Spatial and temporal phosphorylation of a transcriptional activator regulates pole-specific gene expression in *Caulobacter*

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Polar localization of proteins in the *Caulobacter* predivisional cell results in the formation of two distinct progeny cells, a motile swarmer cell and a sessile stalked cell. The transcription of several flagellar promoters is localized to the swarmer pole of the predivisional cell. We present evidence that the product of the *flbD* gene is the transcriptional activator of these promoters. We show that FlbD is distributed in all cell types and in both poles of the the predivisional cell. We also demonstrate that FlbD can be phosphorylated, and that a FlbD kinase activity is under cell cycle control. Cells expressing a FlbD mutant that should activate transcription in the absence of phosphorylation, exhibited an alteration in the temporal pattern of flagellin transcription. Furthermore, predivisional cells expressing the mutant FlbD failed to polarly localize flagellin synthesis. We propose that the phosphorylation of FlbD is restricted to the swarmer compartment of the predivisional cell, and serves as the control point for regulating the spatial transcription of flagellar promoters.

[Key Words: *Caulobacter*; phosphorylation; transcriptional activator; cell cycle; polarity]

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Polar localization of gene products is an integral aspect of cellular differentiation in *Caulobacter*. A programmed sequence of differentiation events occurs within each cell cycle, resulting in the formation of two distinct daughter cells, a motile swarmer cell, and a sessile stalked cell (for review, see Newton and Ohta 1990; Gober and Shapiro 1991; Gober et al. 1991a). These two cell types differ in morphology, the capacity to reinitiate DNA replication, and their program of gene expression. Asymmetry is established in the predivisional cell, resulting in the biogenesis of a polar flagellum at the incipient swarmer cell pole and the specific programming of the stalked cell chromosome to reinitiate DNA replication. The molecular mechanisms underlying the generation of asymmetry in *Caulobacter* are similar to those operating in organisms as diverse as *Bacillus*, *Drosophila*, and the nematode (for review, see Horvitz and Herskowitz 1992).

Spatial localization of proteins in the predivisional cell type dictates the developmental fate of the progeny cells. Two different mechanisms are responsible for polar protein localization in *Caulobacter*. First, proteins are often specifically segregated to progeny stalked or swarmer cells. For example, two heat shock proteins, DnaK and Lon, are targeted to the stalked cell pole of the predivi-

sional cell (Reuter and Shapiro 1987). Conversely, motility-related proteins, such as flagellar and chemotaxis proteins, are targeted to the swarmer pole of the predivisional cell (Nathan et al. 1986; Loewy et al. 1987; Alley et al. 1992). The methyl-accepting chemotaxis receptor McpA is specifically targeted to a discrete location at the pole of predivisional cells (Alley et al. 1992). The process that recruits McpA to the pole is not understood. It is likely, however, that a specialized structure is present at the swarmer pole that serves to direct the assembly of flagellar and chemotaxis gene products. The pole of the predivisional cell is derived from the previous cell division site, suggesting that asymmetry is an intrinsic property of the *Caulobacter* cell cycle. A second mechanism that serves to position gene products is polar gene expression. The gene encoding DNA gyrase-B subunit, an essential DNA replication protein, is expressed exclusively in the stalked pole of the predivisional cell (M. Rizzo, L. Shapiro, and J. Gober, in prep.). Asymmetry in flagellar gene expression is achieved through the localization of flagellar mRNA (Milhausen and Agabian 1983; Gober et al. 1991b; Gober and Shapiro 1992). A consequence of mRNA localization is that the expression of a subset of flagellar genes is confined to the swarmer pole of the predivisional cell. Previously, we have investigated the mechanism of flagellar mRNA localization and found that all the information necessary for the polar localization of gene expression is contained within the

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promoter DNA sequence (Gober et al. 1991b). This experimental finding suggests that polar transcription is responsible for the localization of flagellar mRNA (Gober et al. 1991b; Gober and Shapiro 1992). Swarmer pole-specific transcription occurs in late predivisional cells after a constriction between the swarmer and stalked cell compartments has formed, but before separation of the progeny cells, and is limited to genes that encode external flagellar structures.

The promoters that exhibit polar transcription, the hook operon, *flaNQ*, and the 25-kD flagellin gene, *flgK*, share similar *cis*-regulatory sequences, suggesting that temporal and spatial transcription is accomplished through a common mechanism (Gober et al. 1991c; Gober and Shapiro 1992). All three promoters are expressed late in the cell cycle and are transcribed by σ^{54} -containing RNA polymerase (Mullin et al. 1987; Brun and Shapiro 1992). Expression of σ^{54} promoters is achieved through the action of transcriptional activator proteins that bind to enhancer sequences commonly located ~100 bp from the transcription start site (for review, see Kustu et al. 1989). Each of these flagellar promoters contains a conserved enhancer sequence known as an *ptr* element (Minnich and Newton 1987; Mullin et al. 1987; Mullin and Newton 1989; Gober et al. 1991c). The temporal and spatial transcription of these promoters is probably regulated by proteins that bind to the *ptr* enhancer sequence. Two proteins are known to bind to the *ptr* enhancer, a 95-kD protein, Rf-1, and a 55-kD protein (Gober et al. 1991c; Gober and Shapiro 1992). Both binding activities are under cell cycle control and occur at maximal levels in the predivisional cell type (Gober et al. 1991b; Gober and Shapiro 1992). The *ptr*-containing promoters also contain a binding site for integration host factor (IHF) located between the enhancer and the promoter sequences (Gober and Shapiro 1990; Gober and Shapiro 1992). The IHF-binding site in both the hook and *flaNQ* promoters is required for maximal levels of transcription (Gober and Shapiro 1990, 1992). As is the case with the enhancer-binding proteins, IHF is expressed under cell cycle control (Gober and Shapiro 1992).

In this paper we investigate the mechanism of both temporal and spatial transcription of *ptr*-containing flagellar promoters. We provide both genetic and biochemical evidence that the product of the *flbD* gene is the transcriptional activator of this subset of flagellar promoters. To examine the regulation of FlbD expression, we fused the carboxyl terminus to a 7-amino-acid epitope. Immunoblots prepared from cell cycle extracts showed that FlbD is present in all cell types during the cell cycle and in both compartments of the predivisional cell.

If FlbD is responsible for the temporal and spatial expression of these flagellar genes, then its activity must be modulated in both a cell cycle and spatial manner. We demonstrate that a kinase activity present in *Caulobacter* cell extracts can catalyze the phosphorylation of pure FlbD and is expressed under cell cycle control, with maximal activity occurring in the predivisional cell stage. We constructed a mutant FlbD protein that should be

able to activate transcription in the absence of kinase activity. We then constitutively expressed this protein and assayed the cell cycle expression of *flgK* and swarmer pole-specific expression of flagellins. Predivisional cells expressing the mutant FlbD fail to polarly localize flagellin synthesis. These results are consistent with the view that the phosphorylation of FlbD serves as a control point for regulating the spatial transcription of flagellar promoters.

Results

Genetic evidence for the role of flbD in transcriptional activation of ptr-containing flagellar promoters

To determine the mechanism of compartment-specific transcription of the hook (*flbG*), *flaNQ*, and *flgK* promoters, we set out to genetically identify the transcriptional activator that binds to the *ptr* enhancer element. One consequence of the flagellar regulatory hierarchy is that strains with mutations in many early flagellar structural genes (class I genes) do not transcribe *ptr*-containing promoters such as *flbG* and *flaNQ* (Newton et al. 1989; Xu et al. 1989). Therefore, the phenotype of strains with mutations in class I structural genes is indistinguishable from that of strains having mutations in genes that encode *trans*-acting regulatory proteins. We have now measured the level of transcription of the two other known *ptr*-containing promoters, *flgL* (27-kD flagellin) and *flgK* (25-kD flagellin) using promoter fusions to a promoterless *lacZ* reporter gene. The *flgL-lacZ* transcription fusion generates 8720 units of β -galactosidase in a strain that is wildtype for motility (NA1000) and was not expressed in any class I mutant strains (Table 1). This pattern of expression is identical to that obtained with *flaNQ* and *flbG* promoter fusions (Newton et al. 1989). In contrast, the *flgK-lacZ* fusion differs in its pattern of expression, in that it did not require most of the class I gene products for expression. This result indicates that *flgK* transcription, unlike other *ptr* promoters, is not influenced by flagellar assembly cues. The *flgK* promoter was expressed in all of the class I mutant strains tested, with two notable exceptions. Strains with a Tn5 insertion in either *rpoN* or *flbD* did not express the *flgK-lacZ*

Table 1. Transcription of flagellin genes in class I mutants

Strain	Genotype	β -Galactosidase activity ^a	
		<i>flgL-lacZ</i>	<i>flgK-lacZ</i>
NA1000	<i>syn-1000</i>	8720	6000
SC1131	<i>fliLM</i>	271	5682
SC1132	<i>flbF</i>	160	5538
SC1029	<i>podW</i>	178	4318
SC1048	<i>flaH</i>	252	5952
SC508	<i>fliQ</i>	230	6258
SC1032	<i>flbD</i>	125	224
SC1055	<i>rpoN</i>	138	158

^aExpressed as units (Miller 1972).

fusion (Table 1). *rpoN* encodes the σ^{54} subunit of RNA polymerase (Brun and Shapiro 1992) and is therefore essential for the transcription of these class II and III genes. Sequence analysis has demonstrated that *flbD* would encode a 52-kD polypeptide that is homologous to the transcriptional activators of other σ^{54} promoters (Ramakrishnan and Newton 1990). These data suggest that FlbD may encode the previously identified 55-kD protein that binds to the *ftr* enhancer (Gober et al. 1991c) and may directly activate the transcription of *flgK* as well as other *ftr*-containing promoters.

Purification and characterization of FlbD

To determine whether FlbD is the transcriptional activator of genes containing the *ftr* enhancer element, we subcloned the *flbD* gene from a cosmid that complements motility in a strain containing a Tn5 insertion in *flbD* (SC1032). Using site-directed mutagenesis, a *Bam*HI restriction site was introduced immediately adjacent to the translation initiation ATG codon. A 1800-bp *Bam*HI–*Eco*RI DNA fragment from this construction, which contains the entire *flbD* coding sequence, was then subcloned into the expression vector pTrcHisA, so that a fusion protein was created between the leader sequence of the vector and the full-length FlbD protein. The amino terminus (~3-kD) of this fusion contains both a stretch of histidine residues and a peptide se-

quence from bacteriophage T7 coat protein. The His–FlbD fusion protein was used in all in vitro experiments.

The fusion protein was purified as described in Materials and methods. The His–FlbD preparation eluted from a nickel–Sephacrose column contained a predominant Coomassie blue-stained protein band with an apparent molecular mass of 54 kD (Fig. 1A). A second lower molecular mass polypeptide (~42 kD) was often seen in these fractions. The abundance of this smaller protein increases upon storage of the preparation, suggesting that it is a product of the degradation of FlbD. The 54-kD polypeptide is likely to be the His–FlbD fusion protein, as it reacted in an immunoblot with an antibody directed against the amino terminus of the fusion (Fig. 1B). To demonstrate that purified FlbD binds specifically to *ftr* promoters, two different gel mobility shift experiments were performed. In the first experiment, purified FlbD was incubated with 32 P-end-labeled fragments containing promoter DNA from each of the four *ftr*-containing genes. In all four cases, a shift in the electrophoretic mobility of the promoter DNA was observed when incubated with highly purified FlbD fusion protein, indicating that FlbD binds to all of these promoters (Fig. 1C). To determine whether binding required an intact *ftr* enhancer sequence, a gel mobility shift competition experiment was performed (Fig. 2). A radioactive, double-stranded oligonucleotide containing the *ftr* enhancer sequence from *flbG* was used to assay FlbD-binding

Figure 1. Purified FlbD binds to promoter DNA containing the *ftr* enhancer region. (A) SDS-PAGE of the purification of the His–FlbD fusion protein stained with Coomassie blue. The 1365-bp *flbD*-coding region was cloned in-frame into the vector pTrcHisA, which allowed for the expression of the 52-kD *flbD* product fused to a 3-kD amino-terminal peptide containing a polyhistidine region. (Lane 1) Proteins present after IPTG-induced expression in *E. coli*; (lane 2) proteins present in inclusion bodies from the same IPTG-induced culture; (lane 3) elution of highly purified His–FlbD from nickel–Sephacrose. (B). Immunoblot of the same protein fractions of A using antibody directed against the fusion portion of His–FlbD, and secondary antibody conjugated to alkaline phosphatase. (Lane 1) Crude extract from culture induced with IPTG; (lane 2) inclusion body fraction from culture induced with IPTG; (lane 3) highly purified fraction from the nickel–Sephacrose column. (C) Gel mobility shift analysis performed with *ftr*-containing promoter fragments and highly purified His–FlbD. 32 P-End-labeled fragments containing the promoter sequences of *flbG*, *flgK*, *flgL*, and *flaN* (see Materials and methods) were incubated either with or without pure His–FlbD and were then electrophoresed in a nondenaturing, 8% polyacrylamide gel. (B) DNA complexed with protein; (F) free DNA. (D). Comparison of the *ftr* sequences of *flbG*, *flgK*, *flgL*, and *flaN*. The distance in base pairs relative to the transcription start site is indicated by the number located above each sequence.

column. (C) Gel mobility shift analysis performed with *ftr*-containing promoter fragments and highly purified His–FlbD. 32 P-End-labeled fragments containing the promoter sequences of *flbG*, *flgK*, *flgL*, and *flaN* (see Materials and methods) were incubated either with or without pure His–FlbD and were then electrophoresed in a nondenaturing, 8% polyacrylamide gel. (B) DNA complexed with protein; (F) free DNA. (D). Comparison of the *ftr* sequences of *flbG*, *flgK*, *flgL*, and *flaN*. The distance in base pairs relative to the transcription start site is indicated by the number located above each sequence.

Figure 2. DNA binding competition assay to determine FlbD-binding specificity. 32 P-labeled double-stranded oligonucleotide representing the *flbG* sequence of *flbG* (–80 to –120 bp from the transcription start) (Gober et al. 1991a) was used as a binding substrate (0.1 pmole/lane). Increasing amounts of non-radioactive, competitor plasmid containing either the wild-type *flbG* promoter (pII-Kic) (Gober et al. 1991a) or a *flbG* promoter with a 2-bp mutation in the *flbG* promoter (pGRFII) (Gober and Shapiro 1992) were incubated with His-FlbD and the labeled oligonucleotide and electrophoresed on a nondenaturing, 8% polyacrylamide gel. All lanes contained labeled *flbG* oligonucleotide. (Lane 1) Labeled *flbG* oligonucleotide without protein; (lanes 2,7) pure His-FlbD, no competitor; (lane 3) protein plus 0.5 pmole of wild-type competing plasmid; (lane 4) protein plus 0.2 pmole of wild-type competing plasmid; (lane 5) protein plus 0.1 pmole of wild-type competing plasmid; (lane 6) protein plus 0.05 pmole of wild-type competing plasmid; (lanes 8–11) pure His-FlbD plus 0.5, 0.2, 0.1, and 0.05 pmole, respectively, of mutant *flbG* competitor, pGRFII. Sequences of wild-type *flbG* promoter (pII-Kic) and mutated *flbG* promoter (pGRFII) are shown below.

activity. Plasmids containing either the wild-type *flbG* promoter or an *flbG* promoter that contained a 2-bp mutation in the *flbG* promoter were used as nonradioactive competitors (Fig. 2). This *flbG* mutation completely abolishes *flbG* transcription in vivo (Gober and Shapiro 1992). The wild-type *flbG* promoter effectively competed for DNA-binding activity at one-half the concentration of the labeled oligonucleotide probe. In contrast, a fivefold molar excess of the *flbG* promoter with the mutant *flbG* promoter competed only slightly for FlbD binding (Fig. 2). These experiments indicate that FlbD requires the same *flbG* sequences for binding as are required for in vivo transcriptional activation of the *flbG* promoter. They also indicate that FlbD is likely to be the transcriptional activator of promoters with *flbG* enhancers.

Temporal regulation of *flbD* expression

The timed transcriptional activation of *flbG*-containing promoters may be accomplished by modulating the cellular levels of FlbD during the *Caulobacter* cell division cycle. To test this model, a 7-amino-acid M2 epitope was fused to the carboxyl terminus of FlbD. A *Bam*HI restriction site was introduced by site-directed mutagenesis directly after the final *flbD* codon (Fig. 3A). This construction results in the addition of 9 amino acids to the carboxyl terminus of FlbD. The modified *flbD* was then subcloned into plasmid pJM20, creating an in-frame fusion protein with the M2 epitope. The promoter that expresses *flbD*, from the *flbG* operon, was cloned 5' to this fusion, and the entire construction was mated into *Caulobacter* on a plasmid. To test whether the epitope-tagged FlbD could function properly, a complementation assay was performed (Fig. 3B). The FlbD::M2 fusion plasmid was introduced into a recombination deficient (*rec*[–]), *flbD*::Tn5 mutant (SC1032), and motility was assayed by stabbing the cells into semisolid swarm agar. Motility is characterized by a large zone of growth that surrounds the point where the cells were inoculated. The FlbD::M2 fusion restored motility in the *flbD*–

A pflbD/JM21

B

Figure 3. Construction of a FlbD-epitope tag protein fusion. (A) Schematic map of the construction of a FlbD::M2 fusion. A *Bam*HI restriction site was introduced following the final codon of *flbD* by site-directed mutagenesis. This was ligated to pJM20, creating an in-frame fusion of *flbD* with the 7-amino-acid M2 epitope. A 0.3-kb *Bam*HI–*Hind*III fragment containing the *flbG* operon promoter was then ligated 5' to this fusion protein. The entire construction was then ligated into pRK290/20R for introduction into *Caulobacter*. (B) pflbD/JM20 was introduced into recombination deficient (*rec*[–]) wild-type and *flbD*::Tn5 (SC1032) strains of *Caulobacter*, and motility was assayed in semisolid agar. Motility is indicated by a wide halo of bacterial growth surrounding the initial point of inoculation.

D::Tn5 mutant (Fig. 3B), indicating that the M2 peptide fused to the carboxyl terminus of FlbD does not significantly interfere with FlbD function.

Two different experiments were performed to assay the cell cycle expression of FlbD::M2. First, the cell cycle pattern of synthesis of FlbD was assayed by synchronizing cultures that harbored the FlbD::M2 fusion plasmid and pulse-labeling proteins with [35 S]methionine at different times during the cell cycle. Labeled FlbD::M2 was then immunoprecipitated from extracts using anti-M2 antibody and subjected to polyacrylamide gel electrophoresis (PAGE). The synthesis of FlbD::M2 was under cell cycle control (Fig. 4A), with expression beginning in early stalked cells and peaking slightly in predivisional cells. This pattern is similar to the pattern of expression of mRNA transcribed from this promoter (Van Way et al. 1993). To determine whether the availability of FlbD was temporally controlled or restricted to the predivisional cell type, we performed an immunoblot on extracts prepared from synchronized cells harboring the FlbD::M2 fusion plasmid (Fig. 4B). The level of FlbD::M2 varied slightly during the cell cycle but was present in all cell types, including those that do not express *fla* promoters. The relatively constant level of FlbD::M2 throughout the cell cycle indicates that the rate of turnover of the protein probably increases at times when its rate of synthesis increases. The net result would be a relatively constant level of FlbD throughout the cell cycle. We should note that the epitope fusion at the amino terminus of FlbD may also alter its stability. The cell cycle pattern of FlbD::M2 synthesis did not parallel the temporal pattern of flagellin expression in this experiment (Fig. 4C). These data indicate that FlbD levels are not subject to cell cycle control and suggests that

FlbD activity may be modulated as a function of the cell cycle.

Role of FlbD in regulating polar flagellar gene expression

The polar transcription of *fla*-containing promoters may result from the specific positioning of essential transcription factors to the swarmer pole of the predivisional cell. We performed two different experiments to determine whether FlbD is specifically targeted to the swarmer pole. The intracellular distribution of the FlbD::M2 fusion was first assayed by immunofluorescence microscopy (Fig. 5A). This technique has been employed successfully to examine the targeting of the methyl-accepting chemotaxis receptor to the poles of both *Caulobacter* and *Escherichia coli* (Maddock and Shapiro 1993; M. Alley, J. Maddock and L. Shapiro, unpubl.). This assay demonstrated that the FlbD::M2 fusion was present in both poles of the predivisional cell and was also present in all cell types (Fig. 5A). The latter result agrees with the cell cycle immunoblot experiment presented above (see Fig. 4B). A negative control for this experiment consisted of reacting anti-M2 antibody with cells that did not contain FlbD::M2. In this case, the cells did not fluoresce and were not visible (not shown). To confirm the results obtained by microscopy, we performed an immunoblot on newly divided progeny swarmer and stalked cells. In this experiment, a culture was synchronized and permitted to progress through the entire cell cycle. Immediately following cell division, the progeny swarmer and stalked cells were separated by centrifugation through Ludox LS, and extracts were prepared and subjected to an immunoblot following electro-

Figure 4. Cell cycle pattern of FlbD-M2 expression. (A) Cell cycle synthesis of FlbD::M2. Swarmer cells were isolated, suspended in fresh medium, and allowed to progress through the cell cycle. Periodically, a sample was removed and proteins were labeled with Trans 35 S-label. Samples were labeled at 0, 45, 90, 120, 150, and 180 min. The cell types present at each time point, as assayed by microscopy are represented schematically above each lane. Extracts were prepared, and FlbD::M2 was immunoprecipitated using monoclonal anti-M2 antibody in conjunction with

Staphylococcus aureus cells. Immunoprecipitated protein was subjected to PAGE and visualized on x-ray film following fluorography. (B) Assay of the level of FlbD::M2 throughout the cell cycle by immunoblot. Extracts from the above experiment were electrophoresed, transferred to nitrocellulose, and probed with monoclonal anti-M2 antibody. Antibody-antigen complexes were detected with secondary antibody conjugated to alkaline phosphatase. (C) Cell cycle expression of flagellin protein. Flagellin was immunoprecipitated with anti-flagellin antibody using the 35 S-labeled cell extracts from A.

Figure 5. Localization of FlbD-M2 in the predivisional cell. (A) Immunofluorescence microscopy with anti-M2 antibody. Immunofluorescence was performed on *Caulobacter* cells harboring pFlbD/JM20 using monoclonal anti-M2 antibody. Secondary goat anti-mouse antibody was conjugated to biotin. The complex was detected by using Texas red conjugated to streptavidin. (B) Cell type distribution of FlbD::M2 immediately following cell division. Cells harboring pFlbD/JM20 were synchronized and allowed to divide. The progeny cell types were separated by centrifugation in Ludox. Equal quantities of protein from the stalked and swarmer cell fractions were electrophoresed, and an immunoblot was performed using monoclonal anti-M2 antibody. Secondary antibody was conjugated to alkaline phosphatase. (C) Cell type distribution of flagellin protein immediately following cell division. Immunoblot using polyclonal anti-flagellin antibody was performed on the same extracts used in B.

phoresis. Progeny stalked and swarmer cells contained equal amounts of FlbD::M2 (Fig. 5B), indicating that the protein is not preferentially targeted to either pole of the predivisional cell. As a control, an immunoblot was performed on these same extracts using anti-flagellin antibody. More flagellin was present in the progeny swarmer

cells than in the stalked cells (Fig. 5C). A small amount of flagellin was present in the stalked cell fraction. This is because this fraction was slightly contaminated with predivisional cells that express high levels of flagellin. Unlike FlbD, the flagellins are targeted to the swarmer pole of the predivisional cell where they are assembled into the flagellum structure. These data suggest that the pole-specific expression of *flb*-containing promoters is not the result of polar localization of FlbD.

Temporal control of FlbD activity by phosphorylation

The experimental data presented above demonstrates that FlbD is not under temporal regulation, nor is the protein spatially localized to the swarmer pole of the predivisional cell. The amino terminus of FlbD is homologous to the amino-terminal domain of a large family of regulatory proteins that usually respond to environmental cues (Nixon et al. 1986; for review, see Parkinson and Kofoed 1992). NtrC is the best known σ^{54} transcriptional activator in this family of regulatory proteins and is activated through the phosphorylation (Ninfa and Magasanik 1986; Keener and Kustu 1988; Weiss and Magasanik 1988) of an aspartate residue (D54) in its amino terminus (Sanders et al. 1992). The amino-terminal domain of FlbD possesses the analogous aspartate residue (D52) that is phosphorylated in NtrC. Given that FlbD levels are not temporally or spatially controlled, it is possible that the expression pattern of flagellar promoters is the result of a transient activation of FlbD by phosphorylation.

If FlbD activity is regulated by phosphorylation, the aspartate residue at position 52 could be essential for the activity of the protein. We changed this residue to an alanine by site-directed mutagenesis. The mutant *flbD* (D52A) was introduced into a *flbD*::Tn5 (SC1032) strain, and motility was assayed by light microscopy. The mutant D52A *flbD* was unable to complement motility in this strain (not shown), indicating that the D52 residue is important for FlbD function. We then tested whether FlbD was phosphorylated in *Caulobacter*. To accomplish this, strains harboring the plasmid expressing epitope-tagged FlbD (pFlbD/JM20) were labeled with either Trans³⁵S-label or inorganic ³²PO₄. Two different strains were labeled, a wild-type (NA1000) strain and the mutant *flbD*::Tn5 (SC1032). As a control, a wild-type strain that did not carry plasmid pFlbD/JM20 was also labeled. Extracts were prepared, and the FlbD::M2 fusion was immunoprecipitated using anti-M2 antibody. Immunoprecipitated proteins were then separated by PAGE. A 54-kD ³²P-labeled protein could be immunoprecipitated only from cultures expressing the FlbD::M2 fusion. At least two other ³²P-labeled polypeptides coimmunoprecipitated with FlbD::M2 (Fig. 6A), indicating that other phosphorylated proteins may interact with FlbD. These proteins also coimmunoprecipitated with FlbD::M2 in the cultures that were labeled with ³⁵S. ³²P-labeled proteins could not be immunoprecipitated from cells that did not contain pFlbD/JM20 (Fig. 6A). In addition, labeled protein could not be immunoprecipitated

Figure 6. Phosphorylation of FlbD. (A) In vivo phosphorylation of FlbD::M2. NA1000 (wild type for motility) or SC1032 (*flbD*::Tn5) cultures harboring pFlbD/JM20 (or control cells without plasmid) were grown in low phosphate BM medium (Contreras et al. 1978). Cells were labeled with either Trans- ^{35}S -label or $^{32}\text{P}_4$ (200 $\mu\text{Ci/ml}$) for 15 min. FlbD-M2 was immunoprecipitated with monoclonal anti-M2 antibody as described previously, and the labeled proteins were separated by SDS-PAGE. (B) In vitro phosphorylation of FlbD. Approximately 5 μg of crude cell extract from *Caulobacter* NA1000 was incubated at 30°C in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ either without (-FlbD), or with 1 μg of highly purified his-FlbD (+FlbD). Samples were removed at the time points indicated, passed through a Sepharose G-25 column to remove excess label, and frozen on dry ice. The samples were electrophoresed on a 12% SDS-polyacrylamide gel, and the gel was dried and exposed to film.

from $^{32}\text{P}_4$ labeled cells that carried a methyl-accepting chemotaxis receptor (McpA) fused to the M2 epitope (not shown).

These experiments suggest that FlbD may be phosphorylated in vivo but do not rule out the possibility that the FlbD::M2 fusion protein is not phosphorylated; rather it coimmunoprecipitates with several phosphoproteins. We therefore tested whether cell extracts could catalyze the phosphorylation of pure FlbD in vitro (Fig. 6B). The reaction consisted of crude extract incubated in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and, in some cases, ~ 2.5 μg of pure His-FlbD. Samples were removed after 5, 10, and 25 min of incubation. Because acyl-phosphates are inherently unstable, the reactions were quenched with EDTA, effectively chelating out Mg^{2+} ion, which is required for the dephosphorylation of this class of response regulators (Stock et al. 1992). Phosphorylation of 54- and 56-kD bands was seen in samples in which exogenous His-FlbD was added, with maximal phosphorylation occurring by 10 min (Fig. 6B). The presence of a double band might indicate that FlbD is post-translationally modified in addition to being phosphorylated. In the control reaction in which exogenous FlbD was absent, faint doublets were seen at 10 and 25 min (Fig. 6B). These bands were not seen when extracts were prepared from a *flbD*::Tn5 strain, suggesting that they may correspond to endogenous FlbD (not shown). A control reaction including His-FlbD and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, but lacking *Caulobacter* extract, yielded no labeled proteins corresponding to the molecular mass of FlbD (not shown).

The experiments described above employed cells or cell extracts from mixed, unsynchronized populations that contain all cell types. We therefore prepared cell-cycle, stage-specific extracts from a synchronous culture

of *Caulobacter*. These extracts were then used as a source of FlbD kinase in the in vitro phosphorylation assays. The phosphorylation of FlbD is under cell cycle control. Little or no activity was demonstrable in the early stages of the cell cycle (Fig. 7). The peak in phosphorylation activity occurred between 120 and 150 min into the cell cycle, corresponding to the time when the *flbD*-containing flagellar genes are maximally transcribed (Fig. 7). At 180 min, after the cells had undergone division, phosphorylation activity was diminished (Fig. 7). This experiment supports the model that the cell cycle

Figure 7. Cell cycle phosphorylation of FlbD. Swarmer cells isolated from wild-type strain NA1000 were allowed to proceed synchronously through the cell cycle. The generation time was 180 min. Every 30 min, samples were removed and lysed by sonication in the presence of protease inhibitors. Cell types at the time of sample removal are indicated above each time point. Protein concentrations were determined by Bradford assay. Equal amounts of protein from each of the stage-specific extracts was used in the phosphorylation assay, as described in Materials and methods. Samples were resolved on 12% SDS-PAGE, and the phosphorylated proteins were visualized by autoradiography. The band corresponding to phosphorylated FlbD is indicated at right.

phosphorylation of FlbD regulates the temporal transcription of flagellar genes.

FlbD phosphorylation and the temporal regulation of flagellar gene expression

To assess the contribution of FlbD phosphorylation in regulating temporal transcription, we created a mutant protein that should be active in the absence of phosphorylation. This was achieved by changing a serine in the conserved central domain (S140) to a phenylalanine. A mutation of this type in NtrC (S160F) has been shown to activate transcription in vitro, in the absence of its cognate kinase NtrB (Popham et al. 1989; Weiss et al. 1991). The mutant NtrC, however, still responds to phosphorylation. We placed wild-type and the mutant (S140F) *flbD* under control of a *tac* promoter (which is expressed constitutively in *Caulobacter*) and assayed the cell cycle

pattern of expression of both a *flgK::lacZ* fusion protein and a *flgK*-promoterless *neo* transcription fusion by immunoprecipitation. Cells overexpressing wild-type FlbD exhibited a normal pattern of *flgK* expression (Fig. 8A,C; see Fig. 7 for comparison with flagellin protein expression). Expression of both fusions was maximal in late predivisional cells. Note that expression in swarmer cells is characteristic of *flgK* and is not the result of new transcription; rather, it is the result of translation of mRNA that was transcribed previously in the swarmer pole of predivisional cells (Milhausen and Agabian 1983; Gober et al. 1991c). In cells overexpressing the mutant FlbD (S140F), a slight increase in *flgK* expression in swarmer cells and stalked cells was observed between 0.15 and 0.45 division units (Fig. 8B and D, respectively). The peak in expression occurred at the same time as cells expressing wild-type FlbD, which is expected because the S140F mutant probably can respond to phos-

Figure 8. Role of FlbD activation in regulating cell cycle expression of a flagellin promoter. (A) Cell cycle pattern of *flgK::lacZ* protein fusion expression in a strain harboring wild-type *flbD* on an overexpression vector. Cells were synchronized as described in Materials and methods. Swarmer cells were suspended in fresh M2-glucose medium and allowed to progress through the cell division cycle at 32°C. Samples were removed at 0, 30, 60, 90, 120, 150, and 180 min, labeled with Trans ³⁵S-label, and the FlgK::β-galactosidase fusion was immunoprecipitated with anti-β-galactosidase antibody. For all time points, 1 × 10⁶ cpm of labeled protein was incubated with antibody. Immunoprecipitated protein was subjected to PAGE. The cell types present at each time point are represented schematically above each lane. (B) Cell cycle pattern of *flgK::lacZ* expression in a strain harboring mutant *flbD* S140F on an overexpression vector. Samples were treated as described in A. For all time points, 1 × 10⁶ cpm of labeled protein was incubated with antibody. (C) Cell cycle pattern of neomycin phosphotransferase (NPTII) expression from a *flgK-neo* transcription fusion in a strain harboring wild type *flbD* on an overexpression vector. The experiment was performed exactly as described above, except the cells harbored a *flgK-neo* transcription fusion on plasmid pRK290. The product of the *neo* gene, NPTII, was immunoprecipitated with anti-NPTII antibody (5'-3', Inc., Boulder, CO). For all time points, 1 × 10⁶ cpm of labeled protein was incubated with antibody. (D) Cell cycle pattern of neomycin phosphotransferase (NPTII) expression from a *flgK-neo* transcription fusion in a strain harboring mutant *flbD* S140F on an overexpression vector. Samples were treated as described above. NPTII was immunoprecipitated using anti-NPTII antibody. For all time points, 1 × 10⁶ cpm of labeled protein was incubated with antibody.

A *tac-flbD* (wild type)

B *tac-flbD* (S140F)

C *tac-flbD* (wild type)

D *tac-flbD* (S140F)

phorylation. The temporal pattern of expression in cells harboring the S140F mutant indicates that other factors in addition to the temporal phosphorylation of FlbD may have a role in regulating the cell cycle transcription of these promoters.

Role of FlbD phosphorylation in regulating the spatial pattern of flagellar gene expression

The mutant S140F FlbD was also used to determine the role of FlbD activation in swarmer pole-specific expression of flagellar genes. This experiment was essentially the same as that used previously to demonstrate swarmer pole-specific transcription (Gober et al. 1991; Gober and Shapiro 1992). Cells that overexpressed wild-type FlbD were synchronized and allowed to progress to the predivisional stage. Protein was pulse-labeled with Trans³⁵S-label, the label was chased, and the cells were permitted to divide. Stalked and swarmer progeny were separated by centrifugation in Ludox, and flagellin was immunoprecipitated. The presence of labeled flagellin in progeny cell fractions provides a record of the polar location of flagellin expression in the predivisional cell. When cells overexpressed wild-type FlbD, labeled flagellin was present only in progeny swarmer cells (Fig. 9A). This result is a consequence of localized expression of this protein in the swarmer pole of the predivisional cell, as well as specific targeting to the assembling flagellum. The same experiment was performed on cells harboring a plasmid that overexpresses the mutant FlbD (S140F) (Fig. 9B). In addition to synthesizing the 25-kD flagellin, these cells also reproducibly synthesized both the 29- and 27-kD flagellin at this stage in the cell cycle. In wild-type cells, synthesis of these two minor flagellins usually ceases before the late predivisional stage. Therefore, the mutant FlbD (S140F) apparently affects the cell cycle timing of these two flagellins. Two unknown polypeptides that cross-react with the anti-flagellin antibody were also produced in this strain. These may be either flagellin degradation products or, in the case of the higher molecular mass band, may be post-translationally modified flagellin. Most notably, cells expressing the mutant FlbD (S140F) no longer exhibited swarmer cell compartment-specific flagellin expression. Labeled flagellin was present in both progeny swarmer and stalked cells. These data suggest that the modulation of FlbD activity has an essential role in polar gene expression and that the phosphorylation of FlbD is probably restricted to the swarmer pole of the predivisional cell.

Discussion

In this paper we investigate the mechanism of swarmer pole-specific transcription of a subset of flagellar genes in *Caulobacter*. Both genetic evidence and in vitro DNA-binding experiments presented here indicate that the product of the *flbD* gene is the transcriptional activator of this set of promoters. Although the gene encoding FlbD is expressed under cell cycle control, the levels of FlbD do not change significantly during the *Caulobacter*

Figure 9. Role of FlbD activation in swarmer pole-specific expression and targeting of flagellins. (A). Cells that overexpressed wild-type FlbD were synchronized and allowed to progress to the predivisional stage. Protein was pulse-labeled with [³⁵S]methionine, the label was chased, and the cells were permitted to divide. Stalked and swarmer progeny were separated by centrifugation in Ludox, and flagellin was immunoprecipitated with anti-flagellin antibody. Labeled 25-kD flagellin is indicated. The presence of labeled flagellin in progeny cell fractions records the polar location of flagellin synthesis in the predivisional cell. (B) The same experiment as described in A was performed on cells harboring a plasmid that overexpresses the constitutive mutant FlbD (S140F). ³⁵S-Labeled 25-, 27.5-, and 29-kD flagellins are indicated.

cell cycle. Overexpression of wild-type FlbD does not alter the temporal expression pattern of its target promoters. In addition, FlbD is not localized to the swarmer pole of the predivisional cell. We conclude that the activity of FlbD must be modulated in response to both temporal and spatial cues. A schematic depiction of the temporal and spatial activation of FlbD is shown in Figure 10. Although we presume that FlbD activity is modulated by phosphorylation, we acknowledge that this may not be the only influence on FlbD activity or on the expression of these flagellar genes. As depicted, FlbD is probably first activated in the early predivisional cell before a barrier has formed between the swarmer and stalked cell compartments. We hypothesize that the putative FlbD kinase responds to a morphogenetic cue such as a landmark in cell division or, perhaps, an early event in flagellar biogenesis. We propose that after the cell division constriction physically separates the swarmer and stalked cell compartments, FlbD becomes inactive in the stalked cell pole of the predivisional cell. This conclu-

Figure 10. Model for the role of FlbD in regulating temporal and spatial gene expression in *Caulobacter*. A schematic depiction of the temporal and spatial activation of FlbD is shown. FlbD is represented by D. Activated FlbD is represented by a filled-in D. FlbD is present in all cell types throughout the cell cycle and present in both poles of the predivisional cell. Therefore, the activity of FlbD must be modulated to activate temporal and spatial transcription. Phosphorylation of FlbD first occurs in early stalked cells. This may be attributable to the temporal synthesis of a FlbD kinase or, alternatively, kinase activity may be influenced by a morphogenetic cue. The phosphorylation of FlbD results in the timed transcription of flagellar promoters with *flr* enhancers. After a barrier forms between the two poles, active FlbD is restricted to the swarmer compartment of the predivisional cell. Inactivation of FlbD in the stalked cell pole may be the result of specific targeting of the FlbD kinase to the swarmer pole of the predivisional cell. Alternatively, separation of the swarmer and stalked poles may simply segregate the FlbD kinase in the stalk pole from its morphogenetic stimulus. We hypothesize that segregation of FlbD in the stalked cell pole, from its kinase, results in the spontaneous dephosphorylation of FlbD. After cell division, FlbD returns to a nonphosphorylated form in the progeny swarmer cells.

sion is based on the experimental finding that the strain possessing an FlbD S140F mutant, which should be active in the absence of phosphorylation, no longer exhibits compartment-specific expression of flagellins. Dephosphorylation of FlbD in the stalked cell compartment is not likely to require a specific phosphatase because acyl phosphates of the type presumably present in FlbD hydrolyze spontaneously at a rapid rate. For example, the half-life of phospho-NtrC has been estimated at 3.6–5.0 min (Keener and Kustu 1988). Therefore, inactivation of FlbD in the stalked pole may simply be a consequence of sequestering the FlbD kinase in the swarmer cell compartment. Alternatively, the formation of a barrier between the two cell poles may separate the FlbD kinase in the stalked pole from its morphogenetic stimulus. During *Bacillus* sporulation, the formation of the septum is one of the earliest events that leads to the establishment of differing programs of gene expression in the developing mother and forespore compartments. Gene expression in one compartment is coupled to developmental events in the other compartment (for review, see Losick and Stragier 1992). In *Caulobacter*, we have no evidence that a coupling of gene expression between the swarmer and stalked cell compartments exists. In this case, the barrier formed between the stalked and swarmer cell compartments simply results in separating FlbD in the stalked cell cytoplasm from swarmer pole-specific activation cues.

The experiments using the FlbD S140F mutant demonstrate a critical role for phosphorylation in establishing a polar pattern of transcription. Strains expressing

this mutant FlbD still exhibit cell cycle expression of *flgK*, indicating that phosphorylation of FlbD is not the only regulatory event controlling cell cycle transcription. It is probable that other factors contribute significantly to the temporal pattern of flagellar transcription. Rf-1, a 95-kD protein that binds to the *flr* enhancer, has been isolated (Gober and Shapiro 1992; Gober et al. 1991c). This cell cycle-controlled binding activity may have a role in regulating transcription of these promoters either by acting independently or through interaction with FlbD. Defining the role of Rf-1 in temporal regulation awaits the isolation of the gene that encodes this protein. These promoters also require IHF and the σ^{54} subunit of polymerase for transcription (Gober and Shapiro 1990; Gober and Shapiro 1992; Brun and Shapiro 1992). Both of these *trans*-acting factors are expressed under cell cycle control. Low levels of one or both of these factors in early stalked cells may be responsible for the decreased level of *flgK* transcription exhibited early in the cell cycle in cells that express the S140F mutant.

In contrast to other members of the response regulator family of transcription factors, FlbD is not phosphorylated in response to an environmental or nutritional cue but, rather, in response to a cell cycle-related event. This strategy is utilized in eukaryotic cells in which the phosphorylation of Oct-1, the transcription factor involved in the cell cycle transcription of histone H2B genes, is coupled to mitosis (Roberts et al. 1991). The coupling of FlbD activation to cell cycle events is presumably necessary for successful flagellar biogenesis. What is the nature of the kinase and cell cycle event that results in

FlbD phosphorylation? The simplest mechanism to account for the temporally controlled phosphorylation of FlbD is that the synthesis of the putative FlbD kinase is regulated in a cell cycle fashion. This model would require that the kinase be degraded periodically during the cell division cycle. There is precedence for the timed degradation of proteins within the *Caulobacter* cell cycle. The methyl-accepting chemotaxis receptor McpA is proteolyzed when swarmer cells differentiate into stalked cells (Alley et al. 1993). The timing of this particular protease activity cannot account for the pattern of FlbD expression, but it is possible that other proteases are under temporal control. In an alternative model, the kinase may be present throughout the cell cycle and be active only during specific temporal periods. By analogy to other two-component regulatory systems, the FlbD kinase could be phosphorylated in response to a specific cell cycle cue such as an early polar structure or a cell division event. A similar model has been hypothesized to control cell division events in *Caulobacter*. Two different histidine kinases, *pleC* and *divJ*, have been implicated in regulating cell cycle events in *Caulobacter* (Ohta et al. 1992; Wang et al. 1993). Mutations in *pleC* result in cells that are deficient in the biogenesis of polar structures such as the stalk and phage receptors (Sommer and Newton 1991; Wang et al. 1993). Cells with mutations in *divJ*, a suppressor of *pleC*, exhibit abnormal cell division (Sommer and Newton 1991). Therefore, it has been suggested that these kinases are components of a regulatory system that couples cell cycle events to polar morphogenesis (Ohta et al. 1992; Wang et al. 1993). These kinases probably do not encode the putative FlbD kinase, as strains with mutations in either of these genes still synthesize a flagellum (Sommer and Newton 1991), indicating that the genes encoding components of the flagellum are expressed in these mutants. Interestingly, the flagellum in *pleC* mutants does not function properly. It will be of interest to determine whether PleC and DivJ kinase activities are regulated in the same temporal fashion as the FlbD kinase.

Localization of phosphorylated FlbD to the swarmer cell compartment of the predivisional cell may result from specific targeting of kinase activity to the swarmer pole. Polar protein localization in *Caulobacter* has been documented for components of the flagellum and chemotaxis machinery. The chemotaxis receptor McpA is localized to a specific polar region of the cytoplasmic membrane adjacent to the flagellum (Alley et al. 1992). There is a distinct possibility that the putative FlbD kinase is a membrane protein and, like McpA, is targeted to the swarmer pole of the predivisional cell. Many histidine kinases in the family of two-component regulatory systems are membrane proteins (Parkinson and Kofoed 1992).

Another plausible model is that the FlbD kinase senses the establishment of the polar organizing center that directs the localization of McpA and other proteins to the swarmer pole. This polar organizing center is probably established in the stalked cell shortly after the initiation of DNA replication. The establishment of polar-

ity in the early stalked cell in *Caulobacter* is analogous to events that occur during the development of other organisms. For example, during the establishment of the terminal region in the early *Drosophila* embryo, a ligand of maternal origin is deposited at the termini of the egg (for review, see St. Johnston and Nüsslein-Volhard 1992). The presence of this ligand is sensed by a membrane protein, the product of the *torso* gene, which then transmits a signal to the inside of the egg. The *torso* gene product has homology to transmembrane receptor tyrosine kinases (Sprenger et al. 1989) and presumably represents the first member of a signal transduction pathway that senses the polar localization of the maternally derived ligand. The signal transmitted through this pathway eventually results in the transcription of terminal genes, such as *tailless* and *hüskebein* (Klinger et al. 1988; Strecker et al. 1988; Weigel et al. 1990). As outlined above, a signal transduction pathway may also be operating to establish polar gene expression in *Caulobacter*, with the phosphorylation of FlbD being the final step in the signaling pathway. As is the case with *Drosophila*, the initial event in the establishment of polarity lies embedded within the architecture of the cell itself. In *Caulobacter*, the new pole is always opposite the stalk and is derived from the septum of the previous division. Therefore, the establishment of polarity is obligately coupled to cell morphogenesis.

Materials and methods

Bacterial strains, plasmids, and growth conditions

Synchronizable *Caulobacter crescentus* strain NA1000 was used as a motile, wild-type strain. SC1032 is a *flbD::Tn5* mutant strain (Ely and Ely 1989). *Caulobacter* cells were grown at 32°C in either PYE medium (Poindexter 1964) or minimal M2-glucose medium (Johnson and Ely 1977). For $^{32}\text{PO}_4$ -labeling experiments, cells were grown in low phosphate BM medium supplemented with glucose (Contreras et al. 1978). Promoterless *lacZ* transcription fusions were constructed using plasmid *placZ/290* (Gober and Shapiro 1992; J. Gober and M.R.K. Alley, unpubl.). A *flgK-lacZ* transcription fusion was constructed by ligating a 580-bp *PstI-EcoRI* fragment containing the *flgK* promoter to *placZ/290* (containing DNA sequences from -411 to +169 bp from the transcription start site). The *flgL-lacZ* transcription fusion was constructed by subcloning a 750-bp *EcoRI-HindIII* fragment containing the *flgL* promoter into *placZ/290* (-650 to +110 bp from the transcription start site). A *flgK-lacZ* fusion protein was constructed by ligating the 580-bp *PstI-EcoRI flgK* DNA fragment to pJBZ282 (Alley et al. 1993), creating an in-frame fusion of amino acid 28 of *flgK* to amino acid 8 of *lacZ*. *flgK-neo* contains a *flgK* transcription fusion of the 580-bp *PstI-EcoRI* promoter DNA fragment to a promoterless *neo* gene from Tn5 on plasmid pRK290/20R (Gober et al. 1991c). For overexpression of FlbD in *Caulobacter*, pRK290/20R plasmid containing an *E. coli* *tac* promoter was used (J. Gober, unpubl.).

Cloning of *flbD* and construction protein fusions

A cosmid (T13) was isolated that complemented motility in strain SC1032 (*flbD::Tn5*). A complementing 7.0-kb *BamHI* fragment was subcloned from T13. Finally, an ~2.0-kb *PstI-*

EcoRI fragment containing the entire *flbD*-coding sequence was isolated. To construct a fusion protein for overproduction of FlbD in *E. coli*, a *BamHI* restriction site was introduced directly adjacent to the translation initiation codon. The 1800-bp *BamHI*-*EcoRI* fragment was then subcloned into pTrcHisA (Invitrogen, San Diego, CA) creating an in-frame fusion protein with the amino terminus of FlbD fused to the poly histidine-containing leader peptide of pTrcHisA. To construct a fusion with the M2 epitope, a *BamHI* restriction site was introduced directly adjacent to the final codon of *flbD*. A 1700-bp *PstI*-*BamHI* fragment containing the entire *flbD*-coding region was subcloned into plasmid pJM20 (Alley et al. 1993), creating an in-frame fusion protein with the M2 epitope (Fig. 3A). A 280-bp *BamHI*-*HindIII* fragment containing the promoter for the *fliF*-*flbD* operon was ligated 5' to the FlbD::M2 fusion. This entire construction, including promoter and FlbD::M2 fusion, was then ligated into plasmid pRK290/KS1 (Alley et al. 1993), which replicates in *Caulobacter*.

Overproduction and isolation of FlbD

Two liters of LB containing 50 µg/ml of ampicillin was inoculated with an overnight culture of *E. coli* TG-1 containing plasmid pTrcHis-*flbD*. Cells were grown at 37°C with vigorous shaking to an OD₆₀₀ of 0.2. Isopropylthiogalactoside (IPTG) was added (1 mM final concentration), and growth was continued for an additional 90 min. The cells were harvested by centrifugation for 15 min at 4300g at 4°C. The pellet was suspended in 100 ml of TG buffer (10 mM Tris-HCl at pH 7.4, 10% glycerol) and centrifuged again for 15 min at 12,000g at 4°C. Pellets were resuspended in 30 ml of TG buffer containing 0.1 mM phenylmethanesulfonyl fluoride and 0.05 mM *N*-tosyl-L-lysine chloromethyl ketone and *N*-tosyl-L-phenylalanine chloromethyl ketone. The cells were lysed in a French pressure cell. Comparison of the soluble protein fraction with the insoluble protein fraction after cell lysis showed that the majority of FlbD was deposited in an insoluble form into inclusion bodies. Inclusion body-containing pellets were obtained by centrifugation of the lysate at 18,500g for 30 min at 4°C. By SDS-PAGE analysis, FlbD constituted the majority of protein present in the insoluble fraction. The pellets were resuspended in HGEK (10 mM HEPES-HCl at pH 7.6, 10% glycerol, 0.1 mM EDTA, 100 mM KCl) containing 6 M guanidine-HCl and dialyzed stepwise versus 0.5 liters each of nickel-Sephacrose loading buffer (20 mM sodium phosphate at pH 7.8, 500 mM NaCl) containing 2, 0.5, and 0 M guanidine-HCl. All dialysis buffers contained 0.1% NP-40. The dialysate was centrifuged at 27,000g at 4°C for 30 min to remove precipitated protein. The supernatant containing the renatured protein was loaded onto a 2-ml nickel-Sephacrose column, which was rinsed with 10 ml of nickel-Sephacrose rinse buffer (20 mM sodium phosphate at pH 6.0, 500 mM NaCl). The protein was eluted with a 20 ml of linear gradient of rinse buffer ending in 300 mM imidazole, with 0.5 ml fractions being collected. Samples were run on 12% SDS-PAGE and stained with Coomassie blue. Immunoblot analysis was performed as described by Towbin et al. (1979), using primary antibody (Novagen, Madison, WI) directed against the amino-terminal peptide of the fusion.

DNA-binding assays

The DNA used in gel mobility shift assays was a double-stranded 40-bp oligonucleotide representing the -120 to -80 region of *flbG* (Gober et al. 1991a) that was end-labeled with [γ -³²P]ATP (Amersham, Arlington Heights, IL) with T4 polynucleotide kinase. Promoter DNA containing the *ftr* enhancer el-

ements were also used for DNA-binding studies. For *flbG*, a 120-bp *EcoRI*-*HindIII* fragment containing a 112-bp *SacI*-*DdeI* fragment of the *flbG* promoter, with sequences from -120 to -8 bp from the transcription start site, was used (Gober et al. 1991b). For *flgK*, a 319-bp *NcoI*-*EcoRI* fragment containing sequences from -150 to +169-bp from the transcription start was used. A 195-bp *BamHI*-*HindIII* fragment (restriction sites introduced by PCR) contained *flgL* promoter DNA from -185 to +10 bp. The *flaN* promoter DNA was a 540-bp *EcoRI*-*PstI* fragment from *flaN*/pUC19 containing a *SacI*-*PstI* fragment with sequence from -95 to +435 bp from the transcription start site. All probes were labeled with [α -³²P]dNTPs (Amersham, Arlington Heights, IL) using DNA Polymerase I, Klenow fragment. Gel mobility shift assays were performed as described previously (Gober and Shapiro 1992).

FlbD phosphorylation assay

Approximately 2.5 µg of purified His-FlbD and 5 µg of crude protein extract, prepared by sonicating *C. crescentus* NA1000 cells, were added to assay buffer containing 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM DTT, 0.5 mM EDTA, and 10 mM MgCl₂. The mixture was warmed for 2 min at 30°C, at which time 50 µCi of γ -³²P-labeled adenosine triphosphate (Amersham, Arlington Heights, IL) was added. The reaction was stopped by the addition of 50 mM (final concentration) EDTA. Samples were centrifuged through Sepharose G-25 to remove excess label. SDS-PAGE sample buffer was added, and samples were loaded, without boiling, onto a 12% SDS-polyacrylamide gel. The gel was dried and exposed to x-ray film overnight at -80°C. The cell cycle phosphorylation assay was performed identically, with stage-specific extracts prepared from a synchronized culture of NA1000.

Cell cycle expression experiments

Synchronizable *C. crescentus* NA1000 was grown in M2-glucose medium to an OD₆₆₀ = 1.0–1.4. The cells were harvested by centrifugation, and swarmer cells were isolated as by centrifugation in a Ludox gradient (Evinger and Agabian 1977). Pure swarmer cell populations (>97% pure) were suspended in fresh M2-glucose medium and permitted to progress through the cell cycle at 32°C. At different times thereafter, samples were removed and proteins were labeled for 5 min with 3 µCi/ml of Trans³⁵S-label (ICN, Irvine, CA). Labeled protein was immunoprecipitated as described in Gomes and Shapiro (1984). For cell cycle immunoblots, cells (1 ml) were centrifuged and the pellet was suspended in SDS sample buffer (0.2 ml), boiled and subjected to SDS-PAGE. Immunoblotting was performed as described by Towbin et al. (1979). Monoclonal anti-M2 antibody was obtained from IBI, Inc. (New Haven, CT). Monoclonal anti- β -galactosidase antibody was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Secondary goat anti-mouse antibody conjugated to alkaline phosphatase was obtained from Bio-Rad (Hercules, CA). Immunofluorescence microscopy was performed on mid-log phase cultures essentially as described by Maddock and Shapiro (1993). Antigen-antibody complexes were visualized by the binding of streptavidin Texas Red to biotin-conjugated, goat anti-mouse secondary antibody (GIBCO BRL, Gaithersburg, MD).

DNA manipulations

Site-directed mutagenesis was performed on *Caulobacter* DNA in M13 templates grown in *E. coli* strain CJ236 (*dut ung*) (Kunkel and Roberts 1987). Oligonucleotides for site-directed

mutagenesis were synthesized on a Pharmacia Gene Assembler automated DNA synthesizer. All site-directed mutagenesis was confirmed by DNA sequencing using the dideoxy chain termination method (Sanger et al. 1977). Plasmids were introduced into *Caulobacter* by mating with *E. coli* donor strain S17-1 (Simon et al. 1983). β -Galactosidase assays were performed as described previously (Gober and Shapiro 1990).

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