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FlbT Couples Flagellum Assembly to Gene Expression in *Caulobacter crescentus*

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The biogenesis of the polar flagellum of *Caulobacter crescentus* is regulated by the cell cycle as well as by a *trans*-acting regulatory hierarchy that functions to couple flagellum assembly to gene expression. The assembly of early flagellar structures (MS ring, switch, and flagellum-specific secretory system) is required for the transcription of class III genes, which encode the remainder of the basal body and the external hook structure. Similarly, the assembly of class III gene-encoded structures is required for the expression of the class IV flagellins, which are incorporated into the flagellar filament. Here, we demonstrate that mutations in *flbT*, a flagellar gene of unknown function, can restore flagellin protein synthesis and the expression of *fljK*::*lacZ* (25-kDa flagellin) protein fusions in class III flagellar mutants. These results suggest that FlbT functions to negatively regulate flagellin expression in the absence of flagellum assembly. Deletion analysis shows that sequences within the 5′ untranslated region of the *fljK* transcript are sufficient for FlbT regulation. To determine the mechanism of FlbT-mediated regulation, we assayed the stability of *fljK* mRNA. The half-life ($t_{1/2}$) of *fljK* mRNA in wild-type cells was approximately 11 min and was reduced to less than 1.5 min in a *flgE* (hook) mutant. A *flgE flbT* double mutant exhibited an mRNA $t_{1/2}$ of greater than 30 min. This suggests that the primary effect of FlbT regulation is an increased turnover of flagellin mRNA. The increased $t_{1/2}$ of *fljK* mRNA in a *flbT* mutant has consequences for the temporal expression of *fljK*. In contrast to the case for wild-type cells, *fljK*::*lacZ* protein fusions in the mutant are expressed almost continuously throughout the *C. crescentus* cell cycle, suggesting that coupling of flagellin gene expression to assembly has a critical influence on regulating cell cycle expression.

Cells of the gram-negative, aquatic bacterium *Caulobacter crescentus* possess an intrinsic asymmetry, which upon division results in the formation of two distinct daughter cells: a motile swarmer cell and a sessile stalked cell (reviewed in references 10, 26, and 80). These cell types differ in their programs of gene expression and DNA replication. For example, in the stalked cell, replication of chromosomal DNA initiates immediately following cell division, whereas this process is silenced for a defined period in the newly formed swarmer cell. Following this period of repression, the swarmer cell differentiates into a stalked cell. This differentiation event is accompanied by the degradation of flagellar components, stalk growth, the transcription of genes encoding DNA replication proteins, and the initiation of DNA replication (10, 26, 80).

Differentiation into a stalked cell type also initiates a cascade of events that culminate in the assembly of a single polar flagellum at the pole opposite the stalk. Flagellar biogenesis in *C. crescentus* requires the coordinated expression of approximately 50 genes (17, 20, 39) and is regulated by a complex interplay of both cell cycle and flagellar assembly events. The initiation of DNA replication is required for expression of early class II flagellar genes (13, 75), which encode the MS ring (37, 58), the flagellar switch (58, 83), and a flagellum-specific secretory apparatus (29, 64, 68, 75, 84), as well as the *trans*-acting factors encoded by *flbE* (78) and *flbD* (7, 8, 54, 59, 63, 79, 81) (Fig. 1). Class II promoters are transcribed relatively early in the cell cycle and contain conserved *cis*-acting sequences located between 35 and 10 bp upstream of the transcription start site (29, 58, 68, 75, 84). The transcription of these early flagellar operons is regulated by the global response regulator CtrA, which has been shown to bind to these conserved sequence elements and to activate the transcription of class II flagellar promoters at a specific time in the *C. crescentus* cell cycle (15, 50, 62).

The expression of class III flagellar genes, which encode the rods and rings of the basal body and hook, follows the assembly of class II gene products (11, 12, 28, 30, 40, 46, 51–53). Class III gene expression is regulated by two distinct cellular events: the progression to a specific stage in the cell division cycle and the assembly of class II gene products into the nascent flagellar structure. The transcription of class III flagellar promoters requires the general transcription factors σ^{54}-containing RNA polymerase (3, 9) and integration host factor (27, 28, 46), as well as the flagellar transcription factor FlbD (64). FlbD is a member of the response regulator family of bacterial two-component regulatory systems (64) and binds to a conserved enhancer element called ftr which is located approximately 100 bp from the transcription start site (7, 8, 52–54, 79, 81). Cell cycle expression of class III promoters is accomplished through the temporal phosphorylation of FlbD (8, 79). The product of the *flbE* gene is also required for the temporal expression of class III genes and has been shown to be required for FlbD

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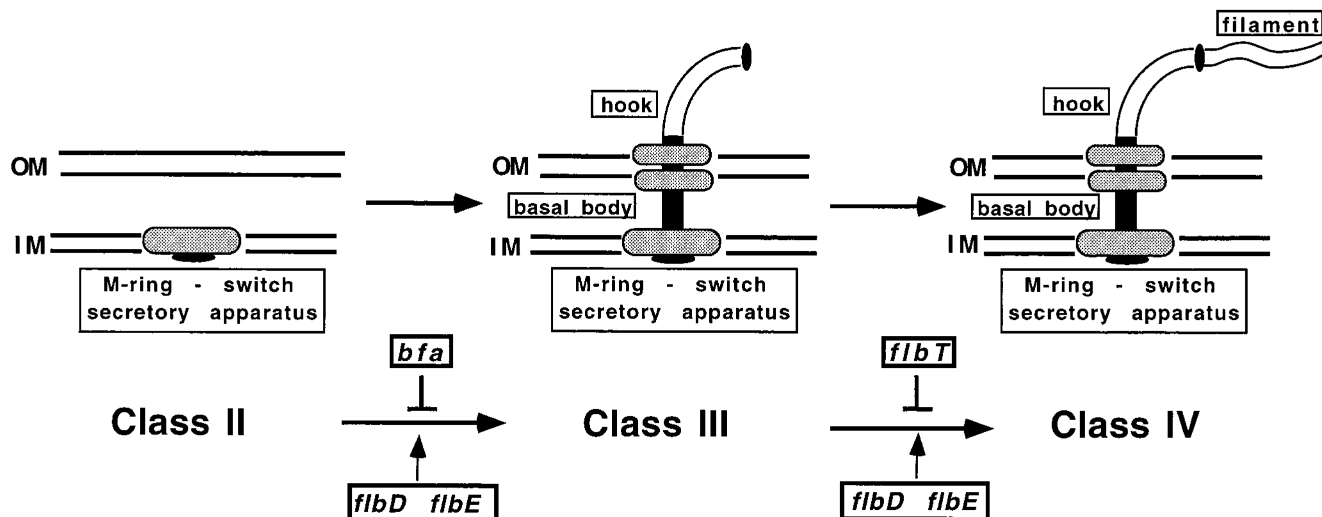

FIG. 1. The *C. crescentus* flagellar regulatory hierarchy (reviewed in references 10, 26, and 80). A schematic of the flagellar regulatory hierarchy is shown. The hypothetical structures are depicted in the order in which they are assembled. Below each intermediate structure is indicated the class of flagellar genes that encode it. Flagellar assembly is coupled to gene expression at two distinct levels of the regulatory hierarchy. A cell cycle cue activates the response regulator CtrA, which in turn activates the transcription of the class II subset of flagellar genes. These genes encode regulatory proteins such as FlbD and FlbE, as well as the MS ring, the flagellar switch, and components of the flagellum-specific class III secretory apparatus. The expression and activation of FlbD and FlbE are required for the transcription of class III genes, which encode components of the basal body and hook structure. In addition, in the absence of assembly of the MS ring-switch secretory complex, the transcription of class III genes is negatively regulated by the *bfa* gene product. The proper assembly of class III genes is, in turn, necessary for the expression of the class IV genes, which encode flagellins. In this report, we describe how FlbT negatively regulates the expression of flagellin in the absence of assembly of the basal body-hook complex. OM, outer membrane; IM, inner membrane.

activity (78). Activation of class III gene expression requires localization of FlbE to the mid-cell site in the predivisional cell (78). Therefore, FlbE is thought to couple an early cell division event to the activation of class III gene expression.

The assembly of class II gene products is also required for the expression of class III genes (Fig. 1). For example, a mutation in any one of the class II flagellar genes results in a lack of transcription of class III genes (11, 45, 56, 59, 82). A mutation, in *bfa* (for bypass of flagellar assembly), that permits expression of class III genes in the absence of an assembled flagellar structure has been isolated (45). Strains containing a mutation in *bfa* also exhibit an alteration in the cell cycle timing of class III gene transcription, indicating that *bfa*, and presumably flagellar assembly, has an influence in regulating temporal expression (45). It is hypothesized that *bfa* encodes a repressor of class III gene expression whose activity or availability is regulated by the assembly of an early flagellar structure. This would be analogous to the anti-sigma factor FlgM, which inhibits σ^{28} activity in response to a flagellar assembly defect in *Salmonella typhimurium* (24, 25, 34, 60). The *bfa* gene has not yet been isolated, and therefore it is not known whether Bfa inhibits FlbE and/or FlbD activity or whether it binds to class III promoters and represses transcription directly.

The incorporation of flagellin monomers into the filament follows the assembly of the hook structure (Fig. 1). *C. crescentus* possesses six different flagellin genes that map in two clusters at distinct chromosomal locations. The α cluster contains genes encoding 27-kDa (*fljL*), 25-kDa (*fljK*), and 29-kDa (*fljJ*) flagellins (21–23, 49). The genetically unlinked β cluster contains three genes, *fljMNO*, each encoding a 25-kDa flagellin (18). The transcriptional regulation of *fljL* and *fljK* has been examined in some detail (49). Like class III flagellar promoters, *fljL* and *fljK* require σ^{54}-containing RNA polymerase (3, 9, 49, 51) and integration host factor (27). In addition, these promoters each contain FlbD binding sites (7, 8, 49, 79). *fljL* transcription requires the assembly of class II gene products (2, 45) and therefore is regulated by *bfa*. Interestingly, although *fljK* transcription requires the same *trans*-acting factors as *fljL* expression, it is not regulated by *bfa* (i.e., the promoter is transcribed in class II flagellar mutants) (2, 79).

Experiments using *bfa* mutants have revealed another level of this *trans*-acting regulatory hierarchy (45). *bfa* mutant strains, which were able to transcribe the 27-kDa flagellin gene, *fljL*, in the absence of flagellar assembly, synthesized no detectable flagellin protein (45). It has also been shown that although flagellin promoters are transcribed in strains with mutations in class III genes, flagellin protein and flagellin-*lacZ* protein fusions are not expressed (2). Furthermore, in the case of the 25-kDa flagellin (*fljK* product), this effect requires the 5′ untranslated region of the mRNA (2). From these experiments, it has been inferred that the assembly of class III flagellar gene products is required for the expression of flagellin, which comprises the final assembled structure of the flagellar filament (2). In contrast to the coupling of class III gene expression to the assembly of class II flagellar structures, which is regulated through the inhibition of transcription, the coupling of the assembly of class III flagellar structures to flagellin synthesis is regulated at the posttranscriptional level.

In this study, we investigated the mechanism of posttranscriptional regulation of *fljK* expression. We show that mRNA sequences that are 5′ to the translation start codon are sufficient to exert regulation and that *fljK* mRNA has a dramatically shorter half-life in the absence of flagellar assembly. Mutations in *flbT*, a well-characterized (16, 68–71) but previously unclassified flagellar gene, can reverse this effect, indicating that the *flbT* gene product may act as a negative regulator of *fljK* gene expression in the absence of flagellar assembly. Strains containing mutations in *flbT* have an altered cell cycle pattern of *fljK* expression, suggesting that posttranscriptional repression and the progression of flagellar assembly have a

TABLE 1. Bacterial strains and plasmids used

Bacterial strain or plasmid	Genotype	Reference or source
E. coli S17-1	Rp4-2 Tc::Mu Km::Tn*7*	74
C. crescentus		
NA1000	*syn-1000*	21
AE8006	*flgE806*::Tn*5*-VB32	11
SC276	*flbT650*	38
SC511	*flgE*::IS*511*	38
SC508	*fliQR153*	38
SC603	*flaF673*	
SC604	*fla-674*	
JG548	*syn-1000 fljK*::*lacZ*	This work
JG549	*flgE806*::Tn*5*-VB32 *fljK*::*lacZ*	This work
JG550	*flbT650 fljK*::*lacZ*	This work
JG551	*flgE806*::Tn*5*-VB32 *flbT650 fljK*::*lacZ*	This work
JG552	*flgE806*::Tn*5*-VB32 *flaF673 fljK*::*lacZ*	This work
JG553	*flgE806*::Tn*5*-VB32 *fla-674 fljK*::*lacZ*	This work
SC1032	*flbD198*::Tn*5 str-152*	57
SC1137	*podF199*::Tn*5 str-152*	77
SC1054	*fliF173*::Tn*5 proA103 str-140* (old *flaO*)	57
SC1055	*rpoN610*::Tn*5 proA103 str-140*	17
SC1060	*flaN176*::Tn*5 proA103 str-140*	57
SC1066	*fliL*::Tn*5 proA103 str-140*	57
SC1114	*flbE607*::Tn*5 str-152*	57
SC1132	*flhA608*::Tn*5 str-152*	57
SC2201	*flbS636*::Tn*5-132 rif-175*	17
SC2204	*flbX639*::Tn*5-132 rif-175*	17
SC3476	*podF199*::Tn*5-132 str-152*	Conversion of the Tn*5* in SC1137 to Tn*5-132*
SC3730	*fliL*::Tn*5 str-140*	Transduction of SC1066
SC3799	*flbT650 fliL179*::Tn*5*	Phage SC3730 × SC276
SC3800	*flbT650 flhA608*::Tn*5*	Phage SC1132 × SC276
SC3801	*flbT650 flaN176*::Tn*5*	Phage SC1060 × SC276
SC3802	*flbT650 flbD198*::Tn*5*	Phage SC1032 × SC276
SC3803	*flbT650 fliF173*::Tn*5*	Phage SC1054 × SC276
SC3804	*flbT650 flbS636*::Tn*5*	Phage SC2201 × SC276
SC3805	*flbT650 flbX639*::Tn*5*	Phage SC2204 × SC276
SC3806	*flbT650 flabE607*::Tn*5*	Phage SC1114 × SC276
SC3807	*flbT650 rpoN610*::Tn*5*	Phage SC1055 × SC276
SC3808	*flbT650 podF199*::Tn*5*	Phage SC3476 × SC276
SC3809	*fliQR153 zzz*::Tn*5 recA101*	Transduction of SC508 to Kan^r *recA*
SC3843	*flbT650 fliQR153 zzz*::Tn*5*	Phage SC3809 × SC276
Plasmids		
pJBZ282	*lacZ* protein fusion vector	M. R. K. Alley
p*fljK*::*lacZ*	505-bp *Pst*I-*Eco*RI fragment containing the *fljK* promoter and 23 codons inserted in frame to *lacZ* in pJBZ282	This work
p*fljK1*::*lacZ*	497-bp *Bam*HI-*Hin*dIII fragment containing the *fljK* promoter and codons 1–14 inserted in frame to *lacZ* in pJBZ282	This work
p*fljK2*::*lacZ*	458-bp *Bam*HI-*Hin*dIII fragment containing the *fljK* promoter and the ATG inserted in frame to *lacZ* in pJBZ282	This work
p*fljK3*::*lacZ*	471-bp *Bam*HI-*Hin*dIII fragment containing the *fljK* promoter region with the upstream leader deleted and 23 codons inserted in frame to *lacZ* in pJBZ282	This work
p*fljK-lacZ*/290	505-bp *Pst*I-*Eco*RI fragment containing the *fljK* promoter region subcloned upstream of a promoterless *lacZ* reporter gene	79

critical role in influencing temporal expression of *C. crescentus* flagellin genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in these experiments are listed in Table 1. *C. crescentus* NA1000, a motile, synchronizable strain, was used as the wild-type strain. AE8006 is a mutant strain with a Tn*5*-VB32 (6) insertion in *flgE* (11). A *flbT flgE* double mutant strain (JG551) was created by transducing *flgE806*::Tn*5*-VB32 into SC276 (*flbT650*) by using phage φCR30 (19). *C. crescentus* cells were grown with shaking at 31°C in either PYE medium (61) or minimal M2-glucose medium (38). *Escherichia coli* strains were grown in Luria-Bertani medium at 37°C (48). To generate a wild-type *fljK*::*lacZ* translational fusion, a 505-bp *Pst*I-*Eco*RI fragment spanning the entire *fljK* promoter region to codon 23 within the gene was fused to the eighth codon of *lacZ* in pJBZ282 (45). The *fljK*::*lacZ* fusions in pJBZ282 were introduced into *C. crescentus* by conjugation, using *E. coli* S17-1 containing the helper plasmid pLVC9 (74). A *fljK-lacZ* transcription fusion (79) contained this same sequence cloned upstream of a promoterless *lacZ* in pRK290 (14) (p*lacZ*/290) (28). To construct a template for site-directed mutagenesis, the 505-bp *Pst*I-*Eco*RI fragment was subcloned first into Bluescript KS (Stratagene) and then into M13-BM21 (Boehringer Mannheim Biochemicals) as a 524-bp *Bam*HI-*Hin*dIII fragment.

Mutagenesis of *fljK* mRNA. The 505-bp *Pst*I-*Eco*RI *fljK* fragment in M13-BM21 (Boehringer Mannheim Biochemicals) was used as a template for site-directed mutagenesis. Single-stranded DNA was isolated from *E. coli* CJ236, and

mutagenesis was performed as described by Kunkel and Roberts (42). Mutants were identified by DNA sequencing (69). To generate p*fljK1*::*lacZ*, a *Hin*dIII site was created at codon 14. Likewise, p*fljK2*::*lacZ* was constructed by creating a *Hin*dIII site after codon 1 of *fljK*. p*fljK3*::*lacZ* was generated by creating a *Bgl*II site immediately following the transcription start site and another *Bgl*II site before the ribosomal binding site in the upstream leader. The resulting mutant was cloned from M13-BM21 into Bluescript KS by using *Bam*HI-*Hin*dIII. The upstream leader was then deleted by digesting with *Bgl*II and religating. All products of site-directed mutagenesis were subcloned in frame to pJBZ282. Expression of wild-type and mutant constructs was assayed by β-galactosidase activity (48) with *C. crescentus* cultures grown to mid-logarithmic phase in PYE medium at 31°C. The reported β-galactosidase values represent mean values from assays performed in triplicate on three separately grown cultures.

Immunoprecipitation of flagellin and β-galactosidase. *C. crescentus* cultures were grown in M2-glucose to an optical density at 660 nm of 1.0 to 1.4. An aliquot was removed and pulse-labeled with Tran^{35}S-label (ICN) for 5 min. Labeled protein was immunoprecipitated with either a polyclonal antiflagellin antibody (33) or a monoclonal anti-β-galactosidase antibody (Boehringer Mannheim Biochemicals).

mRNA stability assay by primer extension. *C. crescentus* NA1000, AE8006, SC276, and JG551 cells were grown in M2-glucose medium to an optical density at 600 nm of 1.0 to 1.2. To initiate the experiment, rifampin was added to the cultures to a final concentration of 200 μg/ml. Aliquots of cells were removed at various times following the addition of rifampin, and RNA was isolated from each sample (29). Primer extension was performed as described by Ausubel et al. (5) with an oligonucleotide primer complementary to *fljK* mRNA coding sequences 53 to 71 nucleotides from the start of translation. The 71-nucleotide extension products were resolved by electrophoresis in a denaturing 8% acrylamide–urea sequencing gel. The dried gel was exposed to X-ray film and subjected to phosphorimager analysis.

Cell cycle expression experiments. *C. crescentus* cultures were grown in M2-glucose to an optical density at 660 nm of 1.0 to 1.4. Swarmer cells were isolated by centrifugation through a Ludox gradient (21). Swarmer cell populations (greater than 97% pure as assayed by light microscopy) were suspended in fresh M2-glucose medium and allowed to progress through the cell cycle. At various times throughout the cell cycle, samples were removed and proteins were pulse-labeled with Tran^{35}S-label (ICN) for 5 min. Labeled protein was immunoprecipitated with the monoclonal anti-β-galactosidase antibody (Boehringer Mannheim Biochemicals) (33) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the dried gel was subjected to fluorography.

RESULTS

Previous experiments have demonstrated that although the *C. crescentus* 25-kDa flagellin gene (*fljK*) is transcribed in strains containing mutations in class II flagellar genes, flagellin protein is not synthesized (79). Furthermore, experiments comparing the expression of flagellin-*lacZ* transcription fusions and protein fusions have shown that class III flagellar mutant strains have a marked reduction in β-galactosidase activity generated from *lacZ* protein fusions, whereas transcription is largely unaffected (2). The conclusion from both of these experiments is that flagellin synthesis is subject to negative posttranscriptional regulation in strains that exhibit defects in flagellar assembly.

To determine whether the rate of β-galactosidase synthesis generated by reporter fusions was regulated in the same manner as that of flagellin protein, cellular proteins were pulse-labeled with Tran^{35}S-label and immunoprecipitation experiments were performed. Wild-type and hook mutant (SC511) strains were assayed for expression of either a *fljK*::*lacZ* protein fusion reporter or a *fljK-lacZ* transcriptional fusion reporter (Fig. 2). Proteins were immunoprecipitated with a polyclonal antiflagellin antibody or a monoclonal anti-β-galactosidase antibody. Flagellin protein was expressed in wild-type cells (Fig. 2, lane 1), but was poorly expressed in hook mutant cells (Fig. 2, lane 2). Likewise, a *fljK*::*lacZ* protein fusion was expressed in wild-type cells (Fig. 2, lane 3) and exhibited a marked decrease in expression in a hook mutant strain (Fig. 2, lane 4). However, a *fljK-lacZ* transcriptional fusion was expressed in both wild-type cells and a hook mutant background (Fig. 2, lanes 5 and 6). These results suggest that a *fljK*::*lacZ* protein fusion is regulated by a posttranscriptional mechanism in the same manner as wild-type flagellin protein.

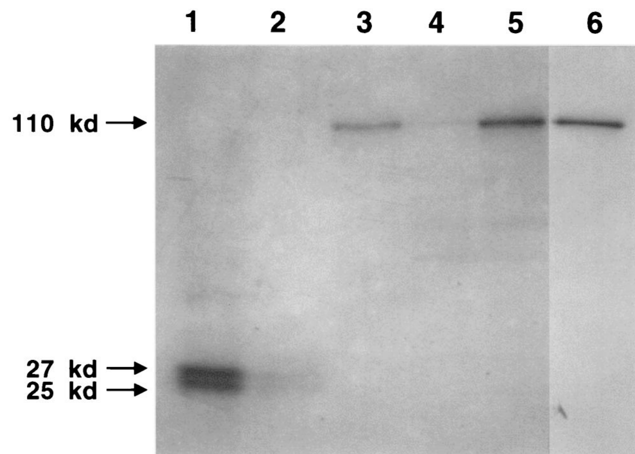

FIG. 2. Posttranscriptional regulation of the 25-kDa flagellin gene, *fljK*. Expression of flagellin protein or *fljK-lacZ* fusions in either wild-type or hook mutant cells was assayed by immunoprecipitation. Cells were grown in M2 medium to mid-log phase, and an aliquot of cells was removed and labeled with Tran^{35}S-label for 5 min. Labeled protein was immunoprecipitated with either antiflagellin (lanes 1 and 2) or anti-β-galactosidase (lanes 3 to 6) antibody and separated by SDS-PAGE as described in Materials and Methods. Lane 1, NA1000; lane 2, SC511; lane 3, NA1000 containing a *fljK*::*lacZ* protein fusion; lane 4, SC511 containing a *fljK*::*lacZ* protein fusion; lane 5, NA1000 containing a *fljK-lacZ* transcription fusion; lane 6, SC511 containing a *fljK-lacZ* transcription fusion. The apparent molecular masses of the immunoprecipitated proteins are indicated with arrows.

Mutations in *flbT* restore flagellin protein synthesis in strains bearing mutations in flagellar structural genes. Previous experiments have demonstrated that mutations in *flbT*, a gene that lies near the α-flagellin gene clusters in *C. crescentus*, increase the level of flagellin expression (69, 72). Therefore, FlbT is an attractive candidate for a negative regulator of flagellin expression. To test this idea, the levels of flagellin in strains containing a mutation in a flagellar structural gene alone or in combination with a *flbT* mutation were assayed by immunoblotting (Fig. 3). Compared to wild-type cells, a strain bearing a *flbT* mutation produced slightly increased levels of the 27-kDa flagellin, encoded by the *fljL* gene, and the 25-kDa flagellin, encoded by *fljK* as well as *fljMNO* of the β cluster. In addition, as previously demonstrated, the mutant *flbT* strain also produced a variant of the 25-kDa flagellin that migrates at an apparent molecular mass of 22 kDa (70, 73). The effect of a *flbT* mutation on flagellin expression in class II and class III flagellar mutants was also examined. All of the class II and III mutant strains tested produced little or no flagellin protein (Fig. 3). If these strains also contained a mutation in *flbT*, 25-kDa flagellin expression was restored in all cases. Therefore, in the case of the 25-kDa flagellins, mutations in *flbT* bypass the requirement for flagellar assembly. In most cases, the mutation in *flbT* could not restore expression of the 27-kDa flagellin. Expression was evident in only two strains: a *flbT flaN* mutant and a *flbT flbX* mutant. These two strains contain mutations in class III flagellar genes, whereas the remainder of the strains tested contain mutations in class II genes. In contrast to the 25-kDa flagellin gene, *fljK*, *fljL* is not transcribed in class II mutants (2, 45) as a consequence of Bfa activity (45) and therefore is not expressed in *flbT*-class II double mutant strains.

Interestingly, mutations in *flbT* were also able to restore flagellin expression in strains containing mutations in genes

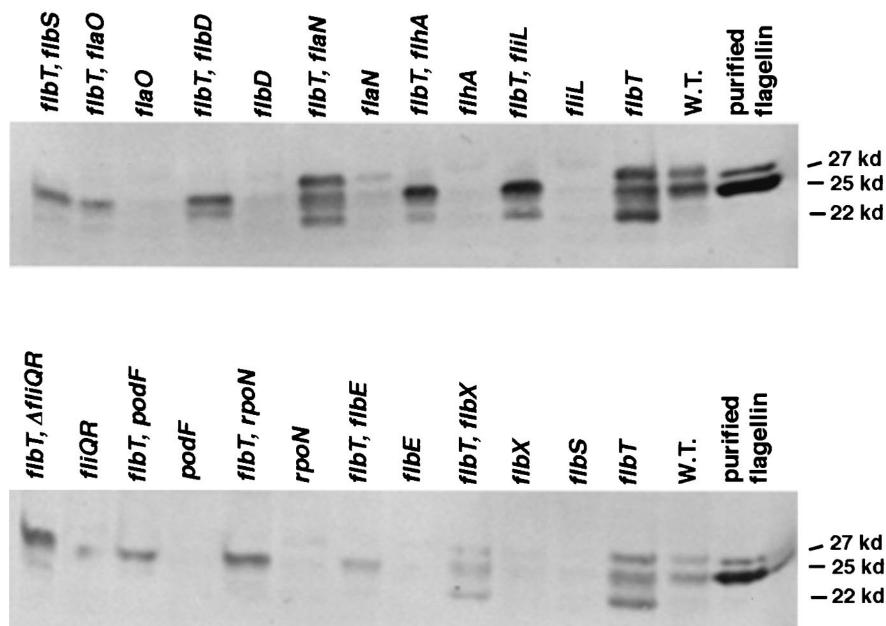

FIG. 3. A mutation in *flbT* restores flagellin expression in flagellar mutants. Proteins from whole-cell extracts from mutant strains were separated by SDS-PAGE and subjected to immunoblotting as described in Materials and Methods. Mutations in a single flagellar gene were introduced into the *flbT* mutant strain SC276 by generalized transduction. The mobilities of flagellins derived from these strains are compared to that of purified flagellin protein. W.T., wild type.

encoding *trans*-acting factors, such as *rpoN* (encoding the σ^{54} subunit of RNA polymerase holoenzyme), *flbD* (encoding a σ^{54} transcriptional activator), and *flbE* (encoding a *trans*-acting factor required for FlbD activity). Previous experiments have shown that these genes are essential for the expression of *fljK* and *fljL*. The simplest interpretation of these results is that the flagellin produced in these double mutant strains derives from *fljMNO* in the β cluster. Recent evidence indicates that the transcription of *fljMNO* is not dependent on *rpoN* or *flbD* (18). However, the fact that this cluster does not produce flagellin in the absence of flagellar assembly probably indicates that protein synthesis is regulated in a fashion similar to that for *fljL* and *fljK*.

Effect of *flbT* mutations on *fljK*::*lacZ* expression. We next tested whether the *flbT* mutant strain had the same effect on *fljK*::*lacZ* reporter expression. To accomplish this, a wild-type *fljK*::*lacZ* protein fusion (Fig. 4) was introduced into wild-type cells, a *flgE* mutant (AE8006), a *flbT* mutant (SC276), and a *flgE flbT* double mutant (JG551) (Fig. 5A). This fusion generated 2,772 U of β-galactosidase activity in wild-type cells. In contrast, in the *flgE* mutant, the *fljK*::*lacZ* fusion was expressed at levels 10% of that in wild-type cells, generating 246 U of β-galactosidase activity (Fig. 5A). This result is consistent with the observation that *fljK* expression is subject to negative posttranscriptional regulation. In a *flbT* mutant, expression of *fljK*::*lacZ* was almost twice that in wild-type cells (5,955 U). This increase in expression is similar to that observed when flagellin protein was assayed in cell extracts derived from wild-type and *flbT* mutants (Fig. 3). To determine whether a mutation in *flbT* could restore *fljK*::*lacZ* expression in a class III mutant, we constructed a strain, JG551, that contained both the *flgE*::Tn*5*-VB32 mutation and the *flbT650* mutation and then assayed β-galactosidase activity. The mutation in *flbT* completely bypassed the requirement for an assembled hook structure; the *fljK*::*lacZ* fusion in this strain possessed approximately 40 times the β-galactosidase activity of strain AE8006 which contains only a mutation in *flgE* (9,253 versus 246 U) (Fig. 5A). This result indicates that *flbT* has a critical role in negatively regulating *fljK* expression in the absence of flagellar assembly.

Since the nature of the *flbT* mutation in strain SC276 is unknown, we tested whether strains containing deletions in

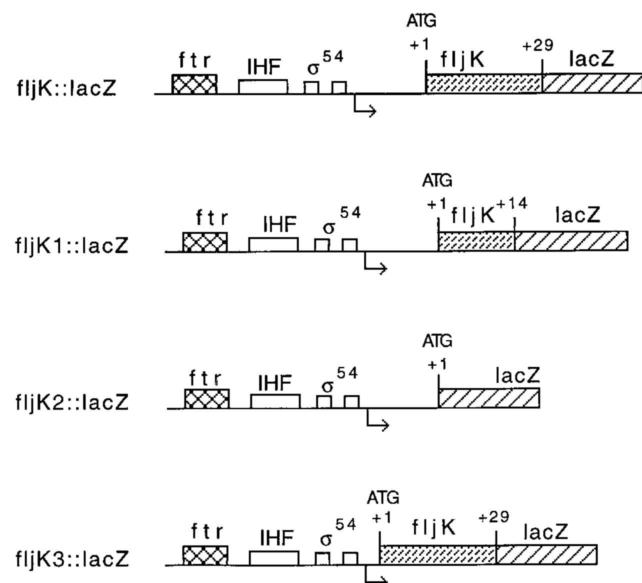

FIG. 4. Schematic diagram of deletion derivatives of *fljK*::*lacZ* translation fusions. Site-directed mutagenesis was used to construct different deletions of *fljK.* The sequence encoding amino acids 1 to 23 was fused in frame to *lacZ* to create *fljK*::*lacZ*. *fljK1*::*lacZ* corresponds to a deletion of amino acids 15 to 23 fused in frame to *lacZ*. The sequence encoding amino acids 2 to 23 was deleted, resulting in an in-frame fusion of the ATG from *fljK* to *lacZ* to create *fljK2*::*lacZ*. *fljK3*::*lacZ* is a deletion of the upstream leader, leaving only the ribosomal binding site intact, fused in frame to *lacZ*. IHF, integration host factor.

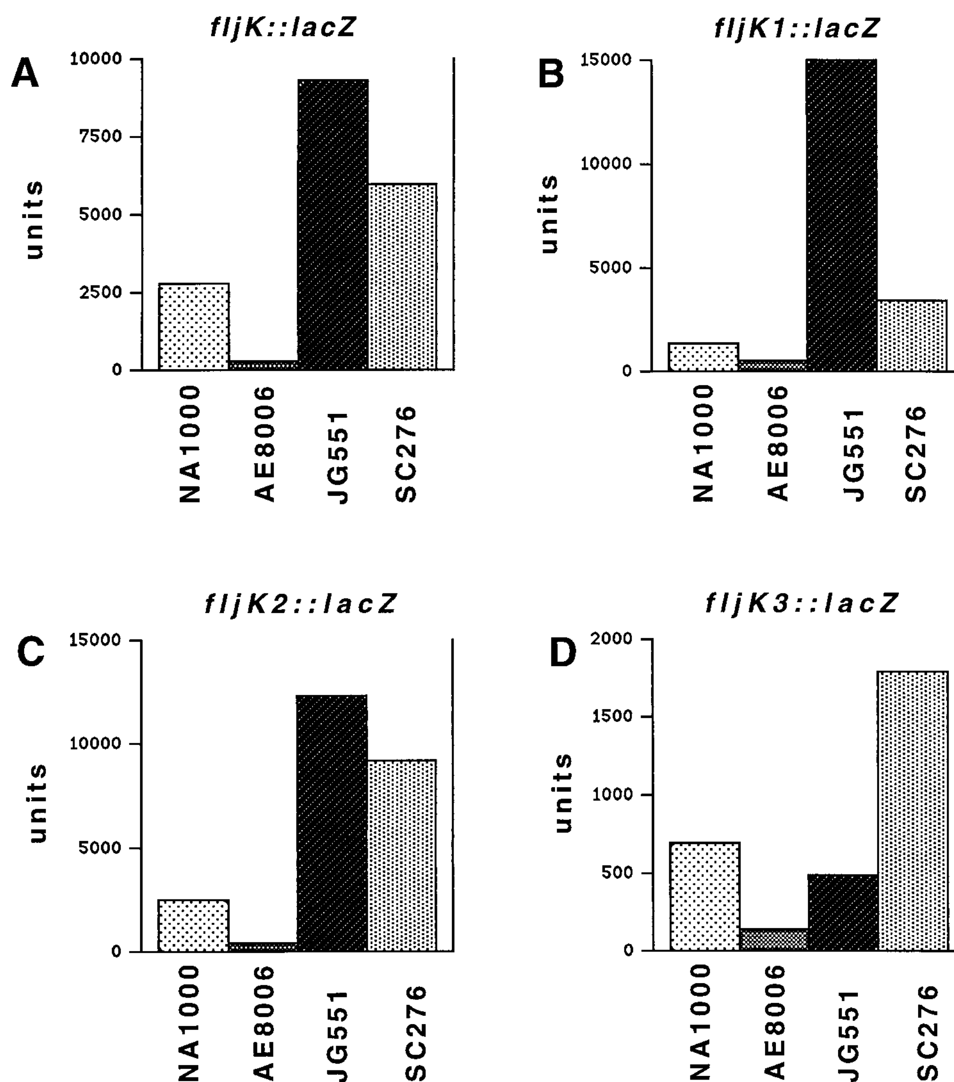

FIG. 5. Effect of deletions on the expression of *fljK*::*lacZ* translation fusions in wild-type and mutant strains. Wild-type and deleted *fljK*::*lacZ* translation fusions were introduced into the mutant strains indicated on the *x* axis. Values on the *y* axis represent β-galactosidase activity, in units (48), assayed in triplicate from three different mid-logarithmic-phase cultures. NA1000 is a synchronizable derivative of wild-type *C. crescentus* CB15. AE8006 contains a Tn*5*-VB32 insertion in *flgE* (hook). JG551 contains a Tn*5*-VB32 insertion in *flgE* and a *flbT650* mutation from strain SC276. SC276 contains the *flbT650* allele. (A) Mean β-galactosidase activity generated from p*fljK*::*lacZ*, which contains the entire 5′ untranslated region of *fljK* and the first 23 codons fused in frame to *lacZ*. The mean β-galactosidase activities were 2,772 U for NA1000, 246 U for AE8006, 9,253 U for JG551, and 5,955 U for SC276. (B) β-Galactosidase activity generated from p*fljK1*::*lacZ*, which contains the entire 5′ untranslated region of *fljK* and the first 14 codons fused in frame to *lacZ*. The mean β-galactosidase activities were 1,328 U for NA1000, 503 U for AE8006, 14,926 U for JG551, and 3,407 U for SC276. (C) β-Galactosidase activity generated from p*fljK2*::*lacZ*, which contains the entire 5′ untranslated region of *fljK* and the first codon fused in frame to *lacZ*. The mean β-galactosidase activities were 2,426 U for NA1000, 373 U for AE8006, 12,279 U for JG551, and 9,135 U for SC276. (D) β-Galactosidase activity (note difference in scale) generated from p*fljK3*::*lacZ*, in which the entire 5′ untranslated region of *fljK* except the ribosome binding site was deleted, fused in frame to *lacZ*. The mean β-galactosidase activities were 685 U for NA1000, 132 U for AE8006, 479 U for JG551, and 1,791 U for SC276. See Fig. 4 for a schematic representation of these fusions.

flbT exhibited a similar effect. The *flgE*::Tn*5*-VB32 mutation was introduced, by transduction, into SC603 and SC604, which have *flmGH flbT flaF* and *flmGH flbT* deleted, respectively (44, 72). *flmG* and *flmH* encode proteins that are homologous to O-linked acetylglucosamine transferases and acetyltransferases, respectively, and are regarded to be required for posttranslational modification of flagellins (44). The function of *flaF* is unknown. Previous experiments have demonstrated that mutations in *flmG*, *flmH*, and *flaF* result in a decrease in the level of flagellin produced (72). In strains SC603 and SC604, the level of β-galactosidase generated by *fljK*::*lacZ* was increased approximately twofold over that in wild-type cells (data not shown). The deletion in *flbT* in both these strains is apparently able to bypass the requirement for a hook structure. When the *flgE*::Tn*5*-VB32 allele was introduced into these strains, the level of β-galactosidase generated by *fljK*::*lacZ* was increased approximately 23 times over that assayed in AE8006 (5,800 versus 246 U) (data not shown). Since the other genes deleted in both of these strains are probably involved in the posttranslational modification of flagellin (44), the simplest conclusion that can be drawn from these results is that the deletion in *flbT* is responsible for the restoration of *fljK*::*lacZ* expression in the absence of a hook structure.

The restoration of *fljK* expression observed in *flbT* and *flgE flbT* mutants could be attributable to an increase in transcription and/or translation. To distinguish between these two pos-

sibilities, we tested the expression of *fljK-lacZ* transcription fusions in the *flbT* mutant strain SC276. In contrast to the high levels of β-galactosidase generated by the *fljK*::*lacZ* protein fusion, expression of the *fljK-lacZ* transcription fusion was dramatically decreased compared to that in wild-type cells (135 versus 3,500 U) (data not shown). This result indicates that FlbT probably functions as a posttranscriptional repressor. In addition, the decrease in transcription and the increase in translation in SC276 suggest that transcription of *fljK* is influenced by the level of translation.

In an attempt to define the region of *fljK* mRNA that is responsible for the posttranscriptional repression in a class III mutant background, a set of deletions were created and in-frame fusions to *lacZ* were constructed (Fig. 4). Mutants in which amino acids 15 to 23 (p*fljK1*::*lacZ*) or 2 to 23 (p*fljK2*::*lacZ*), encoded in the *fljK* coding region, were deleted (Fig. 4) exhibited a pattern of regulation similar to that for the *fljK*::*lacZ* fusion, which encoded all 23 of these amino acids; the fusions were expressed in wild-type cells and had a decreased expression in a hook mutant strain (Fig. 5B and C). The *flbT* mutation increased the expression of both of these fusions and could restore expression in a hook mutant to levels greater than that measured in wild-type cells (Fig. 5B and C). A final deletion mutant was created, in which the entire upstream leader sequence except the ribosomal binding site had been deleted (p*fljK3*::*lacZ*) (Fig. 4). In contrast to fusions with deletions within the coding region, this fusion was expressed poorly in wild-type cells (Fig. 5D [note change in scale]). Expression was reduced an additional fivefold in the hook mutant strain, indicating that this fusion was still subject to posttranscriptional repression. Furthermore, introduction of a *flbT* mutation into this strain partially restored expression; however, the increase in expression compared to that in the hook mutant was less than that observed for fusions containing a wild-type leader sequence. In this case, when the hook mutant strain also contained a *flbT* mutation (JG551), the expression of *fljK3*::*lacZ* was only 3.6 times higher than that in the strain containing a single mutation in the hook. In strains containing a *fljK*::*lacZ* fusion possessing a wild-type leader sequence, a *flbT* mutation increased expression levels by 37-fold. These results indicate that the sequences within the upstream leader are required for the regulation of expression of *fljK* in class III flagellar mutants and, in addition, that the likely site of FlbT-mediated regulation lies within this leader region.

***flbT* regulates *fljK* mRNA stability.** The decreased expression of flagellins and *fljK*::*lacZ* protein fusions in class III flagellar mutants may be a consequence of negative regulation of translation. If this is the case, a block in translation should result in a shorter half-life of flagellin mRNA, as has been observed for other bacterial mRNAs whose translation is negatively regulated (32, 35, 55, 65). To test this idea, we assayed the decay of *fljK* mRNA over time in wild-type cells, and in *flgE*, *flbT*, and *flgE flbT* mutant strains. The cultures were treated with rifampin to inhibit transcription, and total RNA was isolated at 3-min intervals and subjected to primer extension analysis. In wild-type cells, *fljK* mRNA had an estimated half-life of 11 min (Fig. 6). Previous experiments have demonstrated that the steady-state levels of *flgK* mRNA were reduced in a class III flagellar mutant (2). Consistent with this observation, in a strain containing a mutation in *flgE*, the *fljK* mRNA had a greatly reduced half-life of less than 1.5 min. This result suggests that decreased flagellin synthesis in class III mutant cells may be attributable to a decrease in the stability of *fljK* mRNA. *fljK* mRNA stability was increased to greater-than-wild-type levels in a *flbT* mutant background (half-life of greater than 30 min). The same experiment was performed

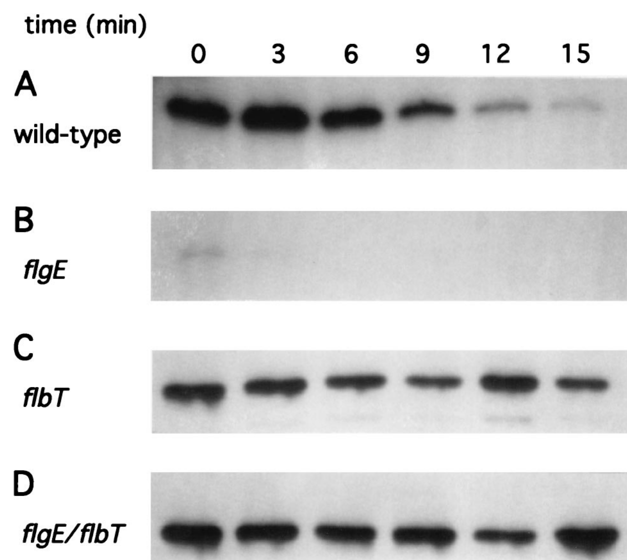

FIG. 6. Effect of flagellar mutations on *fljK*::*lacZ* mRNA stability. *C. crescentus* NA1000 cells were grown to mid-logarithmic phase in M2 medium. At 0 min, 200 μg of rifampin per ml was added to inhibit transcription. At 3, 6, 9, 12, and 15 min, an aliquot was removed, RNA was isolated from each sample, and primer extension was performed with a ^{32}P-labeled oligonucleotide that hybridized to the coding sequence of *lacZ*. The primer extension products were subjected to electrophoresis in a denaturing polyacrylamide gel. (A) *C. crescentus* NA1000 cells. The mRNA half-life is approximately 11 min. (B) Strain AE8006 (*flgE*::Tn*5*-VB32). The mRNA half-life is less than 1.5 min. (C) Strain SC276 (*flbT650*). The mRNA half-life is greater than 30 min. (D) Strain JG551 (*flgE*::Tn*5*-VB32 *flbT650*). The mRNA half-life is greater than 30 min.

with a *flbT flgE* double mutant. Like the *flbT* mutant background alone, a mutation in *flbT* was sufficient to increase *fljK* message stability to greater-than-wild-type levels (half-life of greater than 30 min). We hypothesize that the decrease in mRNA half-life observed in *flgE* mutant strains is a consequence of inhibition of translation, perhaps mediated through the action of the *flbT* gene product.

A mutation in *flbT* alters the temporal pattern of *fljK* expression. One possible function of FlbT in wild-type cells may be to influence the temporal expression of flagellin genes. For example, FlbT activity may exist to shut off flagellin synthesis following the completion of flagellar assembly. To test this hypothesis, the temporal expression of *fljK*::*lacZ* was assayed in wild-type and *flbT* mutant backgrounds. Cell cycle expression of *fljK* was assayed by synchronizing a culture containing an integrated *fljK*::*lacZ* protein fusion. The isolated swarmer cells were suspended in fresh medium and allowed to progress through the cell cycle. At various times thereafter, proteins were pulse-labeled with Tran^{35}S-label, and radioactive β-galactosidase was immunoprecipitated from the cell extracts and subjected to PAGE. In wild-type cells, the *fljK*::*lacZ* fusion was expressed under temporal control. As previously shown (49, 79), expression was observed in swarmer cells (0 cell division units) and was turned off in stalked cells (0.45 cell division unit) (Fig. 7). Later in the cell cycle, when the cells reached the predivisional stage, expression of the *fljK*::*lacZ* fusion returned and continued until cell division occurred. In a *flbT* mutant strain the *fljK*::*lacZ* fusion had an altered pattern of expression. A full-length, labeled FljK::LacZ fusion was synthesized at the same period in the cell cycle as observed in wild-type cells. In contrast to the case for wild-type cells, in newly formed stalked cells and early predivisional cells of the *flbT* mutant strain, a smaller polypeptide of approximately 20 kDa was synthesized.

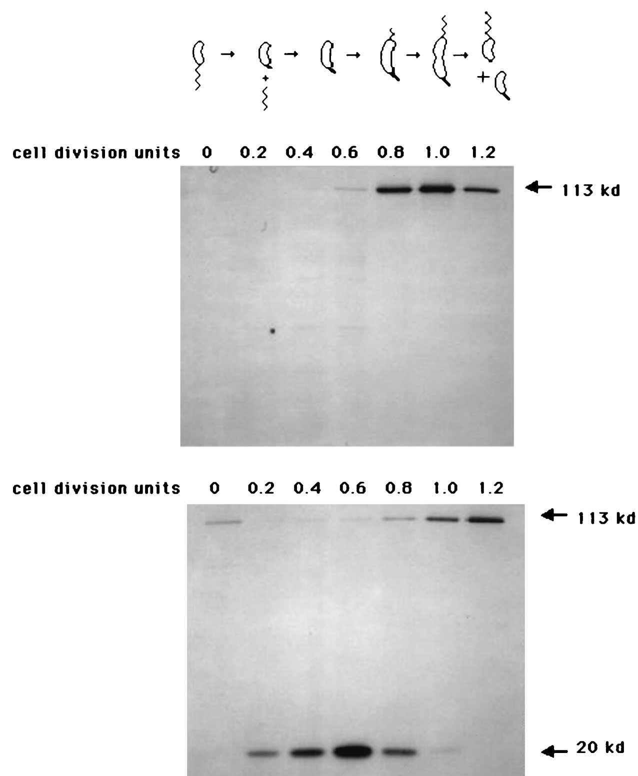

FIG. 7. Effect of a *flbT* mutation on the temporal expression of a *fljK*::*lacZ* translation fusion. The temporal expression of β-galactosidase was assayed with either *C. crescentus* NA1000 or SC276 cells containing a *fljK*::*lacZ* translation reporter fusion. Isolated swarmer cells were suspended in fresh M2 medium and were permitted to progress through the cell cycle. At various times during the cell cycle (0, 30, 60, 90, 120, 150, and 180 min), an aliquot was removed and proteins were labeled with Tran^{35}S-label for 10 min. Labeled protein was immunoprecipitated with a monoclonal anti-β-galactosidase antibody and subjected to SDS-PAGE as described in Materials and Methods. The gel was dried and exposed to X-ray film. (Top) *fljK*::*lacZ* expression in wild-type strain NA1000. The drawing above the fluorogram shows the cell types present at each time point, as determined by light microscopy. Labeled β-galactosidase is indicated by an arrow. (Bottom) *fljK*::*lacZ* expression in a *flbT* mutant strain, SC276. A low-molecular-mass (approximately 20-kDa) proteolytically generated fragment of β-galactosidase is also indicated by an arrow.

Since a monoclonal antibody was employed in these experiments, the most likely source of this polypeptide is β-galactosidase. The best interpretation of this observation is that the *fljK*::*lacZ* fusion is expressed throughout the *C. crescentus* cell cycle but that protein which is expressed in stalked and early predivisional cells is subject to proteolysis. In this case, proteolysis is dependent on the fusion of *fljK* to *lacZ*, since fusions that express full-length *lacZ* at this time of the cell cycle, such as *gyrB*- or *recF*-*lacZ* transcription fusions, do not exhibit proteolysis of β-galactosidase (67). Therefore, we hypothesize that since *fljK* is not transcribed in stalked cells, the extended *fljK* mRNA stability observed in the *flbT* mutant is responsible for *fljK*::*lacZ* expression in stalked cells.

DISCUSSION

Flagellar biogenesis in *C. crescentus* is characterized by cell cycle assembly of a polar flagellar structure. The assembly of the polar flagellum is a complex process that requires over 50 gene products (17) and is regulated both by progression through the cell cycle and by the assembly of the structure itself. In this report, we have identified *flbT* as a key player in coupling flagellar assembly to the expression of flagellin. The gene, encoding FlbT, maps to a region that contains a cluster of late-acting flagellar genes (70), including those encoding proteins involved in the posttranslational modification of flagellin (44) as well as three flagellin genes, *fljL*, *fljK*, and *fljJ*. Mutations in *flbT* have been shown to have pleiotropic effects, including impaired motility, defects in the temporally regulated loss of the flagellum from stalked cells, and a marked increase in flagellin protein synthesis (70, 73). The last property suggests that *flbT* might function in the negative regulation of flagellin expression. The data presented here indicate that FlbT functions to inhibit flagellin expression in the absence of an intermediate flagellar structure into which flagellin monomers can be incorporated. In support of this view, mutations in *flbT* restore the expression of the *fljK* flagellin gene in the absence of a flagellar hook structure (i.e., in a *flgE* mutant strain). Although the precise nature of the *flbT* mutation in strain SC276 is not known, we have found, using anti-FlbT antibodies, that this strain produces no detectable FlbT protein (4). This suggests that the restoration of flagellin expression observed in *flgE flbT* double mutants is a consequence of a loss of FlbT and is probably not attributable to a gain-of-function allele. This is supported by the observation that deletions in *flbT* can also restore *fljK*::*lacZ* expression in a *flgE* mutant.

Immunoblot analysis showed that the *flbT* mutation could restore flagellin synthesis in all class II and class III flagellar mutant strains tested, suggesting that *flbT* is a general regulator that couples assembly to flagellin gene expression. Surprisingly, a mutation in *flbT* could also restore flagellin expression in *rpoN*, *flbD*, and *flbE* mutant strains, which are deficient in essential *trans*-acting factors. Recent analysis has revealed that the β cluster of flagellins, which contains three copies of 25-kDa flagellin genes, is not regulated by *rpoN*, *flbD*, or *flbE* (18), suggesting that the flagellin present in these mutants derives from this cluster. Interestingly, although the flagellins in the β cluster differ in their transcriptional regulation, they apparently have a common mechanism of regulation in response to flagellum assembly.

The negative regulation of flagellin expression in class III mutant strains is most likely mediated through a posttranscriptional mechanism. This conclusion is supported by the observation that although the *fljK* gene is transcribed in mutants that do not assemble a hook structure, there is little, if any, flagellin protein present. Deletion analysis of the *fljK* transcript showed that the expression of *fljK* was reduced in a hook mutant if the entire coding region was absent. Deletion of the 5′ untranslated sequences resulted in a marked decrease of *fljK* expression. Even though expression was decreased in a hook mutant, this fusion exhibited a relatively modest increase in activity in a FlbT-hook double mutant. Based on these data, we hypothesize that FlbT exerts its primary effect through acting on the 5′ untranslated leader of the *fljK* transcript. This is consistent with previous experiments demonstrating that sequences within the 5′ untranslated region of the *fljK* transcript were important for regulation by flagellar assembly (2). The absence of hook assembly presumably results in an increased turnover of *fljK* mRNA. Furthermore, mutations in *flbT* apparently decrease this high rate of mRNA turnover. From the experiments presented here, it is impossible to distinguish whether the apparent increase in mRNA turnover in class III mutants is a direct consequence of message instability in these strains or is brought about by an inhibition of translation, which as a secondary result leads to unstable mRNA. For example, it has been suggested that in some cases in bacteria, message stability is directly proportional to the formation of a translational complex (32, 35, 55). In the absence of ribosome binding,

RNase E sites are exposed, causing rapid degradation of message (66). We envision two possible mechanisms whereby the *flbT* gene product promotes the turnover of flagellin mRNA in class III flagellar mutants. Previous experiments (47), as well as those reported here, have demonstrated that *fljK* mRNA has an unusually long half-life for a bacterial message. One plausible possibility is that a *trans*-acting factor(s) stabilizes *fljK* mRNA, and FlbT functions to antagonize its activity. Alternatively, FlbT may act directly to either destabilize *fljK* mRNA or prevent translation. Either model is consistent with the observation that mRNA sequences upstream of the translation initiation codon are required for FlbT to exert its maximal effect.

This mechanism of posttranscriptional repression of flagellin gene expression contrasts with an analogous regulation of flagellin gene expression in *S. typhimurium*. In *S. typhimurium*, the flagellin and chemotaxis genes are not expressed in mutant strains that have defects in flagellar assembly (41, 43). These genes are transcribed by σ^{28}-containing RNA polymerase. Negative regulation is accomplished through an anti-sigma factor encoded by *flgM* (24, 25, 59). In the absence of flagellar assembly, the intracellular levels of the *flgM* gene product rise and the protein binds to the σ^{28} subunit of RNA polymerase, inhibiting its activity. Once a functional hook structure is assembled, FlgM is exported from the cell via the nascent flagellar structure, thereby relieving repression (34). In *C. crescentus*, as well, the transcription of class III genes (e.g., those encoding the basal body rods, outer rings, and hook) is repressed in the absence of the assembly of a flagellar structure encoded by class II gene products (Fig. 1). A mutation in a single uncharacterized gene, called *bfa*, can restore transcription of class III genes in the absence of flagellar assembly.

The regulation of flagellin gene expression by *flbT* represents another control point in flagellar biogenesis in *C. crescentus*, which is absent in enteric bacteria. What is the logic in possessing two different mechanisms to regulate flagellar biogenesis in response to assembly? The transcription of both class III and class IV flagellar genes is positively regulated by the FlbD transcription factor (7, 8, 55, 79, 81). FlbD is a member of the two-component response regulators (64) and is phosphorylated in response to a cell cycle cue (79), possibly an early stage in cell division (78). Once cells have progressed past this critical cell cycle step, they are competent to transcribe both class III and class IV flagellar genes. We speculate that it is at this stage when FlbT inhibits the expression of the class IV flagellin genes until a hook structure is assembled. With analogy to regulation in enteric bacteria, FlbT repression would then be inactivated, and any newly transcribed flagellin mRNA would be expressed. This model would ensure that flagellin is not synthesized until the correct stage in flagellar assembly is completed.

The *flbT* gene product may have an additional regulatory role later in the cell cycle, following cell division. As noted above, flagellin mRNA possesses an unusually long half-life. We have previously shown that *fljK* is transcribed in the swarmer compartment of the predivisional cell (31, 78, 79). The transcription of *fljK* ceases abruptly upon the completion of cell division; however, the mRNA remains within the progeny swarmer cell and is continually translated (46). Therefore, swarmer compartment-specific transcription of *fljK* results in generation of a supply of *fljK* mRNA for the nascent swarmer progeny cell. This mRNA is translated into flagellin protein, which is assembled in the flagellar filament. Thus, the requirement for continued filament growth in the progeny swarmer cells is fulfilled both by the swarmer compartment-specific transcription of *fljK* and by the unusual stability of flagellin mRNA. We hypothesize that FlbT plays a critical role in shutting off *fljK* translation in swarmer cells once the demand for new flagellin monomers is satisfied. This idea is supported by the observation that *fljK* is synthesized at relatively high levels in swarmer cells possessing mutant *flbT* and continues to be translated after the swarmer cells have differentiated into stalked cells. Therefore, FlbT, and presumably the assembly of the flagellum, contributes to the temporal expression of *fljK*. This result is similar to what occurs in *bfa* mutant cells, where the cessation of transcription of class III promoters during the cell cycle is significantly delayed (45). This suggests that the function of these two gene products in wild-type cells is to repress the synthesis of flagellar genes following the completion of a specific stage of flagellar assembly. Negative regulatory pathways that couple flagellar assembly to gene expression in other organisms, such as those present in enteric bacteria, may serve a similar purpose, perhaps functioning to regulate the number of flagella synthesized by each cell.

As noted above, one consequence of a mutation in *flbT* is the expression of *fljK* at an inappropriate time in the cell cycle. In this mutant strain, the FljK::LacZ fusion protein is degraded when swarmer cells differentiate into stalked cells. This is evidenced by the appearance of a small (approximately 20-kDa) polypeptide that reacts with the monoclonal anti-β-galactosidase antibody employed in this experiment. Proteolysis of β-galactosidase is apparently dependent on the presence of FljK amino acid sequence at the amino terminus. We have shown previously that β-galactosidase is not subject to proteolysis at this stage of the cell cycle (66). Therefore, *C. crescentus* possesses a developmentally regulated protease that can degrade intracellular flagellin specifically in stalked cells. One hallmark of the *C. crescentus* developmental program is the timed degradation of proteins, often in a cell-type-specific fashion. For example, the transcription factor CtrA (15), the FliF protein (MS ring) (37), and the methyl-accepting chemotaxis receptor (1) are all degraded specifically in stalked cells. One documented protease which is involved in this process is the highly conserved ClpXP protease. The ClpXP protease degrades CtrA, an essential response regulator that controls early flagellar gene transcription as well as the initiation of DNA replication (36). The ClpXP protease recognizes disordered C-terminal sequences, which possess some degree of conservation, as its substrates. Therefore, ClpXP is probably not involved in the degradation of FljK::LacZ, since the amino terminus of FljK appears to be the determinant for proteolysis. This indicates that *C. crescentus* probably possesses at least one additional developmentally regulated protease whose activity is restricted to the stalked cell type. In summary, *C. crescentus* utilizes a diverse array of regulatory mechanisms, such as cell cycle- and cell-type-regulated transcriptional activation, flagellar assembly-regulated transcriptional and posttranscriptional repression, and cell-type-specific proteolysis, in order to regulate flagellar biogenesis. The simultaneous operation of these pathways ensures that flagellum synthesis is tightly coupled to the formation of a differentiated daughter cell.

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