A σ^{54} transcriptional activator also functions as a pole-specific repressor in *Caulobacter*

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The differential localization of proteins in the *Caulobacter* predivisional cell leads to the formation of two distinct progeny cells: a motile swarmer cell and a sessile stalked cell. Pole-specific transcription in the predivisional cell is one mechanism responsible for protein localization. Here we show that the σ^{54} transcriptional activator FlbD, which activates swarmer pole-specific transcription of a subset of late flagellar genes, is also capable of functioning as a pole-specific repressor of the early flagellar *flhF* operon. DNase I footprinting and methylation interference assays indicate that FlbD binds to regions of the *flhF* promoter at regions that would be likely to interfere with the binding of RNA polymerase. A mutation that abolishes FlbD binding results in up to a fourfold increase in *flhF* promoter expression. This mutation alters both the spatial and temporal pattern of *flhF* expression resulting in the inappropriate expression of the *flhF* operon in the swarmer pole of the predivisional cell. These results demonstrate that FlbD represses early flagellar gene expression in the swarmer pole of the *Caulobacter* predivisional cell. This is the first instance in which a protein specifically involved in pole-specific repression has been identified in *Caulobacter*. The restriction of FlbD activity to the swarmer pole accomplishes two regulatory missions by simultaneously activating late flagellar gene expression and repressing early flagellar genes.

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The bacterium *Caulobacter crescentus* undergoes a programmed sequence of differentiation events during its cell cycle. Cell division results in the formation of two distinct daughter cells: a motile swarmer cell that contains a single, polar flagellum, and a sessile stalked cell (for review, see Newton and Ohta 1990; Gober and Shapiro 1991; Gober et al. 1991a; Fig. 1A, below). The two progeny cell types differ in their capacity to reinitiate DNA replication and in their pattern of gene expression. The formation of these dissimilar progeny cell types is a consequence of the asymmetric localization of proteins in the *Caulobacter* predivisional cell. The mechanisms that generate asymmetry during the *Caulobacter* cell cycle are analogous to those of other developmental organisms such as *Bacillus*, *Drosophila*, the nematode, and yeast (for review, see Horvitz and Herskowitz 1992).

Two different mechanisms are responsible for polar protein localization in *Caulobacter*. The first involves the targeting of proteins to a specific pole of the predivisional cell. It has been shown that two proteins involved in the heat shock response, Lon and DnaK, are specifically targeted to the stalked pole (Reuter and Shapiro 1987). Conversely, motility-related proteins such as the

methyl-accepting chemotaxis proteins are targeted specifically to the swarmer pole of the predivisional cell (Nathan et al. 1986; Loewy et al. 1987; Alley et al. 1992). Pole-specific protein segregation may involve the presence of specialized polar structures to which the proteins are targeted.

The second mechanism that serves to localize proteins is polar gene expression. As with protein targeting, this mechanism appears to operate in both poles of the predivisional cell. The stalked cell is capable of immediately reinitiating DNA replication after cell division; this ability is silenced in the swarmer cell. The capacity to reinitiate DNA replication may be attributable, in part, to localized gene expression. For example, the gene encoding the B subunit of DNA gyrase, a protein essential for DNA replication, is expressed in the stalked pole of the predivisional cell (Rizzo et al. 1993). Flagellar proteins may also be positioned by pole-specific gene expression. The genes encoding the external components of the flagellum, the hook operon, *flaNQ* and *flgK*, are expressed in the swarmer pole of the late predivisional cell (Milhausen and Agabian 1983; Gober et al. 1991b; Gober and Shapiro 1992). Promoter sequences contain all the information necessary to direct localized expression, indicating that swarmer pole-specific expression is attributable to localized transcription (Gober et al. 1991b;

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Gober and Shapiro 1992). These late flagellar genes share similar *cis*-regulatory promoter sequences, suggesting a common mechanism of transcriptional activation (Gober et al. 1991c; Gober and Shapiro 1992). They all require σ^{54} -containing RNA polymerase and the DNA bending protein, integration host factor (IHF) for expression (Gober and Shapiro 1990, 1992). Like other σ^{54} -activated promoters, these flagellar promoters depend on specific enhancer-binding proteins to activate transcription (for review, see Kustu et al. 1989). They contain an essential, conserved enhancer element termed *ptr*, located ~100 bp from the start site of transcription (Minnich and Newton 1987; Mullin et al. 1987; Mullin and Newton 1989; Gober and Shapiro 1992). The σ^{54} transcriptional activator protein FlbD has been shown to bind specifically to the *ptr* enhancer element (Wingrove et al. 1993). Enhancer mutations that abolish transcription also prevent FlbD from binding, indicating that FlbD is the transcriptional activator of *ptr*-containing promoters (Wingrove et al. 1993).

Spatial transcription of flagellar genes could be accomplished through the specific targeting of FlbD to the swarmer compartment of the predivisional cell. To test this idea, we assayed the subcellular localization of FlbD using immunofluorescence microscopy (Wingrove et al. 1993). In contrast to chemotaxis and flagellum proteins, which are specifically targeted to the swarmer pole (Alley et al. 1992), FlbD is present in both compartments of the predivisional cell. This result suggests that FlbD activity is restricted to the swarmer pole of the predivisional cell. FlbD is homologous to the response regulator proteins of two-component systems such as NtrC (for review, see Parkinson and Kofoed 1992). This class of protein requires the phosphorylation of a conserved aspartate residue in the amino terminus to become active (Sanders et al. 1992). We have shown that FlbD is capable of being phosphorylated both in vivo and in vitro. FlbD is only phosphorylated late in the cell cycle, coincident with the activation of the *ptr*-containing flagellar genes (Wingrove et al. 1993). Additionally, the expression of a mutant version of FlbD that is constitutively active in the absence of phosphorylation results in the inappropriate expression of *ptr*-containing flagellar genes in the stalked pole of the predivisional cell (Wingrove et al. 1993). Thus, it is apparent that the phosphorylation and activation of FlbD is a key element in controlling the expression of *ptr*-containing flagellar genes.

In this paper we show that in addition to being a swarmer compartment-specific transcriptional activator, FlbD is capable of acting as a pole-specific repressor of the *fliF* operon. The *fliF* operon contains five genes including *flbD* and *fliF*, which encodes the M-ring of the flagellum (Ramakrishnan et al. 1992; Fig. 1B). The *fliF* operon shares conserved *cis*-regulatory sequences with other early flagellar gene promoters (class II) such as *fliQR* and *fliLM* (Stephens and Shapiro 1993; Van Way et al. 1993; Fig. 1A). These elements located at -10 and -35 bp are thought to be the binding site of a yet unidentified unique σ -factor termed σ^R (Stephens and Shapiro 1993). Mutations in the conserved -10 and -35

elements of this type of promoter effectively eliminate transcriptional activity (Stephens and Shapiro 1993; Van Way et al. 1993). Directly adjacent to the *fliF* promoter are DNA sequences that, when mutated, result in an increase of transcription, suggesting that this promoter is also subject to negative regulation (Van Way et al. 1993).

The *fliF* promoter region contains several repeated sequences that are similar to the *ptr* element of σ^{54} promoters (Fig. 1C). We show that FlbD specifically binds to two sites on the *fliF* promoter adjacent to the σ^R regulatory element. The DNase I protection pattern indicates that FlbD binding in vivo may interfere with the binding of RNA polymerase to promoter sequences. The addition of a phosphodonator, acetyl phosphate, to DNase I footprinting reactions resulted in a higher binding affinity of FlbD for the *fliF* promoter, suggesting that the activated form of FlbD might bind to the *fliF* promoter with greater affinity. Footprinting by methylation interference identified six guanine residues that, when methylated by DMS, inhibited the binding of FlbD to the promoter. Utilizing site-directed mutagenesis, we altered one of the critical guanine residues at -3 bp. This mutation not only abolished FlbD binding to the *fliF* promoter, as determined by DNase I footprinting, but also resulted in a fourfold increase in promoter expression in vivo. The analysis of the mutant promoter revealed an altered pattern of spatial and temporal expression. The level of expression from the mutant *fliF* promoter was approximately sevenfold higher than that observed from the wild-type *fliF* promoter in late predivisional cells. At this stage of the cell cycle, expression of the *fliF* promoter is restricted to the stalked cell pole of the predivisional cell. In contrast, the *fliF* promoter with a mutant FlbD-binding site is expressed in both poles of the predivisional cell. These results suggest that FlbD acts as a negative regulator of *fliF* expression specifically in the swarmer pole. Therefore, the localization of FlbD activity to the swarmer pole of the predivisional cell simultaneously accomplishes the transcriptional activation of late flagellar genes and the repression of early flagellar genes.

Results

FlbD binds to the fliF promoter

The *fliF* operon is one of the earliest sets of flagellar genes expressed in the *Caulobacter* cell cycle (Ohta et al. 1984; Fig. 1A). Mutations in this operon abolish the expression of flagellar genes found lower in the flagellar hierarchy (Ohta et al. 1984). One of the gene products of the *fliF* operon is the σ^{54} transcriptional activator FlbD (Ramakrishnan and Newton 1990; Fig. 1B). We have shown previously that the activation of FlbD, which occurs through phosphorylation, is responsible for the temporal and spatial regulation of a subset of class III and IV σ^{54} -dependent flagellar genes (Wingrove et al. 1993). The expression of these genes occurs later in the *Caulobacter* cell cycle and is restricted to the swarmer pole of the

Figure 1. (A) Diagram of the *C. crescentus* cell division cycle and regulation of flagellum biogenesis (for review, see Newton and Ohta 1990; Gober and Shapiro 1991; Gober et al. 1992). The oval or theta structures within the cells represent the chromosome. Motile swarmer cells (depicted at the far left) are unable to initiate chromosomal DNA replication. After a defined period that is governed by an as yet unknown internal timing mechanism, a series of global morphogenetic changes take place in the swarmer cell. The flagellum is shed, a stalk is synthesized in its place, and DNA replication initiates. Replication triggers the flagellar biogenesis cascade, which is regulated by a transacting hierarchy. Transcription of early flagellar genes (class II) is thought to be triggered by the synthesis of a novel σ -subunit of RNA polymerase (σ^R). Class II genes encode early flagellar structures (*fliF*, M-ring; *fliLM*, switch), components of the flagellar assembly apparatus (*fliP*), and transcription factors (*flbD*). (Note that only a subset of the genes required for flagellar biogenesis is represented here.) The expression of class II genes and the assembly of early flagellar structures are required for the expression of late flagellar genes (classes III and IV). The late flagellar genes encode a portion of the basal body and the external components of the flagellum, such as the hook and the filament (*flgL* and *flgK*). Class III and IV gene promoters are transcribed by σ^{54} RNA polymerase, and those shown here, the hook operon, *flaNQ*, *flgL*, and *flgK* are directly activated by the transcription factor, FlbD. (B) Schematic map of the *fliF* operon (GenBank accession no. M98855). The *fliF* operon is located adjacent to the *flaNQ* and hook operons (Ohta et al. 1984; Mullin et al. 1987). The arrows denote the direction of transcription. (C) Sequence of the *fliF* promoter region. The sequence of the *fliF* promoter region is shown from -100 to +47 bp relative to the transcription start site. This sequence has been published previously (Mullin et al. 1987). The start site of transcription (Mullin et al. 1987) is denoted by the +1 and the arrow showing the direction of mRNA synthesis. Several *ftr* half-sites are indicated by *ftr'*.

predivisional cell (Gober et al. 1991b; Gober and Shapiro 1992; Fig. 1A). Unlike FlbD-activated flagellar genes, the expression of the *fliF* operon is repressed approximately threefold in the swarmer pole of the predivisional cell compared with its expression in the stalked pole (Gober et al. 1991c).

We have shown previously through gel mobility shift assays that purified FlbD binds specifically to the *ftr* elements located in the promoters of *flbG*, *flgK*, *flgL*, and *flaN* (Wingrove et al. 1993). The *ftr* enhancer contains a conserved consensus sequence with a dyad symmetry (GCCG_nCGGC) and is usually located ~100 bp adjacent to the transcription start site of flagellar σ^{54} promoters. The *fliF* promoter contains several repeated *ftr* half-sites (GCCG or CGGC) scattered throughout the promoter region from -100 to +35 bp from the transcription start site (Fig. 1C). Previous epistasis experiments have shown that *fliF* transcription is increased in strains with mutations in class II flagellar genes, including an *flbD* mutant strain (SC1032) (Newton et al. 1989; see Fig. 4A, below). These data, in conjunction with the presence of FlbD half-binding sites in the *fliF* promoter region, suggests that FlbD might in fact serve as a negative regulator of *fliF* expression.

To determine whether FlbD specifically binds to the

promoter region of the *fliF* operon, both DNase I and methylation interference footprinting assays were utilized. In the first experiment, DNase I protection was performed using a 294-bp *Bam*HI-*Hind*III fragment (-112 to +72 bp relative to the start site of transcription) uniquely end-labeled with 32 P at the *Hind*III site. Increasing concentrations of the purified His-FlbD fusion protein (2.4–60 nM) resulted in strong protection from DNase I attack, with the strongest protected region ranging from -19 to +16 bp (Fig. 2). In addition, a protected region between -52 and -35 bp was observed when the highest concentrations of His-FlbD were used (12 and 60 nM), suggesting the presence of a weaker binding site for FlbD. A third, weaker protected region located from +16 to +72 bp is also detected when high concentrations of His-FlbD were used in the assay. This experiment indicates that FlbD binding to the *fliF* promoter would effectively obscure the promoter region and may therefore directly regulate *fliF* transcription.

As noted above (Fig. 1C), there are several *ftr* half-sites located in the *fliF* promoter region. To determine the residues critical for FlbD binding to this sequence, methylation interference was performed utilizing both strands of the promoter. To facilitate the footprinting of the bottom strand, a *Bam*HI restriction site was intro-

Figure 2. DNase I analysis of FlbD binding to the *fliF* promoter. One pmole of a uniquely ^{32}P -end-labeled fragment corresponding to -112 to $+74$ bp of the *fliF* promoter was subjected to digestion with DNase I in the presence of increasing amounts of pure FlbD (2.4 – 60 nM). Reactions in which no protein was present are denoted by $-$. A strong binding site exists between $+8$ and -19 bp, whereas a weaker site is present between -33 and -49 bp. Positions of the conserved elements in these binding sites in relationship to the -10 - and -35 -bp positive regulatory sequences are indicated at right. The same probe was subject to chemical sequencing (G and G+A reactions) (Maxam and Gilbert 1977).

Figure 3. Identification by methylation interference assay of residues critical for the binding of FlbD to the *fliF* promoter. Uniquely ^{32}P -end-labeled probes representing both strands of the *fliF* promoter were treated with DMS. Modified probes were subjected to a gel mobility shift assay using 120 nM of pure FlbD. Shifted and nonshifted bands were excised and cleaved with piperidine, and the resulting products were electrophoresed on an 8% acrylamide-urea sequencing gel. (A) Results of methylation interference analysis of the bottom strand of the *fliF* promoter. (Lane 1) Shifted probe; (lane 2) probe that was not shifted. Sequence positions in relation to the start site of transcription are indicated at right. (B) Results of methylation interference analysis of the top strand of the *fliF* promoter. (Lane 1) Shifted probe; (lane 2) probe that was not shifted. Sequence positions are indicated at right. (C) The sequence of the *fliF* promoter shown in A and B. Positions on the sequence in relation to the transcriptional start site are given above the top strand. Arrows indicate residues that, when methylated, interfered with FlbD binding to the *fliF* promoter.

duced by the polymerase chain reaction (PCR) at the -72 -bp position. DNA strands were uniquely end-labeled with ^{32}P either at the *Bam*HI or *Hind*III site. Treatment of these promoter fragments with dimethylsulfate (DMS) resulted in the identification of six guanine residues critical for FlbD binding: four on the top strand located at positions -14 , -13 , -12 , and -3 bp, and two on the bottom strand located at positions -2 and -1 bp (Fig. 3). Surprisingly, these residues did not constitute a *ftr* consensus sequence. The regions required for binding, -14 , -13 , -12 bp and -3 , -2 , -1 bp, are, however, probably functionally analogous to *ftr* half-sites. This sequence does contain elements similar to the *ftr* consensus, most notably a conserved GG sequence with dyad symmetry (Fig. 3). However, the spacing between the inverted repeat of this GG sequence is separated by one extra base pair when compared to the *ftr* consensus sequence. In addition to these critical bases, a slightly hypersensitive site was seen at position -35 bp, corresponding to the region identified by DNase I protection as the weaker FlbD-binding site. These sequences represent unorthodox FlbD-binding sites as they diverge significantly from the sequences found in *ftr* enhancers. Nevertheless, these experiments show that purified FlbD binds to the *fliF* promoter in vitro, in a region that could possibly interfere with the binding of RNA polymerase.

Mutagenesis of the *fliF* promoter

To determine the significance of FlbD binding on *fliF* promoter expression in vivo, we created two different mutations within this region. The guanine residue at

position -3 bp identified by methylation interference as necessary for FlbD binding was changed to a cytosine (OM-6; Fig 4A). In addition, as a control, two guanine residues at positions $+5$ and $+6$ bp, which are not required for FlbD binding, were changed to thymines (OM-3; Fig 4B). These two residues were chosen because of their similarity to the conserved *ftr* elements found in the *flgK*, *flaN*, *flbG*, and *flgL* promoters (Mullin and Newton 1989; Gober and Shapiro 1992). The promoter fragments containing these mutations were ligated into plasmid *placZ*/290, which contains a promoterless *lacZ* reporter gene. These transcription fusions were introduced into the wild-type *Caulobacter* strain NA1000, and β -galactosidase levels were measured. In wild-type *Caulobacter*, the *fliF* promoter generated 2513 units of β -galactosidase activity (Fig. 4A). In contrast, the mutation introduced in OM-6 resulted in 10,689 units of β -galactosidase activity, almost four times the activity generated by the wild-type promoter (Fig. 4A), suggesting that this residue is critical for *fliF* repression. The mutation introduced in OM-3 resulted in no change in promoter activity when compared with the wild-type *fliF* promoter (2515 vs. 2513 units) (Fig. 4A), demonstrating that these residues are not involved in the negative regulation of the *fliF* promoter in vivo. To test whether FlbD could regulate these mutant promoters, the *lacZ* fusions were also introduced into the *flbD*::Tn5 strain,

SC1032 (Ohta et al. 1984). In this *flbD* mutant strain, the expression of the wild-type and OM-3 promoter fusions increased four- to fivefold (Fig. 4A). OM-6 *fliF*-*lacZ* expression increased only slightly, indicating that this mutant promoter is not regulated by FlbD (Fig. 4A).

To show that purified FlbD does not bind to promoter fragments containing the OM-6 mutation, we compared the ability of FlbD to bind to the wild-type *fliF* promoter and the two mutant promoters OM-3 and OM-6 as determined by DNase I protection assay. As shown previously (Fig. 2), FlbD provided strong protection from DNase I attack on the wild-type promoter (Fig. 4B). Although the DNase digestion pattern was slightly different on the OM-3 promoter, it too was strongly protected by FlbD from DNase I cleavage (Fig. 4B). The change of the guanine at position -3 bp to a cytosine in OM-6, however, greatly weakened the affinity of FlbD for the promoter, with slight protection from DNase I attack being evident only at the highest concentration of FlbD (30 μ M) (Fig. 4B). These results indicate that FlbD binds to *fliF* promoter in vivo and acts as a negative regulator of *fliF* expression.

The effect of phosphorylation on FlbD binding to the *fliF* promoter

Recently, it has been shown that the phosphorylation of

Figure 4. (A) Mutagenesis of the *fliF* promoter. Site-directed mutagenesis was utilized to create two mutant versions of the *fliF* promoter. In the first mutant (OM-3), the two guanine residues located at $+5$ and $+6$ bp were changed to two thymine residues. In the second mutant (OM-6) the guanine residue at position -3 bp, identified above as critical for FlbD binding, was changed to a cytosine. The fragments containing these mutant promoters were ligated into a promoterless *lacZ* plasmid (Gober and Shapiro 1992), as diagramed at the top. These constructs, including one containing a wild-type *fliF* promoter, were introduced into both wild-type (NA1000) and *flbD*::Tn5 (SC1032) *Caulobacter*, and expression was determined by assaying β -galactosidase activity. The activities of the different promoters, given in units of β -galactosidase activity, are shown at right. (B) Binding of FlbD to mutant promoters. DNase I footprinting analysis was used to compare the ability of FlbD to bind to the wild-type *fliF* and the two mutant promoters, OM3 and OM6. In each case, 1 pmole of uniquely 32 P-end-labeled fragment was subjected to DNase I digestion either in the absence of FlbD (denoted as $-$), or with increasing concentrations of FlbD (1.2–12 μ M) present. The resulting footprints were visualized on an 8% denaturing acrylamide sequencing gel. The area protected by FlbD is given right. G and G + A sequencing reactions (Maxam and Gilbert 1977) are shown.

NtrC in *Salmonella typhimurium* (Weiss et al. 1992; Porter et al. 1993) and of SpoOA in *Bacillus subtilis* (Balduis et al. 1994) enhances DNA binding through promoting oligomerization. Because FlbD is a member of the NtrC family of response regulators, it is probable that the phosphorylation of FlbD also enhances its oligomerization, and possibly also its binding. We have shown previously that FlbD is capable of being phosphorylated in vitro, and that the phosphorylation of FlbD in vivo is restricted to the swarmer pole of the predivisional cell in *Caulobacter* (Wingrove et al. 1993). *fliF* expression is threefold lower in the swarmer pole of the predivisional cell as compared with expression in the stalked pole (Gober et al. 1991). It is therefore possible that the pole-specific phosphorylation of FlbD enhances its ability to repress *fliF* expression in the swarmer pole through an enhancement of binding.

To investigate the possibility that phosphorylated FlbD has a higher affinity for the *fliF* promoter, we attempted to analyze the effect that phosphorylation had on FlbD binding by DNase I footprint analysis. It has been demonstrated that high energy phosphoamidites such as acetyl phosphate and carbamyl phosphate can serve as phosphodonors for response regulators homologous to FlbD (Deretic et al. 1992; Feng et al. 1992; Lukat et al. 1992). Because the kinase responsible for activating FlbD has yet to be identified, we used acetyl phosphate to test whether the phosphorylation of FlbD could influence binding to the *fliF* promoter. Purified His-FlbD was preincubated with 20 mM acetyl phosphate for 5 min prior to the addition of the *fliF* probe and subsequent treatment with DNase I. The presence of acetyl phosphate had no effect when lower concentrations (1.2–2.4 nM) of His-FlbD were used (Fig. 5A); however, as judged by densitometry, up to a 15% increase in binding was seen when higher levels (12–120 nM) of His-FlbD were used (Fig. 5A,B). A hypersensitive site at position –33 bp (indicated by an arrow in Fig. 5A) is also seen in the

presence of high concentrations (120 nM) of His-FlbD plus acetyl phosphate. These results suggest that the phosphorylation of FlbD enhances its binding to the *fliF* promoter. The effect of the acetyl phosphate on FlbD binding is modest, perhaps indicating that acetyl phosphate is not an efficient phosphodonor for FlbD and possibly that the FlbD is not being fully phosphorylated in our assay. Phosphorylation by a kinase specific for FlbD might be more efficient and provide a higher degree of phosphorylation. It has been shown that the phosphorylation of SpoOA, a response regulator homologous to FlbD involved in *B. subtilis* sporulation, permits the protein to bind more efficiently to weak binding sites (Balduis et al. 1994). Phosphorylation also enhances the oligomerization of NtrC, allowing it to bind more efficiently to the upstream enhancer of the *glnA* promoter (Porter et al. 1993). These results suggest that the phosphorylation of FlbD might also enhance its ability to oligomerize and subsequently bind to the weaker, distal binding site (–52 to –35 bp) found in the *fliF* promoter (Fig. 5A).

Effect of FlbD mutations on *fliF* expression

Several mutations that affect the activity of σ^{54} activator proteins have been described. For example in NtrC, an alteration in aspartate 54 to glutamate resulted in a protein that was active in the absence of phosphorylation (Klose et al. 1993; Moore et al. 1993). Likewise, a mutation in the conserved central domain (serine 160 to phenylalanine) also resulted in constitutive NtrC activity (Popham et al. 1989; Weiss et al. 1991). Both of these mutations also increase the ability of NtrC to bind to enhancer sequences (Klose et al. 1993; Porter et al. 1993). *Caulobacter* cells that contain an analogous mutation in FlbD (S140F) inappropriately express swarmer compartment-specific genes in the stalked pole, suggesting that

Figure 5. Effect of acetyl phosphate on the ability of FlbD to bind to the *fliF* promoter.

(A) Various concentrations of FlbD (1.2–60 nM) were incubated in the presence or absence of 20 mM acetyl phosphate (final concentration). One pmole of uniquely 32 P-end-labeled probe was then added, and the mixture subjected to digestion with DNase I. The reactions were then electrophoresed on an 8% acrylamide–urea sequencing gel. Reactions not including FlbD are denoted –. The area protected by FlbD is given at right. The arrow indicates a hypersensitive band that is induced by FlbD binding. (B) Densitometry analysis of data in A. Several bands within the protected region were scanned with a densitometer and were normalized against scanned bands residing outside of the protected region to correct for differences in loading. (●) – Acetyl phosphate; (■) + acetyl phosphate. Data are plotted as percent occupancy as a function of FlbD concentration. Calculations were performed as described by Porter et al. (1993).

this mutation also renders FlbD constitutively active (Wingrove et al. 1993). To test the hypothesis that the activation of FlbD also regulates repression of *fliF* transcription, we assayed the expression of a *fliF-lacZ* fusion in cells that contained mutant versions of FlbD (Fig. 6). Strains were constructed by integrating a plasmid containing either wild-type or mutant versions of *flbD* by homologous recombination. This results in a single crossover event so that each strain contains two copies of *flbD*, the original wild-type copy plus *flbD* on the integrated plasmid. As a control, a plasmid containing wild-type *flbD* was also integrated. Cells containing a mutant FlbD (D52A) that cannot be phosphorylated, expressed slightly higher levels (20% increase) of *fliF-lacZ* (Fig. 6). In contrast, *fliF-lacZ* expression decreased in strains carrying constitutive FlbD mutants. The strain with a S140F mutation in the conserved central domain of FlbD expressed *fliF-lacZ* at approximately one-half the level of the wild-type control (Fig. 6). We also constructed a double mutant with mutations in both the amino-terminal and central domains of FlbD (D52E/S140F). The analogous NtrC mutant (D54E/S160F) has been shown to possess a high level of constitutive transcriptional activity (Klose et al. 1993). Strains containing the double mutation in FlbD expressed the lowest levels of *fliF-lacZ* (30% of wild type). As a control, we also examined the effect of *flbD* mutations on the expression of the mutant OM-6 *fliF* promoter. In contrast to the wild-type *fliF* promoter, the FlbD mutants had little effect on expression of the OM-6 *fliF* promoter (Fig. 6). This experiment suggests that activation of FlbD may have a role in regulating the repression of the *fliF* promoter.

Cell cycle regulation of the *fliF* promoter

The *fliF* operon is expressed relatively early in the *Caulobacter* cell cycle. To examine the role of FlbD in the

temporal regulation of the *fliF* operon, we compared the level of expression of the wild-type *fliF* promoter with that of the mutant OM-6 promoter at different time points throughout the cell cycle. Wild-type NA1000 cells harboring either the wild-type *fliF-lacZ* reporter construct or the OM-6 *fliF-lacZ* reporter construct were synchronized by Ludox density centrifugation. Isolated swarmer cells were allowed to proceed through the cell cycle. Samples were removed at the indicated time points and pulse-labeled with Trans³⁵S-label. Levels of *fliF* expression were determined by immunoprecipitation with anti- β -galactosidase antibody, followed by SDS-PAGE and fluorography. The wild-type *fliF* promoter was not expressed in the early stages of the cell cycle corresponding to the swarmer cell and early stalked cell types (0 and 0.2 units), with slight expression in the late stalked cell (0.4 units) (Fig. 7A). With the transition to the early predivisional cell type (0.6 units), a 17-fold increase in *fliF* expression was seen (Fig. 7A,B). The level of *fliF* expression then decreased in the late predivisional cell type (0.8 and 1 unit), approaching the levels seen in the swarmer cell (Fig. 7A). Expression from the OM-6 mutant *fliF* promoter was markedly different. A slightly higher level of expression (approximately twofold) was seen early in the cell cycle (0, 0.2, and 0.4 unit) compared with the wild type. With the transition to early predivisional cell type (0.6 units), an increase in *fliF* promoter expression, almost 1.5 times higher than that seen from the wild-type *fliF* promoter was observed (Fig. 7B). The most notable difference in expression levels from the wild-type and mutant OM-6 promoters, however, occurred in the late predivisional cell type (1.0 unit). Expression from the mutant OM-6 promoter remained relatively high, a sevenfold difference compared with the wild-type *fliF* promoter (Fig. 7A,B). These results suggest that FlbD is an important negative regulator of *fliF* expression in the latter part of the *Caulobacter* cell cycle.

Figure 6. Effect of *flbD* mutations on the expression of *fliF-lacZ* transcriptional fusions. An extra copy of either wild-type or mutant *flbD* was integrated into the *Caulobacter* chromosome by homologous recombination. Wild-type *fliF-lacZ* or mutant OM-6 *fliF-lacZ* expression was then assayed in each of these strains by measuring β -galactosidase activity. Relative activity in mutants is indicated as a percentage of β -galactosidase activity compared with that obtained with control cells carrying wild-type *flbD*. Note that in this experiment the presence of an extra copy of *flbD* decreases the level of β -galactosidase expressed. In strains carrying a wild-type *fliF-lacZ* fusion and two copies of wild-type *flbD* (w.t.), 600 units of β -galactosidase was

expressed. This is approximately a fourfold decrease compared with the level expressed in wild-type cells (see Fig. 4A). In strains carrying mutant OM-6 *fliF-lacZ* and two copies wild-type *flbD*, 4000 units of β -galactosidase was expressed. This represents an approximately twofold decrease compared with the level in wild-type cells (see OM-6 in Fig. 4A).

Figure 7. A mutation in the *fliF* promoter that abolishes FlbD binding also alters the cell cycle expression of the *fliF* promoter. The cell-cycle pattern of expression of the wild-type *fliF* promoter was compared with that of the mutant OM6 *fliF* promoter. *Caulobacter* harboring either the wild-type *fliF-lacZ* plasmid or the mutant OM6 *fliF-lacZ* plasmid were synchronized by Ludox density centrifugation, and isolated swarmer cells were allowed to proceed through the cell-cycle. Samples were labeled with Trans-³⁵S-label at 0, 45, 90, 120, 150, and 180 min. The stages of the cell cycle at which time samples were removed are indicated schematically above. Extracts were prepared, and promoter expression was assayed by immunoprecipitation using anti- β -galactosidase antibody. Equal amounts (1×10^6 cpm) of labeled proteins were incubated with antibody. Immunoprecipitated proteins were subjected to SDS-PAGE and visualized by fluorography. (A) The cell cycle expression pattern generated by the wild-type *fliF-lacZ* promoter construct. The band corresponding to *fliF-lacZ* product is indicated at right. Below is shown the cell-cycle pattern of expression generated by the OM6 mutant *fliF-lacZ* promoter construct. The band corresponding to the OM6 *fliF-lacZ* product is indicated at right. (B) Data presented in A and B were analyzed by densitometry. Results are presented as relative band intensity as a function of the cell cycle.

Swarmer pole-specific repression of the *fliF* promoter by FlbD

FlbD activates the transcription of *ftr*-containing σ^{54} promoters in the swarmer cell compartment of the predivisional cell. The phosphorylation of FlbD is limited to the swarmer pole and this results in swarmer pole-specific transcription. To determine whether FlbD is responsible for the swarmer pole-specific repression of *fliF*, we compared the pole-specific expression of a wild-type *fliF-lacZ* promoter fusion with an OM-6 *fliF-lacZ* promoter fusion. This experiment was performed as described previously (Wingrove et al. 1993). Cells expressing either the wild-type *fliF* reporter construct or the OM-6 promoter construct were synchronized utilizing Ludox density gradient centrifugation. The isolated swarmer cells were allowed to proceed through the cell cycle. Immediately prior to division, after a cell division constriction had formed, the cells were pulse-labeled with Trans-³⁵S-label, followed by a cold chase of methionine and were then permitted to divide. The progeny

cells were isolated by Ludox centrifugation, and the levels of β -galactosidase were assayed by immunoprecipitation. This method serves as an assay of *fliF* promoter expression at the poles of the predivisional cell. At the time of labeling, the predivisional cell has formed two distinct compartments so that labeled protein synthesized in one pole, is not free to diffuse into the other pole. Therefore, the presence of labeled β -galactosidase in progeny cell fractions is a record of the polar location of expression in the predivisional cell. This type of experiment has been successfully used to demonstrate swarmer pole-specific transcription of *ftr*-containing flagellar promoters (Gober et al. 1991; Gober and Shapiro 1992) and the stalked pole-specific transcription of the DNA gyrase B subunit gene, *gyrB* (Rizzo et al. 1993). The *fliF* promoter is subject to localized expression in the incipient stalked cell pole. Expression from the wild-type *fliF* promoter was approximately three- to fourfold higher in the stalked pole compared to the swarmer pole as judged by densitometry (not shown; Fig. 8A). This is

Figure 8. The OM-6 mutation alleviates the ability of FlbD to repress *fliF* expression in the swarmer pole. (A) *Caulobacter* cells containing the wild-type *fliF-lacZ* reporter plasmid were synchronized by Ludox density centrifugation. The culture was permitted to proceed to the late predivisional stage in the cell cycle, at which point proteins were pulse-labeled for 10 min with Trans³⁵S-label, followed by a chase of cold methionine. Cells were allowed to divide, and the resultant progeny cells were reisolated. Extracts were prepared and β-galactosidase was immunoprecipitated using anti-β-galactosidase antibody. As a control, newly synthesized flagellin was also immunoprecipitated using anti-flagellin antibody. Equal amounts of labeled protein (1×10^6 cpm) were incubated with antibody. Immunoprecipitated proteins were resolved by SDS-PAGE and visualized by fluorography. (B) The same experiment in A was performed on *Caulobacter* strain harboring the mutant OM6 *fliF* promoter. ³⁵S-Labeled β-galactosidase or labeled 25-kD flagellin is indicated at right. (C) The same experiment in A was performed on a *Caulobacter* strain containing a constitutive FlbD mutant (D52E/S140F) (see Fig. 6) and a wild-type *fliF-lacZ* transcriptional fusion. Labeled β-galactosidase is indicated at right. Immunoprecipitation of flagellins from this strain showed little difference in the quantity of flagellin synthesized in the swarmer and stalked cell fractions (not shown).

in contrast to swarmer pole-specific transcription of late flagellar genes. The mutant *fliF* promoter, which cannot be bound by FlbD (OM-6), no longer exhibited stalked pole-specific expression. The mutant promoter expressed equal levels of β-galactosidase in both the swarmer and stalked poles of the predivisional cell (Fig. 8B). Also shown for both experiments is a control in which 25-kD flagellin was immunoprecipitated with anti-flagellin antibody. It has been demonstrated previously that the gene encoding this flagellin (*flgK*) is transcribed preferentially in the swarmer compartment of the predivisional cell (Gober et al. 1991). In both cases, more of the labeled flagellin was present in progeny swarmer cells.

To test the role of FlbD activation in the spatial repression of *fliF*, we assayed for compartment-specific expression of *fliF-lacZ* in a strain carrying the double constitutive (D52E/S140F) FlbD mutant (Fig. 8C). The level of expression of *fliF-lacZ* is much lower in this strain than in wild-type cells. In addition, labeled β-galactosidase is present in approximately equal but lower levels in both progeny swarmer and stalked cells, indicating that expression of the constitutively active FlbD mutant apparently represses transcription in both poles of the predivisional cell. These results suggest that stalked pole-specific expression of *fliF* is attributable to repression of transcription in the swarmer cell pole of the predivisional cell and may be regulated by the restriction of

FlbD activity to the swarmer compartment. Repression is relieved in the mutant promoter, which cannot be bound by FlbD (Fig. 6), suggesting that this protein has a dual role in flagellar biogenesis, serving as both a pole-specific activator and a repressor of developmentally regulated flagellar genes.

Discussion

In this paper we present evidence that FlbD, a σ^{54} transcriptional activator, can also function as a repressor of its own promoter. FlbD-mediated repression is developmentally regulated, occurring late in the cell division cycle and almost exclusively in the swarmer pole of the predivisional cell. In vitro experiments presented here show that purified FlbD binds primarily to a site that overlaps the -10 bp region of the *fliF* operon promoter. Methylation interference experiments indicate that FlbD recognizes a *ptr*-like binding site that deviates significantly from the consensus enhancer sequence found in σ^{54} flagellar promoters. A single-base-pair change in a guanosine residue, demonstrated to be required for binding by methylation interference, increased *fliF* transcription fourfold. Transcription of the mutant promoter, OM-6, did not increase in a *flbD* mutant strain and could no longer be bound by FlbD in vitro. These data suggest that FlbD binds to and represses the *fliF* promoter in *Caulobacter*.

The *fliF* promoter has been subject to intensive analysis by site-directed mutagenesis. Previous experiments defined the sequences within the promoter region that were essential for transcriptional activity (Van Way et al. 1993). This analysis, as well as similar studies of *fliLM* and *fliQR*, two other early class II flagellar promoters, have defined a new developmentally transcribed promoter sequence (Stephens and Shapiro 1993). This sequence is unlike any known bacterial promoter and is considered to be the recognition sequence for a novel σ -subunit of RNA polymerase, called σ^R . These promoters are the first flagellar promoters expressed in the *Caulobacter* cell cycle and control the transcription of flagellar genes that encode early assembled flagellar structures and flagellar specific export proteins. Experimental data suggest that the promoter sequence itself is sufficient to direct the cell cycle expression of these promoters (Stephens and Shapiro 1993; Van Way et al. 1993). Therefore, the timing of *fliF* transcription is most likely attributable to the temporally regulated synthesis or activity of the σ^R protein, which is yet to be identified.

The repression of the *fliF* promoter by FlbD represents another level of regulation, in addition to cell cycle-activated transcription. The *fliF* promoter is apparently unique in this respect as we have not been able to demonstrate FlbD binding to other known class II σ^R promoters, including *fliLM* and *fliP* (J. Wingrove and J. Gober, unpubl.). These promoters, however, are subject to some form of negative regulation. The transcription of most σ^R promoters increases when assayed in class II mutant backgrounds (Newton et al. 1989; Xu et al. 1989). The mechanism responsible for this increase in transcription is not known but is unlikely to directly involve FlbD. Repression of the *fliF* promoter by FlbD may simply be a means of autoregulation. *flbD* is the final gene in the *fliF* operon, and binding of FlbD to the promoter may be a mechanism of regulating the cellular levels of FlbD. We have shown previously that the levels of FlbD do not vary greatly during the cell division cycle even though the transcription of the FlbD promoter is under strict temporal control. The repressive activity of FlbD may be partially responsible for maintaining the constant level of FlbD during the cell cycle. The σ^{54} activator NtrC also controls its own expression by repressing the transcription of the σ^{70} *glnAp1* promoter in *Escherichia coli* (Reitzer and Magasanik 1985).

The FlbD-mediated pattern of repression of the *fliF* promoter mirrors the temporal and spatial pattern of expression of FlbD-activated promoters. For example, *fliF* promoter transcription decreases in the late predivisional cell, whereas the transcription of *ptr*-containing flagellar promoters increases at this stage. Mutation of the primary FlbD-binding site in the *fliF* promoter causes an increase in transcription but does not significantly alter the timing of promoter activity. This suggests that FlbD does not control the overall timing of *fliF* transcription but, rather, exerts repression late in the cell cycle. Earlier experiments, in which several promoter-up mutants of *fliF* were isolated, showed that one mutant promoter had both increased levels of *fliF* mRNA and an

altered temporal pattern of expression (Van Way et al. 1993). Like our experiments, the up-regulated mutant promoter was also expressed late in the cell cycle, as well as in newly formed swarmer cells. Based on our methylation interference data, the mutant promoter isolated in these previous experiments would be predicted not to bind FlbD. The cell cycle experiments presented here indicate that FlbD exerts its greatest influence on *fliF* transcription just before cell division. At the cell cycle peak of *fliF* transcription, relieving FlbD-mediated repression resulted in an approximately twofold increase in *fliF* expression levels relative to that of a wild-type promoter. In late predivisional cells, however, there was a sevenfold difference among the mutant, nonrepressed promoter, and wild-type promoter expression levels (see Fig. 7B).

The flagellar promoters that are subject to transcriptional activation by FlbD are transcribed preferentially in the swarmer compartment of late predivisional cells (Gober et al. 1991; Gober and Shapiro 1992). In contrast, the *fliF* promoter is expressed preferentially in the stalked cell compartment. This conclusion is based on previous experiments demonstrating pole-specific expression of *fliF*-luciferase (*lux*) reporter gene fusions (Gober et al. 1991), and from similar experiments with *fliF*-*lacZ* transcription fusions reported here (Fig. 8). Pole-specific transcription is thought to be one mechanism utilized by *Caulobacter* cells to position gene products in the swarmer and stalked poles of the predivisional cell (for review, see Gober and Shapiro 1991). *fliF* is the first flagellar gene for which stalked pole transcription has been observed. Mutation of the FlbD-binding site in *fliF* resulted in equal levels of transcription in both the swarmer and stalked poles of the predivisional cell. This result indicates that *fliF* transcription is limited to the stalked pole as a result of FlbD repression and is not attributable to the pole-specific targeting of σ^R or another essential transcription factor.

How does FlbD simultaneously participate in both swarmer pole-specific transcriptional activation and repression? Specific targeting of FlbD to the swarmer pole could hypothetically accomplish both regulatory missions. However, previous experiments have demonstrated that this clearly does not occur. Immunofluorescence microscopy and immunoblots of proteins extracted from newly divided cells showed that FlbD is not preferentially targeted to the swarmer cell compartment and is present in equal quantities in both incipient cell poles (Wingrove et al. 1993). These data suggest that the activity of FlbD must be limited to the swarmer pole of the predivisional cell. FlbD is a member of a family of σ^{54} transcriptional activators that are regulated by the phosphorylation of a conserved aspartate residue in their amino-terminal domain (Parkinson and Kofoid 1992). The regulation of transcriptional activation of σ^{54} promoters is ultimately controlled by regulating the level of phosphorylated activator protein (Ninfa and Magasanik 1986). Experiments with the enteric bacterial protein NtrC have shown that phosphorylation of the amino-terminal domain stimulates ATP hydrolysis by the con-

served central domain, which in turn stimulates open complex formation catalyzed by σ^{54} -containing RNA polymerase (Weiss et al. 1991). A FlbD kinase activity is expressed under cell cycle control, suggesting that temporal phosphorylation of FlbD regulates the cell cycle transcriptional activation of its target promoters (Wingrove et al. 1993). There are several mutant NtrC proteins that can activate transcription both in vivo and in vitro in the absence of kinase activity (Weiss et al. 1991; Klose et al. 1993; Porter et al. 1993). We introduced an analogous mutation into FlbD by altering a conserved serine at residue position 140 to phenylalanine (S140F) (Wingrove et al. 1993). Cells that expressed the constitutive FlbD S140F mutant no longer exhibited swarmer pole-specific expression of *ftr*-containing σ^{54} promoters. In this strain, flagellin protein was expressed at almost equal levels in the swarmer and stalked cell poles of the predivisional cell (Wingrove et al. 1993). This result indicates that the phosphorylation of FlbD in wild-type cells is restricted to the swarmer pole of the predivisional cell and this is the major regulatory event that results in swarmer pole transcription.

The fact that FlbD is not spatially localized suggests that pole-specific phosphorylation also regulates repression of the *fliF* promoter. Unlike σ^{54} promoters, in which transcriptional activation requires both binding to enhancer sequences and ATP hydrolysis, the control of *fliF* repression is most likely to be solely regulated by the binding of FlbD to the promoter region. The pattern of *fliF* transcription can be attributed to the net effect of the two competing binding activities of σ^R -containing RNA polymerase and FlbD. FlbD binding to the *fliF* promoter presumably occludes these sequences, thus preventing binding of RNA polymerase. The spatial pattern of *fliF* expression suggests that only the phosphorylated form of FlbD competes effectively with RNA polymerase for binding to the *fliF* promoter. We hypothesize that the temporal and spatial pattern of FlbD phosphorylation regulates *fliF* promoter repression. Phosphorylation in this case may influence the affinity of FlbD for the *fliF* promoter. Phosphorylation has a dual role in modulating the activity of σ^{54} transcriptional activators; in addition to stimulating ATPase activity, there is growing evidence that binding of NtrC to the *glnA* promoter is enhanced by phosphorylation (Weiss et al. 1992; Porter et al. 1993). The *glnA* enhancer is composed of two strong NtrC-binding sites separated from each other by an estimated three helical turns. An NtrC dimer binds to each of these sites (Porter et al. 1993). Binding to the enhancer is cooperative (Weiss et al. 1992; Porter et al. 1993), which results from protein-protein interactions between the two bound dimers (Porter et al. 1993). Phosphorylation of NtrC increased cooperative binding but did not increase the affinity of a single dimer molecule for a single binding site (Porter et al. 1993). These experiments show that phosphorylation functions to stimulate oligomerization of NtrC bound to the enhancer.

The phosphorylation of FlbD may enhance its ability to bind to the *fliF* promoter region. We have shown here that FlbD binds primarily to two sites in the *fliF* pro-

moter. The center of the primary site as defined by methylation interference is located at -8 bp from the transcription start site (Fig. 3). Both methylation interference and site-directed mutagenesis show that this site is essential for the binding of FlbD to the *fliF* promoter. The next strongest site as indicated by DNase footprinting is located approximately three turns of the DNA helix upstream at -41 bp (Fig. 2). By analogy to the *glnA* enhancer, phosphorylation of FlbD may stimulate oligomerization of FlbD bound to these two sites in the *fliF* promoter. In support of this hypothesis, mutations in FlbD that would be predicted to enhance the oligomerization of FlbD increased repression of *fliF* (Fig. 6). In NtrC, both analogous mutations have been demonstrated to increase the ability of NtrC to bind to enhancer sequences (Klose et al. 1993; Porter et al. 1993). Furthermore, the double constitutive D52E/S140F FlbD mutant no longer exhibited swarmer compartment-specific repression of *fliF*. The low level of β -galactosidase present in both progeny cells (see Fig. 8C) indicates that repression occurred in both poles of the predivisional cell. The fact that the constitutive FlbD mutants had no effect on the expression of the mutant OM-6 *fliF* promoter (Fig. 6) also strengthens the suggestion that the binding of FlbD to the *fliF* promoter may be regulated by phosphorylation. Oligomerization of FlbD probably cannot occur as efficiently on the OM-6 promoter, as it contains only a single FlbD-binding site. We also have attempted to experimentally test the idea that phosphorylation can enhance FlbD-binding activity by using acetyl phosphate as a phosphodonor (Fig. 5). Our experiments show that FlbD incubated in the presence of acetyl phosphate can form a complete DNase footprint at relatively lower concentrations of FlbD. The effect of acetyl phosphate on binding is marginal and may be attributable to the fact that acetyl phosphate is not an ideal phosphodonor. Future identification of the FlbD kinase will resolve this important issue.

What role does FlbD-mediated repression have in the generation of two cell types? The swarmer pole-specific activity of FlbD determines the relative programs of flagellar gene expression in the progeny swarmer and stalked cells (Fig. 9). Following cell division the swarmer cell flagellar filament continues to lengthen (Langenauf and Agabian 1978). Filament lengthening is the result of the translation of flagellin (*flgK*) mRNA that was transcribed previously in the swarmer compartment before cell division (Milhausen and Agabian 1983; Gober et al. 1991). Expression of *flgK* in the swarmer compartment is a direct consequence of the pole-specific activation of FlbD. Therefore, the pole-specific activation of FlbD permits the completion of assembly of late flagellar structures in the newly divided swarmer cell (Fig. 9).

In contrast, FlbD-mediated repression prevents early flagellar components from being expressed in the swarmer pole of the predivisional cell. We speculate that repression of the *fliF* operon in the swarmer pole is essential for late flagellar gene expression. Early flagellar components such as the M-ring, if expressed inappropriately in the swarmer pole would not be assembled be-

cause of the presence of a pre-existing M-ring structure. It is possible that the accumulation of unassembled M-ring monomers in the cytosol is recognized as a block in flagellar assembly. Because many spatially expressed promoters are also regulated by flagellar assembly, M-ring synthesis at this stage of flagellar biogenesis may prevent late gene expression. On the other hand, in the stalked cell pole, the lack of FlbD-mediated repression permits the expression of the *fliF* operon. Because the *fliF* operon encodes the earliest assembled flagellar structure (M-ring), this sets the stage for the initiation of new flagellum synthesis after the stalked cell has divided (see Fig. 9). Therefore, the differential activities of FlbD at two different promoter classes dictates the stage of flagellar biogenesis in the two progeny cell types.

Materials and methods

Bacterial strains, plasmids, and growth conditions

C. crescentus strain NA1000 was used as a wild-type, synchronizable motile strain. SC1032 is a mutant strain with a Tn5 insertion in the *flbD* gene (Ohta et al. 1984). Unless noted otherwise, strains were either grown in PYE media (Poindexter 1964) or minimal M2-glucose media (Contreras et al. 1978) at 30°C. The *fliF-lacZ* transcription fusion was created by ligating a 294-bp *Bam*HI–*Hind*III fragment (–212 to +72 bp from transcription start) into a plasmid containing a promoterless *lacZ* gene, *placZ/290* (J. Gober and M. Alley, unpubl.). The 294-bp *fliF* promoter fragment was also subcloned into Bluescript KS, and was utilized for FlbD–DNA-binding assays. *flbD* mutants in Bluescript KS were introduced into a β -lactamase negative (*bla*) *C. crescentus* NA1000 by electroporation.

DNA manipulations

To facilitate methylation interference footprinting of the bottom strand of the *fliF* promoter, PCR was used to introduce a

*Bam*HI site at –72 bp. The resulting 150-bp fragment was subcloned into Bluescript KS. Oligonucleotide site-directed mutagenesis was performed as described by Kunkel and Roberts (1987). The 294-bp *fliF* promoter fragment was introduced into M13BM21 (Boehringer Mannheim, Indianapolis, IN), and mutagenesis was performed on the isolated single strands. Mutagenesis of *flbD* was performed as described previously (Wingrove et al. 1993). All mutagenesis was confirmed by dideoxy DNA sequencing using a primer complementary to bases +90 to +108 bp of the *fliF* promoter. The resulting mutant promoter fragments were ligated into *placZ/290* and Bluescript KS as described above. Oligonucleotides used in DNA sequencing and in site-directed mutagenesis were synthesized on a Pharmacia Gene Assembler automated DNA synthesizer. β -Galactosidase assays were performed as described previously (Gober and Shapiro 1990).

Purification of FlbD and preparation of DNA probes for binding assays

Pure histidine-tagged FlbD (His–FlbD) was prepared as described by Wingrove et al. (1993), with the following modifications. The supernatant obtained from French pressure cell lysis containing soluble His–FlbD was applied to a 10-ml nickel–Sepharose column. The column was first rinsed with nickel–Sepharose rinse buffer (20 mM sodium phosphate at pH 6.0, 500 mM NaCl), followed by 500 mM imidazole (30 ml). A final elution using 1 M imidazole (30 ml) yielded His–FlbD that was >95% pure as judged by SDS-PAGE. Samples containing pure His–FlbD were pooled and dialyzed extensively against nickel–Sepharose loading buffer (20 mM sodium phosphate at pH 7.6, 500 mM NaCl).

End-labeled probe for DNase I footprint analysis was prepared by digesting plasmid containing either the wild-type or mutant *fliF* promoter fragments with *Hind*III, followed by end-labeling that utilized DNA polymerase I, Klenow fragment (Boehringer Mannheim) and [α - 32 P]dGTP (Amersham, 3000 Ci/mmol). The Klenow fragment was heat-inactivated for 10 min at 72°C, fol-

lowed by digestion with *Bam*HI. The desired fragment was isolated by polyacrylamide gel electrophoresis and then subjected to electroelution, phenol/chloroform extraction, and ethanol precipitation. The probes for the methylation interference assay were prepared in a similar manner, utilizing the *Hind*III site for top-strand analysis, and the *Bam*HI introduced at position -72 bp for analysis of the bottom strand.

DNA-binding assays

DNase I footprinting assays (Heberlein et al. 1985) were performed by incubating pure His-FlbD (1.2–120 nM) with labeled DNA fragment (1 pmole) for 5 min at 4°C in a buffer containing 12.5 mM HEPES at pH 7.6, 50 mM KCl, 0.05 mM EDTA, 6.25 mM MgCl₂, 5% (vol/vol) glycerol (HEMG), and 2% (vol/vol) polyvinyl alcohol (PVA), final reaction volume of 50 µl. Reactions were placed at room temperature, and an equal volume of a 10 mM MgCl₂, 5 mM CaCl₂ solution was added. Following a 1-min incubation, 1.25 ng of DNase I (Boehringer Mannheim) was added and digestion was allowed to proceed for 60 sec. Reactions were terminated by the addition of 90 µl of stop solution containing 20 mM EDTA at pH 8.0, 1% (wt/vol) SDS, 200 mM NaCl and 250 µg/ml carrier RNA. Samples were extracted immediately with equal volumes of phenol/chloroform and chloroform, followed by ethanol precipitation. The pellets were washed twice with 70% ethanol, dried, and resuspended in 80% (vol/vol) formamide, 1× Tris/boric acid/EDTA (TBE), 0.05% Bromphenol Blue, and 0.05% Xylene Cyanol FF. The samples were heated for 5 min at 95°C, placed on ice, and then electrophoresed in 8% acrylamide-urea sequencing gels in 1× TBE. Sequencing reactions were performed as described by Maxam and Gilbert (1977). Reactions utilizing acetyl phosphate were performed in a similar manner, except that samples containing His-FlbD in HEMG were preincubated with 20 mM acetyl phosphate (final concentration) for 5 min prior to the addition of the DNA probe.

Methylation interference assays were performed essentially as described by Hendrickson and Schleif (1985). End-labeled probe (1 pmole) was incubated for 1 min with 1 µl of DMS in a buffer containing 50 mM sodium cacodylate at pH 8.0 and 1 mM EDTA at pH 8.0 (210 µl final volume). The reactions were terminated by the addition of 400 µl of stop buffer containing 1.5 M sodium acetate at pH 7.0, 1.0 M β-mercaptoethanol and 100 µg/ml of tRNA. Samples were precipitated twice with ethanol, rinsed twice with 70% ethanol, dried, and resuspended in 10 µl of sterile TE. The modified probe was used in a gel mobility shift assay, using 120 nM of pure His-FlbD (Wingrove et al. 1993). Shifted and nonshifted bands were cut from the gel, electroeluted, extracted with phenol/chloroform, precipitated with ethanol, and dried. The recovered probe was resuspended in 90 µl of 10% piperidine and incubated at 90°C for 30 min. Samples were dried, resuspended in 200 µl of TE, precipitated with ethanol, and then rinsed twice with 70% ethanol. This was repeated, and the samples were dried and then subjected to acrylamide-urea electrophoresis as described above.

Cell cycle expression experiments

Synchronizable NA1000 *C. crescentus* harboring either the wild-type *fliF-lacZ* plasmid or the OM6 *fliF-lacZ* plasmid were grown in 500 ml of M2-minimal glucose media to an OD₆₀₀ of 0.8–1.2. Swarmer cells were isolated by Ludox density centrifugation as described previously (Contreras et al. 1978). Swarmer cells were resuspended in minimal-M2 glucose media and allowed to proceed through the cell cycle at 30°C. At the indicated time points, samples were removed and pulsed for 10 min with

3 µCi/ml of Trans-³⁵S-label (ICN, Irvine, CA). Labeled protein was immunoprecipitated as described previously in Gomes and Shapiro (1984), using anti-β-galactosidase antibody (Boehringer Mannheim). Equal quantities of protein (1×10⁶ cpm) were incubated with antibody. Precipitated protein was subjected to SDS-PAGE and visualized by autoradiography. To assay compartment-specific gene expression, cells were synchronized as above and were allowed to proceed through the cell cycle. At the late predivisional stage, cells were pulsed for 10 min with 6 µCi/ml of Trans-³⁵S-label, followed by the addition of cold methionine. Cells were allowed to divide and the subsequent progeny cells were isolated by Ludox density centrifugation. Proteins were immuno precipitated and visualized as described above.

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A sigma 54 transcriptional activator also functions as a pole-specific repressor in *Caulobacter*.

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