200 mM purified σ<sub>54</sub>-dependent factor. The concentrations (nM) of FlbD<sub>S140F</sub> protein are indicated above the lanes.
both FlbD\(^{S140F}\) 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 flf (Fig. 6, lanes 6 and 9) would have predicted that NtrC, which presumably is functioning nonspecifically with the \(C.\) crescentus promoters, would generate more transcripts from the flgK template than from the flf template, as was the case with FlbD\(^{S140F}\). Thus, in our in vitro transcription system, the efficiency of transcription is unlikely to be a consequence of \(E\)\(^{54}\) interaction with the respective promoters, and we speculate that the efficiency of FlbD interactions with the respective fr 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 fr sequence elements

### TABLE 3. Effect of fr mutations on expression of flgFp-lacZ promoter fusions

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>(fr^a)</th>
<th>lacZ expression*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC9906</td>
<td>Wild type</td>
<td></td>
<td>173</td>
</tr>
<tr>
<td>PC9907</td>
<td>Wild type</td>
<td>fr5 (G92T)</td>
<td>21</td>
</tr>
<tr>
<td>PC9908</td>
<td>Wild type</td>
<td>fr5* (G106T)</td>
<td>32</td>
</tr>
<tr>
<td>PC9909</td>
<td>Wild type</td>
<td>fr5** (G120T)</td>
<td>69</td>
</tr>
<tr>
<td>PC9910</td>
<td>flbD: Tn5</td>
<td>Wild type</td>
<td>8</td>
</tr>
<tr>
<td>PC9911</td>
<td>flbD: Tn5</td>
<td>fr5 (G92T)</td>
<td>12</td>
</tr>
<tr>
<td>PC9912</td>
<td>flbD: Tn5</td>
<td>fr5* (G106T)</td>
<td>10</td>
</tr>
<tr>
<td>PC9913</td>
<td>flbD: Tn5</td>
<td>fr5** (G120T)</td>
<td>9</td>
</tr>
</tbody>
</table>

* See the legend to Fig. 4.

* Data are Miller units, corrected for vector (pAN5) activity of 34 Miller units.

### FIG. 6. The role of fr sequence elements in determination of the transcriptional efficiency of fla gene promoters. Single-cycle transcription assays were performed by using either a mixture of two supercoiled plasmids, pAKC8 containing the class IV fljK gene promoter and pAKC28 containing the class III flg 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 RNAP core enzyme and \(\sigma^{54}\)-dependent factor were present at final concentrations of 100 and 200 nM, respectively. NtrC, NtrB, and FlbD\(^{S140F}\) 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.
ca. 100 bp from the transcriptional start sites (Table 1). Our results also demonstrate that transcription from class III fetB, fetF, and fetG promoters and the class IV fetK promoter is activated by the FliB protein and that FliB-dependent transcription requires pairs of intact fet 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 FliB functions at fet sequence elements as a global activator of class III and class IV fla gene transcription in the C. crescentus cell cycle. 

FliB is a transcriptional activator of class III and class IV genes. We have extended our initial studies of FliB activity on the class III fetB promoter (3, 4, 32, 59) by examining the function of FliB on a wide range of ς^III^-dependent promoters, including the promoters of the fetG, fetF, and fetB operons at class III and the fetK gene at class IV. Our results show (Fig. 3) that purified FliB is sufficient for the transcriptional activation of each of these promoters. The only identified ς^III^-dependent class III fla gene promoters we have not examined are fetH and fetK. These promoters also contain pairs of fet sequence elements at ca. 100 bp from the transcription start sites (Table 1), and the fet2 and fet3 sequence elements of fetK are known from previous work to be required in vivo for normal regulation of this fla gene (15, 31). We predict that both the fetK and fetH promoters would be recognized by purified FliB in our reconstituted Ear^− transcription system.

Regulation of FliB activity. The FliB 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 carboxyl terminal, 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 Ear^− into the open complex (42, 57).

FliB 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 FliB proteins activate transcriptional initiation from fetB (2, 4, 5), fetF (Fig. 2A), and fetK (Fig. 2B) promoters without any apparent phosphorylation. These observations suggest that unmodified FliB contains a basal level of activity or that it uses a unique mechanism to activate the initiation of transcription from ς^III^-dependent fla genes in C. crescentus. Other lines of evidence indicate, however, that FliB can be phosphorylated in vivo (59) and that the high-energy-phosphate-containing molecule phosphoramidate stimulates FliB activity in vitro (4). These results suggest a role for phosphorylation in governing FliB function. It may be that the biologically relevant activity of FliB requires phosphorylation or some other form of modification. Establishing a direct role for phosphorylation in the transcription regulation will rely on identification of a FliB kinase and demonstration of its effects on FliB activity.

Organization of cis-acting sequences. The organization of the −100 fet sequence elements and the −24, and −12 promoter sequences in the C. crescentus fla genes is highly reminiscent 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 FliB, 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 fet1, fet1* (3, 32), and fet4 (3, 32, 58) sequences. Mutation of the fet1 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 fet sequences presented here, demonstrate that the conserved guanine residues are critical for FliB function at these promoters. Our results also suggest that pairs of fet sequence elements may maximize oligomerization of the FliB protein near the ς^III^-dependent promoters. This conclusion is supported by the symmetry of FliB binding to the arms of the fet dyad (3), the organization of many class III gene fet sequence elements in pairs (Table 1), and the observation that fet 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, FliB 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 Ear^+ complex into the open complex (42, 57). Further biochemical experiments will be necessary to demonstrate that FliB activates transcription by a similar mechanism.

In addition to the fet elements studied in this report, 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 fetH gene promoter (12), and a second conserved sequence element termed RF-3 has been reported upstream of the fetI (18) and the fetF (51) promoters. The RF-3 sequences have not been examined genetically, but alignment of these sequences with the fet sequences in Table 1 suggests that they are in fact fet elements: the RF-3 in fetF corresponds to fet5*, and the RF-3 in fetH corresponds to fet6* (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 fetH regulation. The fetH gene has been shown previously, however, to be transcribed from both its own promoter and by read-through transcription from the fetG operon (11), whose promoter we have examined here. Thus, the fetH promoter may represent a secondary means for expression of fetH that is independent of FliB control. Although our transcription studies suggest that FliB is sufficient to modulate Ear^−-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 fet 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 fine-tuning of transcriptional efficiency would be one way to assure the relative levels of expression. The center-to-center spacing between adjacent fet sites is different in individual promoters (Table 1): (i) three turns between fet1 and fet1* (fetB) and fet6
and ftr6 (flgF), 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 ftr9 (flgF) and ftr1 and ftr7 (fljK), which is similar to that of upstream NtrC binding sites found in the nifL/A promoter (25, 60); and (iii) one and one-half turns between ftr3 and ftr9 (flgF) and ftr8 and ftr4 (fljL), 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 σ70-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 (Fig. 6).

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 higher-affinity 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.

ACKNOWLEDGMENTS

We are grateful to A. Ninfa and J. Feng for kindly supplying purified E. coli proteins used in the in vitro transcription assays and to G. Hecht for making the PCR products used in constructing the transcriptional templates.

This work was supported by Public Health Service grant GM22299 from the National Institutes of Health. A.K.B. was supported by Public Health Service postdoctoral fellowship GM5290-01 from the National Institutes of Health.

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