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The *Caulobacter crescentus* FlbD protein acts at *ftr* sequence elements both to activate and to repress transcription of cell cycle-regulated flagellar genes

(RNA polymerase/σ^{54} promoters/DNA footprinting)

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ABSTRACT **The flagellar genes (*fla* genes) in *Caulobacter crescentus* are organized into a regulatory hierarchy of four levels, I–IV, in which transcription of the class III and class IV genes late in the cell cycle from σ^{54}-dependent promoters depends on expression of the class II genes above them. Timing of *fla* gene expression has been attributed to sequential activation and repression by specific transcription factors. Here we report that purified FlbD activates transcription *in vitro* from the σ^{54}-dependent class III *flbG* promoter and represses transcription from the class II *fliF* promoter by binding to *ftr* (flagellar transcription regulator) sequence elements required for their transcriptional regulation *in vivo*. The FlbD protein makes symmetrical base-specific contacts at three highly conserved guanine nucleotides in each half site of *ftr1* and *ftr1** at *flbG* and the single *ftr4* site at *fliF*. The dual function of FlbD in activation of class III genes and repression of the class II *fliF* promoter is consistent with a central role of FlbD as a switch protein mediating the transition from level II to level III *fla* gene expression.**

During each cell division, the bacterium *Caulobacter crescentus* differentiates to produce a sessile stalked cell and a motile swarmer cell. Swarmer cell differentiation is marked by flagellum biosynthesis at one pole of the predivisional cell through sequential activation and repression of flagellar genes (*fla* genes) during the cell cycle. The order of *fla* gene expression corresponds to the sequence of their assembly into the flagellum. The *fla* genes are organized into a transcriptional hierarchy of levels I–IV in which expression of genes at each level depends upon genes at levels above them in the hierarchy (1–3). Level I genes at the top of the hierarchy have yet to be identified, but they are presumed to encode products that respond directly to cell cycle signals and are required for expression of class IIA and IIB genes at the next lower level (3). Components of the flagellar basal body, as well as proteins that are involved in flagellar protein-specific export, are encoded by genes at level II (refs. 4–6; G.R., J. Zhao, and A.N., unpublished work), whereas genes encoding the axial filament, hook, and flagellar filament occupy the two lowest levels of the hierarchy (levels III and IV; refs. 7–13).

A role for specialized RNA polymerase holoenzymes in governing the timing of *fla* gene transcription was originally based on the observation that genes at levels III and IV of the hierarchy are transcribed by σ^{54} RNA polymerase ($E\sigma^{54}$) (7–15). Class IIB promoters, which are transcribed earlier in the cell cycle, bear little or no resemblance to σ^{54}, σ^{70}, or other described bacterial promoters (3, 5, 6, 9, 16) and are presumably recognized by a novel RNA polymerase holoenzyme, designated here $E\sigma^{Y}$.

Transcription by $E\sigma^{54}$ in *Salmonella typhimurium* and other bacteria depends on its interaction with specific activator proteins that typically bind to DNA sequence elements ≈100 bp upstream of the transcriptional start site (17). Genetic analysis has identified analogous cis-acting sequences termed *ftr* (flagellar transcription regulator) elements in *C. crescentus* that control the timing of transcription from the σ^{54}-dependent level III and IV *fla* genes (7–12, 18, 19). Another member of this *ftr* family, *ftr4*, overlaps the level II *fliF* promoter and is required to turn off *fliF* transcription at the end of its synthetic period (3). The FlbD protein described by Ramakrishnan and Newton (20) is one candidate for the transcription factor acting at these *ftr* sequences. The *flbD* gene is essential *in vivo* for transcription regulation of level III and IV *fla* genes (1), it encodes a predicted protein homologous to the σ^{54}-dependent gene transcription activator NtrC (17), and it is sufficient to activate transcription from the cloned *flbG* promoter in *Escherichia coli* (20). Mobility-shift experiments (21) also suggest that the FlbD protein binds to an *ftr* sequence within the *flbG* promoter.

Here we show that FlbD is in fact a sequence-specific DNA-binding protein that recognizes conserved bases in *ftr* sequences. We have used a purified *C. crescentus* RNA polymerase that initiates transcription from the *fliF* promoter and a reconstituted $E\sigma^{54}$ from *E. coli* that recognizes the *flbG* promoter to demonstrate that FlbD acts at *ftr* sequence elements both to activate transcription from the level III σ^{54}-dependent *flbG* promoter and to repress transcription from the level II σ^{Y}-dependent *fliF* promoter. Thus, FlbD is a key switch protein in the *fla* gene hierarchy mediating the transition from level II to level III transcription.

EXPERIMENTAL PROCEDURES

Protein Purification. *E. coli* core RNA polymerase (22) and σ^{54} (23) were purified as described. Integration host factor (IHF) was purified by the procedure of Surette and Chaconas (24). The *flbD* gene was overexpressed in a T7 expression system using plasmid pET3-b (25). FlbD was purified from ≈30 g (wet weight) of cells by disruption in a French pressure cell, ammonium sulfate precipitation at 43% saturation, and column chromatography on DEAE-Sephacel and heparin-Sepharose in 20 mM Tris, pH 7.9/5% (vol/vol) glycerol/0.1 mM EDTA/0.1 mM dithiothreitol. The purified protein was

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Abbreviations: $E\sigma^{54}$, RNA polymerase holoenzyme containing σ^{54}; IHF, integration host factor.

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>95% pure as judged by Coomassie blue staining of SDS/polyacrylamide gels and identified as FlbD by N-terminal amino acid sequence analysis (G.R., unpublished data).

C. crescentus RNA polymerase was extracted (22) from ≈100 g (wet weight) of cells and purified by chromatography on heparin-Sepharose, Bio-Rad A-1.5M, and single-stranded-DNA-cellulose. The DNA-cellulose fractions were tested for activity on the 290-bp *Bam*HI–*Hin*dIII fragment of the *fliF* promoter by run-off transcription assays (Fig. 1*B*).

Footprinting Procedures. Footprinting probes (Fig. 1*B*) were derived from pNJ5 (*flbG–flgK* promoter region; ref. 14) and pGIR125 (*fliF* promoter). Derivatives of the 290-bp *Bam*HI–*Hin*dIII fragment of *fliF* carrying mutations in the *ftr4* element were constructed by site-directed mutagenesis using oligodeoxynucleotide AB3 (5′-GGATTTACCCCGTACAAGGT-3′) to construct pAKC1 and oligodeoxynucleotide AB5 (5′-GCAGGATTTACAGTACAAGG-3′) to construct pAKC2.

DNase I and dimethyl sulfate footprinting reactions were performed essentially as described (26, 27). Binding reactions were carried out in 20 μl at 34°C in transcription buffer (50 mM Tris, pH 7.5/10 mM $MgCl_2$/50 mM KCl/0.1 mM EDTA/1 mM dithiothreitol). The cleavage products were resolved in 6% polyacrylamide sequencing gels and visualized by autoradiography.

Transcription Assays. Transcription assays on supercoiled plasmid templates were performed at 34°C as described (28). Purified reagents were added in the following concentrations: core RNA polymerase, 100 nM; σ^{54}, 200 nM; NtrB, 160 nM; NtrC, 400 nM; template, 5 nM. FlbD and IHF were added sequentially after the addition of template DNA and RNA polymerase. Run-off transcription assays were performed in 50 μl of transcription buffer with 3 μg of the *C. crescentus* RNA polymerase preparation. After 20 min of incubation at 34°C, 5 μCi (185 kBq) of [α-^{32}P]UTP was added along with GTP, ATP, CTP (0.4 mM each), UTP (0.2 mM), and heparin (100 μg/ml). The reactions were terminated by ethanol precipitation after an additional 10 min of incubation. FlbD was added to the reactions after the addition of template and RNA polymerase. The transcripts were resolved in 8% polyacrylamide denaturing gels and visualized by autoradiography.

RESULTS

FlbD Binds Specifically to *ftr* Elements *ftr1*, *ftr1, and *ftr4*.** The 19-bp sequence elements *ftr1*, *ftr2*, *ftr3*, and *ftr4* share residues that are either invariant or highly conserved at 12 of 17 positions (Fig. 1). To determine whether the FlbD protein specifically recognizes *ftr1* of *flbG* and *ftr4* of *fliF*, we used both DNase I footprinting and dimethyl sulfate protection/enhancement assays. Binding of FlbD to the *ftr1* sequence element of the Eσ^{54}-dependent *flbG* promoter was monitored with a restriction fragment of the divergent *flbG–flgK* promoter region (Fig. 1*B*). Our FlbD preparation showed strong DNase I protection at three distinct regions (Fig. 2*A*): (*i*) a 40-bp region extending from −126 to −86 of *flbG* which includes the *ftr1* sequence identified genetically by Mullin and Newton (10, 12) at −117 to −101, (*ii*) a region adjacent to *ftr1* at −155 to −133 which we have designated *ftr1**, and (*iii*) a region of protection that corresponds to the location of *ftr2* and *ftr3* in the *flgK* promoter region where FlbD would also be expected to bind (12, 18). Inspection of the *ftr1** sequence has shown that it can be aligned with *ftr1*, *ftr2*, *ftr3*, and *ftr4*; its possible role is considered in the *Discussion*. We could not determine the precise extent of protection at *ftr2* and *ftr3* in this experiment due to lack of resolution near the top of the gel.

Dimethyl sulfate methylation protection/enhancement assays identified several FlbD contacts at *ftr1* and *ftr1** (Fig. 2*B*). Methylation-dependent cleavage of guanine residues at −104, −113, and −114 of *ftr1* and at −137, −146, and −147 of *ftr1** was reduced by bound FlbD, whereas cleavage at adenine at position −119, which lies between *ftr1* and *ftr1**, became hypersensitive. These FlbD–guanine contacts at *ftr1* and *ftr1** are illustrated in Fig. 1*C*.

Repression of transcription from the class IIB *fliF* promoter depends upon the *ftr4* sequence that extends from −14 to +3 (Fig. 1 and ref. 3) and the gene encoding FlbD (1), which lies at the end of the *fliF* operon. DNase I footprinting assays (Fig. 3*A*) showed that FlbD protected a 20-bp region (−15 to +5) that includes the *ftr4* sequence. The specificity of the FlbD interaction with *ftr4* was confirmed by examining the effects on FlbD binding of *ftr4* mutations known to derepress *fliF* expression *in vivo* (3). Mutant promoters with a T → G change at residue −15 (AKC1) and a deletion of the guanine residues at −13 and −12 (AKC2) decreased the ability of FlbD to protect *ftr4* from DNase I cleavage (Fig. 3*A*). Further analysis of FlbD binding at *ftr4* by dimethyl sulfate protection assays (Fig. 3*B*) revealed a pattern of protection (positions −14, −13, −12, and −3) that was similar to that observed at *ftr1* and *ftr1** (see Fig. 1*C* for comparison). The similarity of the FlbD contacts at *ftr1*, *ftr1**, and *ftr4* provides strong evidence that their function *in vivo* depends upon binding of the FlbD protein.

FlbD Is an Activator of σ^{54}-Dependent Transcription *in Vitro*. The *C. crescentus flbG* and *flgK* promoters are recognized by *E. coli* Eσ^{54}, and in the presence of NtrB and NtrC

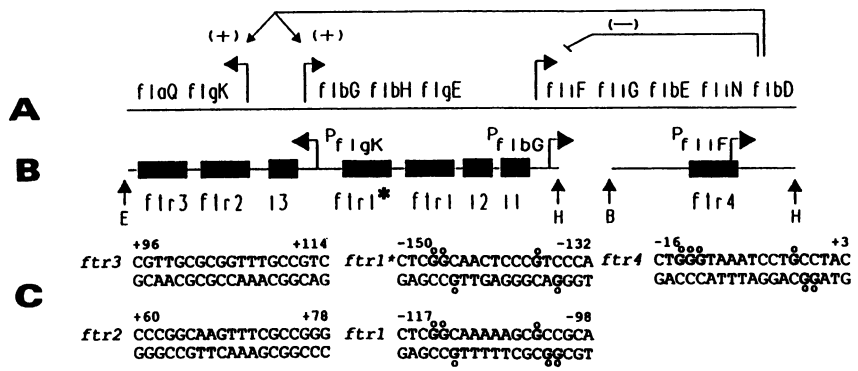

FIG. 1. *ftr* sequences of the *flbG–flgK* and *fliF* promoter regions. (*A*) Organization of the *fliF*, *flbG*, and *flgK* transcription units. Arrows drawn from the *flbD* coding region illustrate positive (+) and negative (−) regulation. (*B*) Maps of the 650-bp *Eco*RI (E)–*Hin*dIII (H) fragment of the *flbG–flgK* promoter region and the 290-bp *Bam*HI (B)–*Hin*dIII (H) fragment of the *fliF* promoter used in these studies. (*C*) Nucleotide sequence of the *ftr* elements *ftr1*, *ftr2*, *ftr3*, and *ftr4* with their positions relative to the transcriptional start sites labeled. Symmetrical guanines of the *ftr* sequences that are protected from methylation with dimethyl sulfate by FlbD are indicated by a circle above or below the base (data for bottom strand not shown).

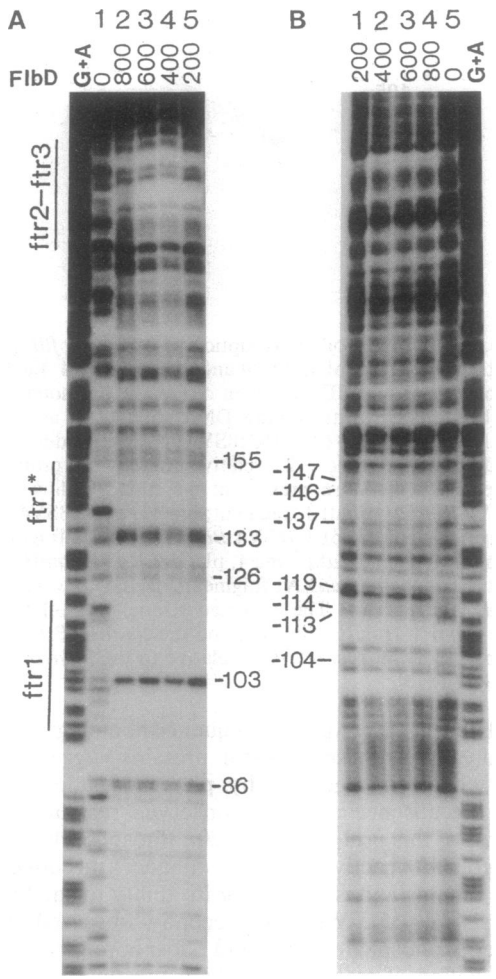

FIG. 2. Footprinting of the purified FlbD protein to the *flbG–flgK* promoter region. The probe for *A* and *B* was the 650-bp *Eco*RI–*Hin*dIII fragment of pNJ5 labeled at the *Hin*dIII site with Klenow DNA polymerase. (*A*) DNase I protection pattern in the absence (lane 1) or presence (lanes 2–5) of FlbD. (*B*) Dimethyl sulfate methylation protection/enhancement assays in the absence (lane 5) or presence (lanes 1–4) of FlbD. The final concentration of FlbD (nM) is indicated above each lane. Lanes labeled G+A contained products of Maxam–Gilbert G + A sequencing reactions. Nucleotides numbered in *A* are the endpoints of the regions protected from DNase I cleavage whereas those in *B* correspond to those guanine and adenine residues where dimethyl sulfate-dependent methylation was either inhibited or enhanced.

transcription is initiated *in vitro* from the same start utilized by *Caulobacter* RNA polymerase *in vivo* (14). Because genetic experiments have shown that *flbG* transcription requires *flbD* and an intact *ftr1* and is stimulated ≈5-fold by the presence of the *ihf1* and *ihf2* binding sites (10, 12, 18, 19, 29), we examined whether transcription from the *flbG* promoter in our *in vitro* assay system would depend on purified FlbD, the *ftr1* sequence, and IHF.

Single-cycle transcription assays were performed with supercoiled template plasmids that carried the restriction fragment used for footprinting of *ftr1* (Fig. 1*B*). In plasmid pNJ5, the *flbG* transcription start site lies 365 nt upstream from the T7 Rho-independent terminator (14). Addition of NtrB and NtrC to the reaction mixtures gave the expected 365-nt transcript (Fig. 4, lane 1). When FlbD was substituted for NtrB and NtrC (lanes 3–9), it also activated transcription by the *E. coli* Eσ^{54} and generated a transcript that was identical in size. The presence of NtrB, which is essential for NtrC-dependent activation, had no effect upon the ability of FlbD to activate *flbG* transcription (compare lanes 3 and 4).

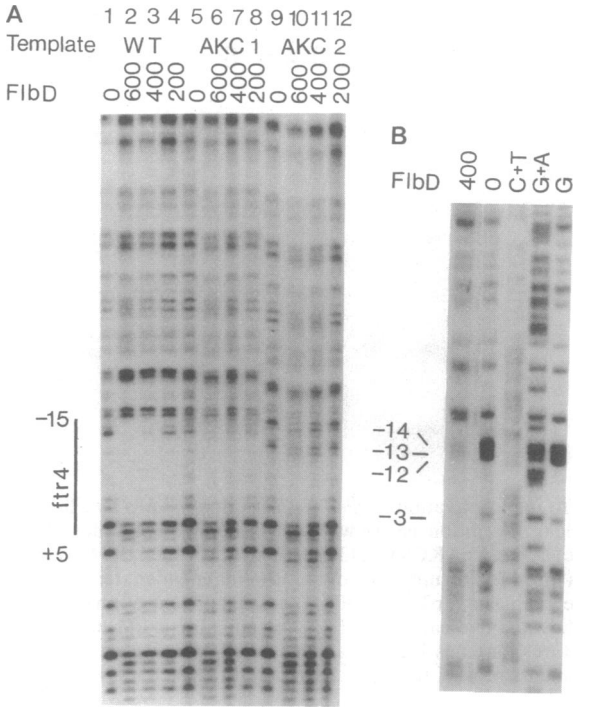

FIG. 3. Footprinting of the FlbD protein at the *fliF* promoter region. (*A*) DNase footprinting. Probes for these assays were the 290-bp *Bam*HI–*Hin*dIII *fliF* promoter fragments from pGIR125 (wild type, lanes 1–4), pAKC1 (−15 T→ G mutant, lanes 5–8), and pAKC2 (deletion of guanines at −14 and −13, lanes 9–12) labeled at their *Hin*dIII sites with Klenow DNA polymerase. The final concentration of FlbD (nM) is indicated above the appropriate lanes. Nucleotides corresponding to the endpoints of DNase I protection relative to the transcriptional start site of *fliF* are shown. (*B*) Dimethyl sulfate methylation protection/enhancement assay using the wild-type fragment in *A*. FlbD was added to a final concentration of 400 nM. Shown in the three lanes at right are products of Maxam–Gilbert sequencing reactions that were derived from the same labeled fragment. The numbered nucleotides at left correspond to the positions of guanine residues protected from methylation by FlbD.

Based on intensity of the labeled bands, addition of *E. coli* IHF along with FlbD resulted in ≈5-fold higher levels of the transcript than with the FlbD protein alone (Fig. 4, lanes 7–9).

Introduction of a single base change in *ftr1* (G→ T at −113 in pAKC5), which dramatically reduces *flbG* transcription *in vivo* (10, 12), eliminated FlbD-dependent transcription from the *flbG* promoter *in vitro* (Fig. 4*B*, lanes 3 and 4), whereas activation by NtrC was unaffected (compare Fig. 4*B*, lane 1 with Fig. 4*A*, lane 1). Activation of *flbG* transcription by high concentrations of NtrC does not depend upon the *ftr1* sequence (14) and probably results from NtrC binding nonspecifically to the template DNA.

FlbD Inhibits Transcription of the *fliF* Promoter *in Vitro*. A simple model for negative autoregulation of *fliF* transcription is that binding of FlbD to *ftr4* (Fig. 3), which overlaps the *fliF* transcriptional start site, inhibits transcription. To test this model directly, we examined the ability of FlbD to inhibit transcription from the *fliF* promoter *in vitro*. Because the *fliF* promoter and other class IIB promoters appear to be different from other prokaryotic promoters (3, 5, 6, 16), we purified an RNA polymerase activity from *C. crescentus* cells based on its specificity for transcription from the σ^{Y}-dependent promoter of *fliF* (*Materials and Methods*).

The specificity of *fliF* promoter recognition was tested by run-off transcription assays with two *fliF* templates (3) that differed at their 3′ termini by 18 nt with respect to the *in vivo* transcription start site (Fig. 5). The transcripts generated from these template fragments migrated very close to the

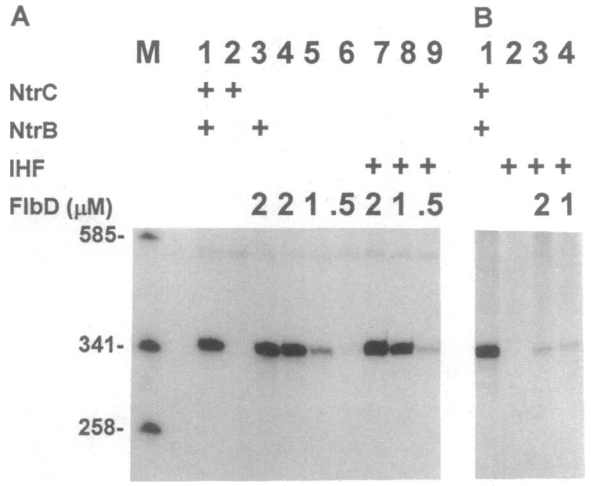

FIG. 4. Activation of *flbG* transcription *in vitro* by FlbD. Single-cycle transcription assays were performed with plasmids pNJ5 (wild type) (*A*) and pAKC5 (−113 G → T) (*B*) in which the *flbG* promoter was fused 365 bp upstream of the Rho-independent terminator of the parental plasmid pTE103 (30). The final concentration of FlbD (μM) is shown above each lane. IHF was added (100 nM) to the reaction mixtures for lanes 7–9 of *A* and lanes 2–4 of *B*. End-labeled *Sau*3 A1 fragments of pUC18 were run in lane M as size markers.

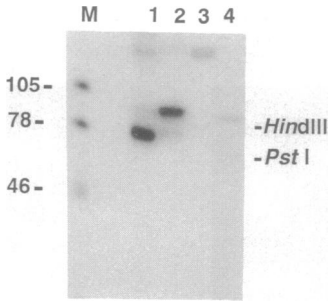

FIG. 5. *In vitro* run-off transcription assays using *fliF* promoter fragments derived from pUC18SV4 and pUC18SV4#4. Each carries the 290-bp *Bam*HI–*Hin*dIII fragment of the *fliF* promoter (Fig. 1*B*) that has been filled in with Klenow DNA polymerase and ligated to the *Hin*cII site of pUC18 (3). pUC18SV4#4 carries a deletion of the TA residues at −24 and −25 relative to the *fliF* promoter (3). Template fragments were present at ≈5 nM as follows: lane 1, pUC18SV4 *Bam*HI–*Hin*dIII fragment; lane 2, pUC18SV4 *Bam*HI–*Pst* I fragment; lane 3, pUC18SV4 *Bam*HI–*Hin*dIII fragment with FlbD protein added at 1 μM; lane 4, pUC18SV4#4 *Bam*HI–*Hin*dIII fragment. End-labeled *Sau*3 A1 fragments of pUC18 were loaded in lane M as size markers. The expected sizes of run-off transcripts from the *Bam*HI–*Hin*dIII fragment and the *Bam*HI–*Pst* I fragment are 102 nt and 84 nt, respectively, relative to the *in vivo* transcriptional start site (3).

expected 102-nt and 84-nt lengths (Fig. 5, lanes 1 and 2). Promoter specificity was examined with a mutant template (deletion of nucleotides −24 and −25) that substantially reduces *in vivo* transcription of *fliF* (3). The amount of the expected 102-nt run-off transcript from the mutant template (Fig. 5, lane 4) was reduced relative to the wild type (Fig. 5, lane 1). Thus, the *Caulobacter* RNA polymerase preparation used in our studies requires sequence elements in the *fliF* promoter region that are identical to those required *in vivo*.

When FlbD was included in the run-off transcription reaction with a wild-type template (Fig. 5, lane 3), the 102-nt transcript that was observed in its absence (Fig. 5, lane 1) was eliminated. These results are consistent with previous genetic findings that *flbD* negatively autoregulates *fliF* expression (1) and support the conclusion that FlbD acts during the cell cycle as a negative switch to turn off synthesis of flagellar basal body proteins encoded by the *fliF* operon.

DISCUSSION

These studies provide compelling evidence that FlbD is a key regulator in the periodic expression of flagellar genes in the *C. crescentus* cell cycle. The FlbD protein turns off transcription from the σ^{Y}-dependent *fliF* promoter at level II and activates transcription from σ^{54}-dependent *flbG* promoter at level III of the *fla* gene hierarchy. The function of FlbD as a positive and negative regulator is mediated by a family of cis-acting DNA sequences designated as *ftr*.

FlbD Is Both an Activator and a Repressor of *fla* Gene Transcription. FlbD-dependent activation of *flbG* transcription by the reconstituted Eσ^{54} required an intact *ftr1* sequence and was stimulated by IHF (Fig. 4). These results are in complete agreement with previous genetic analyses of the functions of the *ftr1*, *ihf1*, and *ihf2* sequence elements (9, 10, 12, 18, 19, 29). The activity of FlbD as a repressor was demonstrated by using an RNA polymerase purified from *C. crescentus* that recognizes the σ^{Y} promoter of the *fliF* gene (Fig. 5). Moreover, full inhibition of *fliF* transcription by FlbD depended on an intact *ftr4* sequence element located at −15 to +5 (unpublished data). These observations are consistent with genetic results showing that mutations in either *flbD* or *ftr4* increase *fliF* transcription by 5- to 10-fold (refs. 1 and 3; G.R., J. Zhao, and A.N., unpublished results).

Regulation of FlbD Activity. Sequence homology of FlbD to the family of response-regulator proteins suggests that its activity could be regulated by phosphorylation (31, 32), despite the absence of three conserved residues that are signatures of this protein family (20). Phosphorylation of the homologous Eσ^{54} activator, NtrC, enhances its cooperativity in DNA binding and is critical for its ability to catalyze ATP hydrolysis (33–35). We have recently observed that the high-energy phosphate compound phosphoramidate potentiates activation of transcription from the *flbG* promoter by FlbD (36) and that FlbD^{S140F}, which contains a substitution (Ser^{140} → Phe) corresponding to that in the constitutively active NtrC protein NtrC^{S160F} (33), is a better activator of transcription *in vitro* than wild-type FlbD (36). In addition, Wingrove *et al.* (21) have recently detected the phosphorylation of an epitope-tagged FlbD protein late in the *C. crescentus* cell cycle, when class III and IV *fla* genes are transcribed.

The capacity of FlbD to activate *flbG* transcription and repress *fliF* transcription is subject to two distinct pathways of control. Activation of level III *flbG* transcription requires, in addition to *flbD*, synthesis of the switch proteins, the M-ring protein, and all other level II genes (refs. 1 and 2; G.R., J. Zhao, and A.N., unpublished results). Mutations in any of them prevent Eσ^{54}-dependent transcription of genes at levels III and IV, presumably because basal-body assembly cannot be completed. In contrast, only the level II *fliF*, *fliG*, and *flbE* genes appear to be directly involved in negative regulation of *fliF* by *flbD*: mutations in any of these genes results in a 5- to 10-fold increase in the levels of *fliF* transcription (ref. 1; G.R., J. Zhao, and A.N., unpublished results). Thus, FlbD activity *in vivo* must be governed by a device(s) that senses the expression of other basal-body genes and/or their assembly into a complete structure. Whether these controls are exerted through covalent modification, such as phosphorylation, or another mechanism will require both biochemical and genetic analyses.

FlbD Makes Base-Specific Contacts with *ftr1*, *ftr1, and *ftr4*.** Dimethyl sulfate footprinting reveals specific interactions of bound FlbD with cis-acting *ftr* sequences that have been shown genetically (3, 9, 10, 12, 19) to govern transcription from levels II and III promoters. The dimethyl sulfate footprints of FlbD at *ftr1*, *ftr1**, and *ftr4* demonstrate that FlbD

binds near to conserved symmetrical guanine residues on both strands of these sequences (see marked guanine residues in Fig. 1*C*), and the contact sites within each *ftr* sequence lie in regions of near-dyad symmetry [C(C/G)CGGCA(G/A)A] that span consecutive major grooves. The symmetry of methylation protection suggests that FlbD, which contains a putative helix–turn–helix motif, binds to the *ftr* sequence as a dimer with each monomer contacting one half-site; such a mechanism is supported by crystallographic analysis of several bacterial transcription factors containing helix–turn–helix motifs (37–39).

The helical arrangement of the adjacent *ftrI* and *ftrI**, which are spaced 33 bp from center to center of the adjacent elements, is strikingly similar to that of the two adjacent high-affinity binding sites for NtrC at the *glnAp2* promoter (40, 41). Binding of NtrC to these sites at *glnAp2* generates maximum activation of transcription by $E\sigma^{54}$ and may constitute a mechanism for the formation of an oligomeric NtrC complex that catalyzes ATP hydrolysis and potentiates isomerization of the closed $E\sigma^{54}$ complex into the open complex (33–35). We have now obtained evidence *in vitro* that *ftrI** is required for *flbG* transcription (unpublished results) and believe that the *ftrI*/*ftrI** pair plays a similar role in activator binding and function.

Role of FlbD in Regulation of the *fla* Gene Hierarchy. Based on our analysis of FlbD binding at *ftrI*, *ftrI**, and *ftr4*, we have now identified 19-bp sequence elements that are very similar to these FlbD binding sites at about −100 of each level III and IV *fla* gene promoters (36). Highly conserved nucleotides include the guanine residues that are specifically contacted by the FlbD protein at *ftrI*, *ftrI**, and *ftr4* and the A+T-rich central core. With the exception of the *fljL* promoter, these putative *ftr* sequences are organized in pairs with approximately three helical turns between centers of the adjacent sites. It is our hypothesis that FlbD functions at these sequences as a key component of the developmental switch whose activity during a specific stage of the cell cycle is required to establish repression of class II genes transcribed from the *fliF* promoter and to act as a global activator of transcription from the $E\sigma^{54}$-dependent promoters of class III and class IV genes.

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1. Newton, A., Ohta, N., Ramakrishnan, G., Mullin, D. & Raymond, G. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 6651–6655.
2. Xu, H., Dingwall, A. & Shapiro, L. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 6656–6660.
3. VanWay, S. M., Newton, A., Mullin, A. H. & Mullin, D. A. (1993) *J. Bacteriol.* **175**, 367–376.
4. Ramakrishnan, G., Zhao, J.-L. & Newton, A. (1991) *J. Bacteriol.* **173**, 7283–7292.
5. Yu, J. & Shapiro, L. (1992) *J. Bacteriol.* **174**, 3327–3338.
6. Dingwall, A., Zhuang, W. Y., Quon, K. & Shapiro, L. (1992) *J. Bacteriol.* **174**, 1760–1768.
7. Chen, L.-S., Mullin, D. & Newton, A. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 2860–2864.
8. Minnich, S. A. & Newton, A. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 1142–1146.
9. Mullin, D., Minnich, S., Chen, L.-S. & Newton, A. (1987) *J. Mol. Biol.* **195**, 939–943.
10. Mullin, D. A. & Newton, A. (1989) *J. Bacteriol.* **171**, 3218–3227.
11. Dingwall, A., Gober, J. W. & Shapiro, L. (1990) *J. Bacteriol.* **172**, 6066–6076.
12. Mullin, D. & Newton, A. (1993) *J. Bacteriol.* **175**, 2067–2076.
13. Dingwall, A., Garman, J. D. & Shapiro, L. (1992) *J. Mol. Biol.* **228**, 1147–1162.
14. Ninfa, A. J., Mullin, D. A., Ramakrishnan, G. & Newton, A. (1989) *J. Bacteriol.* **171**, 383–391.
15. Brun, Y. V. & Shapiro, L. (1992) *Genes Dev.* **6**, 2395–2408.
16. Stephens, C. L. & Shapiro, L. (1993) *Mol. Microbiol.* **9**, 1169–1179.
17. Kustu, S., Santero, E., Keener, J., Popham, D. & Weiss, D. (1989) *Microbiol. Rev.* **53**, 367–376.
18. Gober, J. W. & Shapiro, L. (1992) *Mol. Biol. Cell* **3**, 913–926.
19. Gober, J. W., Champer, R., Reuter, S. & Shapiro, L. (1991) *Cell* **64**, 381–391.
20. Ramakrishnan, G. & Newton, A. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 2369–2373.
21. Wingrove, J. A., Mangan, E. K. & Gober, J. W. (1993) *Genes Dev.* **7**, 1979–1992.
22. Burgess, R. R. & Jendrisak, J. J. (1975) *Biochemistry* **14**, 4634–4638.
23. Hunt, T. P. & Magasanik, B. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 8453–8457.
24. Surette, M. G. & Chaconas, G. (1989) *J. Biol. Chem.* **264**, 3028–3034.
25. Studier, F. W., Rosenberg, A. H., Dunn, J. J. & Dubendorf, J. W. (1990) *Methods Enzymol.* **185**, 60–89.
26. Galas, D. & Schmitz, A. (1978) *Nucleic Acids Res.* **5**, 3157–3170.
27. Ogata, R. T. & Gilbert, W. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 5851–5854.
28. Ninfa, A. J., Reitzer, L. J. & Magasanik, B. (1987) *Cell* **50**, 1039–1046.
29. Gober, J. W. & Shapiro, L. (1990) *Genes Dev.* **4**, 1494–1504.
30. Elliot, T. & Geiduschek, E. P. (1984) *Cell* **36**, 211–219.
31. Stock, J. B., Ninfa, A. J. & Stock, A. M. (1989) *Microbiol. Rev.* **53**, 450–490.
32. Parkinson, J. S. & Kofoid, E. C. (1992) *Annu. Rev. Genet.* **26**, 71–112.
33. Weiss, D. S., Batut, J., Klose, K. E., Keener, J. & Kustu, S. (1991) *Cell* **67**, 155–167.
34. Weiss, V., Claverie-Martin, F. & Magasanik, B. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 5088–5092.
35. Porter, S. C., North, A. K., Wedel, A. B. & Kustu, S. (1993) *Genes Dev.* **7**, 2258–2273.
36. Benson, A. K., Wu, J. & Newton, A. (1994) *Res. Microbiol.*, in press.
37. Otwinowski, Z., Schevitz, R. W., Zhang, R. G., Lawson, C. L., Joachimiak, A., Marmostein, R. Q., Luisi, B. F. & Sigler, P. B. (1988) *Nature (London)* **335**, 321–329.
38. Jordan, S. R. & Pabo, C. O. (1988) *Science* **242**, 893–899.
39. Shultz, S. C., Shields, G. C. & Steitz, T. A. (1990) *J. Mol. Biol.* **213**, 159–166.
40. Hirschman, J., Wong, P.-K., Sei, K., Keener, J. & Kustu, S. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 7525–7529.
41. Sasse-Dwight, S. & Gralla, J. D. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 8934–8938.