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Posttranscriptional Regulation of *Caulobacter* Flagellin Genes by a Late Flagellum Assembly Checkpoint

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Flagellum formation in *Caulobacter crescentus* requires ca. 50 flagellar genes, most of which belong to one of three classes (II, III, or IV). Epistasis experiments suggest that flagellar gene expression is coordinated with flagellum biosynthesis by two assembly checkpoints. Completion of the M/S ring-switch complex is required for the transition from class II to class III gene expression, and completion of the basal body-hook structure is required for the transition from class III to class IV gene expression. In studies focused on regulation of the class IV flagellin genes, we have examined *fljK* and *fljL* expression in a large number of flagellar mutants by using transcription and translation fusions to *lacZ*, nuclease S1 assays, and measurements of protein stability. The *fljK-lacZ* and *fljL-lacZ* transcription fusions were expressed in all class III flagellar mutants, although these strains do not make detectable 25- or 27-kDa flagellins. The finding that the *fljK-lacZ* translation fusion was not expressed in the same collection of class III mutants confirmed that *fljK* is regulated posttranscriptionally. The requirement of multiple class III genes for expression of the *fljK-lacZ* fusion suggests that completion of the basal body-hook is an assembly checkpoint for the posttranscriptional regulation of this flagellin gene. Deletion analysis within the 5′ untranslated region of *fljK* identified a sequence between +24 and +38 required for regulation of the *fljK-lacZ* fusion by class III genes, which implicates an imperfect 14-bp direct repeat in the posttranscriptional regulation of *fljK*. Our results show that *fljL* is also regulated posttranscriptionally by class III and unclassified flagellar genes, apparently by a mechanism different from the one regulating *fljK*.

The stalked cell of the dimorphic bacterium *Caulobacter crescentus* divides repeatedly and asymmetrically to generate two functionally and structurally distinct progeny cells, an old nonmotile stalked cell and a new motile swarmer. The swarmer cell carries surface structures at one cell pole that are sequentially assembled during the course of the previous cell cycle. One of these structures is the flagellum; as in the flagella of enteric bacteria, it is composed of the basal body, which is embedded in the cell envelope, and the external hook and flagellar filament (reviewed in reference 20). The flagellum is assembled from the inside of the cell to the outside of the cell, and in *C. crescentus* the order of assembly corresponds generally to order of flagellar (*fla*) gene expression during the cell cycle (reviewed in references 4, 11, and 30).

Formation of a functional *C. crescentus* flagellum requires ca. 50 *fla* genes (10), many of them organized into a regulatory hierarchy containing four classes of genes (I to IV [Fig. 1]). Expression of genes at each level of the hierarchy is required for expression of genes below them in the hierarchy. Thus, the class II transcription units, which encode components of the M/S ring-switch complex, are near the top of the hierarchy, and their expression early in the cell cycle is required for expression of the class III genes (31, 39, 46). The class III genes, which encode components of the outer basal body and hook (7, 8, 33), are required in turn for expression of the class IV flagellin genes at the end of the cell cycle (15, 36). The class I designation has been reserved for the gene or genes at the top of the hierarchy that regulate class II gene expression, presumably in response to DNA replication (9, 41) or another cell cycle cue. The recently identified response regulator CtrA has been shown to regulate transcription from several class II promoters in vivo (37), one of the characteristics expected of a class I gene. Another group of *fla* genes has not yet been assigned within in the hierarchy, but these unclassified genes (Fig. 1) have been shown to regulate expression of the class IV *fljK* and *fljL* flagellin genes or assembly of the flagellin proteins into the flagellar filament (16, 40).

Most *fla* genes encode structural proteins, and the requirement of multiple class II and class III genes for the expression of *fla* genes at the next lower levels in the hierarchy (Fig. 1) has suggested a model in which flagellum assembly in *C. crescentus* is coordinated with *fla* gene expression by two assembly checkpoints (39). Assembly of the M/S ring-switch complex is required for expression of class III genes, and then assembly of the completed basal body-hook structure is required for expression of the class IV flagellin genes at the end of the cell cycle (Fig. 1). This regulation of gene expression by assembled structures is similar to that proposed originally for *Escherichia coli*, where a single assembly checkpoint, completion of the basal body-hook, regulates the last stage of flagellum biosynthesis (17) by providing for the export of the anti-σ^{28} factor FlgM and allowing for transcription of late *fla* genes (14, 19).

Promoter analysis of the *C. crescentus fla* genes has also provided insights into the molecular basis for the class II-to-class III transition. The class II genes are transcribed early in the cell cycle from a unique class of promoters which contain a conserved DNA motif recognized by the response regulator CtrA (37). The class III and class IV genes, by contrast, are transcribed from specialized σ^{54}-dependent promoters (27, 32). Their expression depends on the σ^{54}-containing RNA polymerase (1, 5) and the transcriptional activator FlbD (38), which functions at *ftr* (flagellar transcription regulation) sequences located ca. 100 bp upstream or downstream from the transcription start sites of all class III and IV promoters (2, 28, 29, 42, 43). With the exception of *rpoN* and *flbD*, which encode σ^{54} and FlbD, the requirement of class II genes for transcrip-

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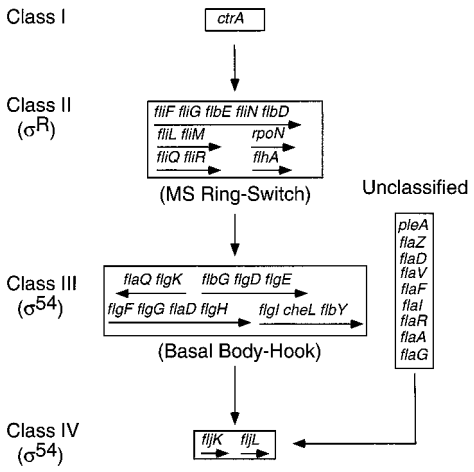


FIG. 1. Regulatory hierarchy of the *C. crescentus fla* genes. In this model, expression of genes at lower levels in the hierarchy requires expression of genes above them. The sigma factor required for transcription of class II genes (σ^R) has not been identified. The two proposed assembly checkpoints, M/S ring-switch complex and the basal body-hook structure, are indicated in parentheses under the class II and class III genes, respectively, which are required for completion of their assembly. The transcriptional organization of the class III *flaH* gene, unlike the class II, III, and IV genes shown, has not been determined and is consequently not diagrammed in the hierarchy. Genes at the right have not been placed in the hierarchy, but they are known to be required for regulation of the class IV flagellin gene expression or flagellin assembly into the filament.

tion from class III promoters can be relieved by a mutation in a negative regulatory gene, *bfa* (21).

Regulation of the transition from class III to class IV gene expression is less well defined. The requirement of class III genes for synthesis of the 25- and 27-kDa flagellins (16, 33) supports the assignment *fljK* and *fljL* as class IV genes (Fig. 1). It is also known that the two flagellin genes are periodically transcribed near the end of the cell cycle at the time of flagellin synthesis (24), and it had been assumed that expression of *fljK* and *fljL* was regulated exclusively at the transcriptional level. More recently, S1 assays revealed that a class III *flgE* mutant, which does not make detectable flagellin proteins, synthesizes nearly wild-type levels of the *fljL* transcript (25, 26). These results suggested that *fljL* might also be subject to posttranscriptional regulation. A similar conclusion was reached from the observations that *fljK* is transcribed in several class II *fla* mutants (42) and that *fljL* is also transcribed in class II mutants when the strains carry a mutation in *bfa*, although none of the strains makes detectable flagellins (21).

In the studies reported here, we have analyzed the regulation of the class IV flagellin genes *fljK* and *fljL* in a large number of *fla* mutants by using transcription and translation fusions, nuclease S1 assays, and measurements of hybrid protein stability. The results of these experiments demonstrate that the 25-kDa flagellin gene *fljK* is transcribed but not translated in all class III *fla* mutants examined. The requirement of multiple class III genes for *fljK-lacZ* expression leads us to propose that *fljK* is regulated posttranscriptionally by the basal body-hook assembly checkpoint. Deletion analysis within *fljK* identified a sequence element in the 5′ untranslated region (UTR) that may be required for this regulation. The 27-kDa flagellin gene *fljL* is also subject to posttranscriptional regulation by class III *fla* genes, but apparently by a mechanism different from the one controlling *fljK* expression. We discuss the role of posttranscriptional regulation in the *fla* gene hierarchy and possible mechanisms for its control.

MATERIALS AND METHODS

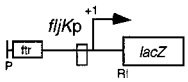
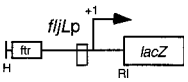
Bacterial strains and culture conditions. *C. crescentus* strains were all derived from strain CB15 (ATCC 19089). The PC strains were isolated or constructed in this laboratory, and SC strains were isolated by Johnson and Ely (15). Recombinant plasmids were introduced into *C. crescentus* by conjugation (36). *C. crescentus* strains were routinely grown in M2 medium or PYE medium supplemented, where indicated, by tetracycline (2 μg/ml) as described previously (36). *E. coli* strains were grown in ML medium supplemented with ampicillin (50 μg/ml) or tetracycline (15 μg/ml).

Site-directed mutagenesis. All site-directed mutagenesis was carried out by using an Altered Sites mutagenesis kit (Promega) according to the manufacturer's instructions.

Construction of *fljK* and *fljL* transcription and translation fusions to *lacZ*. Restriction fragments containing the 5′ regions of *fljK* or *fljL* were obtained from a larger clone of the chromosomal region, pCN200, which contains the complete 25- and 27-kDa flagellin gene transcription units (26). A 550-bp *Pst*I-*Eco*RI fragment containing ca. 380 bases upstream of the transcription start site and the first 23 codons of the *fljK* open reading frame (ORF) was subcloned into pBluescript II KS (Promega). The *Bam*HI-*Hin*dIII fragment was subcloned in the transcription fusion vector pRKlac290 (12) to create pDKA087 (Table 1). After site-directed mutagenesis, the same DNA fragment was fused in frame to the *lacZ* reporter gene as a *Kpn*I-*Hin*dIII fragment in the translation fusion vector pANZ13 (35) to generate plasmid pDKA104 (Table 2).

The 680-bp *Hin*dIII-*Eco*RI fragment containing ca. 580 bases upstream of the transcription start site and the first 18 codons of the *fljL* ORF was subcloned into pBSKS (Promega). This fragment was then moved into pRKlac290 to create the

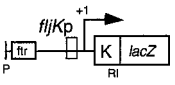
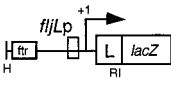
TABLE 1. Regulation of *fljK* and *fljL* transcription fusions to *lacZ* by the *fla* gene hierarchy

Class and strain	Mutation	β-Gal activity[a]	
			
		pDKA087	pDKA089
II			
CB15	WT[b]	1.00	1.00
PC5515	*flhA177*::Tn*5*	1.5	0.10
PC8843	*fliL196*::Tn*5*	1.64	0.07
SC508	*fliQ153*	1.85	0.09
PC8742	*fliF462*	1.65	0.11
PC8750	*fliG470*	0.26	0.04
PC9264	Δ*fliN*	2.75	0.08
PC1106	Δ*flbE*	1.85	0.01
PC5510	*flbD198*::Tn*5*	0.09	0.02
PC5794	*rpoN610*::Tn*5*	0.08	0.04
III			
SC272	*flgK128*	0.41	0.98
SC269	*flaJ125*	1.52	0.97
SC298	*flgE144*	1.05	2.7
SC511	*flgE*::IS*511*	0.97	2.95
SC243	*flgF110*	1.00	1.95
SC252	*flaD115*	1.00	1.60
SC1117	*flgH174*::Tn*5*	1.71	1.90
SC295	*flgI141*	1.35	1.98
SC284	*flaH135*	1.54	1.05
Unclassified			
SC229	*flaA104*	0.84	0.79
SC270	*flaI126*	1.38	0.87
SC175	*flaZ102*	0.92	0.74
SC305	*flaR148*	0.91	0.91
SC278	*flaG131*	1.12	1.75
SC1121	*flaY183*::Tn*5*	0.58	0.84
SC1062	*flaE174*::Tn*5*	0.60	0.83
SC1065	*flbA604*::Tn*5*	0.82	0.89
PC5236	*pleA306*::Tn*5*	1.45	2.55

[a] β-Galactosidase (β-gal) activities are normalized to 1.00 in wild-type strain CB15, where activities in Miller units (23) were 2,059 for *fljK-lacZ* and 840 for *fljL-lacZ*.

[b] WT, wild type.

TABLE 2. Regulation of *fljK* and *fljL* translation fusions to *lacZ* by the *fla* gene hierarchy

Class and strain	Mutation	β-Gal activity[a]	
			
		pDKA104	pDKA106
II			
CB15	WT[b]	1.00	1.00
PC5515	*flhA177*::Tn*5*	0.06	
PC8843	*fliL196*::Tn*5*	0.13	
SC508	*fliQ153*	0.11	0.06
PC8742	*fliF462*	0.08	0.07
PC8750	*fliG470*	0.08	0.06
PC9264	Δ*fliN*	0.08	0.06
PC5510	*flbD198*::Tn*5*	0.02	
PC5794	*rpoN610*::Tn*5*	0.01	
III			
SC272	*flgK128*	0.24	1.74
PC5506	*flbG602*::Tn*5*	0.03	
SC269	*flaJ125*	0.15	
SC298	*flgE144*	0.05	0.79
SC243	*flgF110*	0.09	1.09
SC252	*flaD115*	0.08	
SC1117	*flgH174*::Tn*5*	0.06	
SC295	*flgI141*	0.10	0.95
SC284	*flaH135*	0.09	0.36
Unclassified			
SC270	*flaI126*	0.08	
SC305	*flaR148*	0.85	0.53
SC278	*flaG131*	1.18	0.84
SC229	*flaA104*	0.89	0.53

[a] β-Galactosidase (β-gal) activities are normalized to 1.00 in wild-type strain CB15, where activities in Miller units (23) were 3,113 for *fljK-lacZ* and 188 for *fljL-lacZ*.

[b] WT, wild type.

transcription fusion plasmid pDKA089 (Table 1). The same fragment was then fused in frame to the *lacZ* of pANZ13 to generate plasmid pDKA106 (Table 2).

β-Galactosidase activity assays. All β-galactosidase assays were carried out in liquid culture by the method of Miller (23). The values reported represent the averages of three independent assays in which the activity in an individual assay differed by no more than 7% of the average.

S1 nuclease protection assays. RNA was isolated from CB15 and the *fla* mutant strains as previously reported (33). S1 nuclease assays were carried out essentially as described by Berk and Sharp (3). Chromosomal *fljK* transcripts were detected by using the same *Pst*I-*Eco*RI fragment of pCN200 described above and yielded a 131-nucleotide (nt) partially protected fragment after nuclease S1 digestion. Chromosomal *fljL* transcripts were detected by using the *Hin*dIII-*Eco*RI fragment and gave a 123-nt partially protected product. DNA probes were 5′ end labeled (22) with [γ-^{32}P]ATP and hybridized at 55°C (24) with 100 μg of total cellular RNA isolated from the indicated mutant strains. After treatment with nuclease S1 nuclease Boehringer Mannheim), the resistant DNA fragments were separated by electrophoresis on a polyacrylamide gel and visualized by autoradiography.

Transcripts derived from plasmids pDKA104 and pDKA110 were specifically detected by using as a 5′-end-labeled probe the *Kpn*I-*Hin*dIII DNA fragment, which contains the entire *Pst*I-*Eco*RI fragment used to detect chromosomal *fljK* transcripts and flanking DNA sequences derived from polylinker sequences of plasmid pDKA104. This 5′-end-labeled probe detects only plasmid-derived transcripts containing the same polylinker sequences. The protected product corresponding to the plasmid transcript should also be slightly larger than the one that would be detected for the chromosomal transcript (142 nt versus 131 nt [data not shown]).

Radioimmune precipitation assays. Radioimmune assays for β-galactosidase fusion proteins and hook protein were carried out as described previously (34). Cells were grown in M2 medium (36) to early log phase and labeled for 7 min with 20 μCi of [^{35}S]methionine per ml. The cultures were brought to a final concentration of 75 mM methionine–0.2% peptone to stop further incorporation of [^{35}S]methionine, and 1-ml samples were taken at the times indicated. After incubation with anti-β-galactosidase monoclonal antibody (Promega) and polyclonal rabbit anti-hook protein serum, the resulting immunocomplexes were precipitated with *Staphylococcus aureus* cells and washed as described previously (18). The samples were then boiled in sodium dodecyl sulfate sample buffer (22), and the proteins were separated by electrophoresis on 10% polyacrylamide gels. The gels were then dried and exposed to film.

Construction of 3′ deletions of the *fljK* ORF and internal deletions of the 5′ UTR. The fragment containing the *fljK* promoter and 5′ ORF was progressively shortened by using site-directed mutagenesis to add in frame *Hin*dIII sites. These altered DNA fragments were cloned into pANZ13 as *Kpn*I-*Hin*dIII fragments. The deoxyoligonucleotides used for site-directed mutagenesis were ATC GCC CTG CAA GCT TTG AAT GGC ACG AAT; ATC AAT ACG AAA GCT TGC GCG ATG ATC, and TCT TTC GTT ATG CAA GCT TAC AGC ATC AAT, which correspond to fusion constructs in plasmids pDKA120, pDKA121, and pDKA122, respectively. The same DNA fragment was then used to introduce small internal deletions in the 5′ UTR of *fljK* by site-directed mutagenesis. The mutated fragments were moved into pANZ13 as *Kpn*I-*Hin*dIII fragments. The deoxyoligonucleotides used for site-directed mutagenesis were GCC AAA ATG GCG TCG TTG AAA AGG ACT CTT TCG, ATG CTC CCG GCA GAA TTC GAC CTT GAA AAG GAC T, and GTC CTA CCG AGC AAA CCT TGA AAA GGA CTC, which correspond to deletions Δ38-43, Δ24-42, and Δ10-43, respectively. These fragments were used to construct the transcription and translation fusions (described in the legend to Fig. 6).

RESULTS

Regulation of *fljK-lacZ* and *fljL-lacZ* transcription fusions. The assignment of flagellin genes *fljK* and *fljL* to class IV at the bottom of the hierarchy (Fig. 1) was originally based on the results of radioimmune precipitation assays which showed that 25- and 27-kDa flagellin proteins could not be detected in most of the *fla* mutants (16, 36) (Fig. 1). We have reexamined the regulation of the class IV flagellin genes by using transcription fusions in which the intact *fljK* or *fljL* promoter was fused to the *lacZ* reporter gene to generate plasmids pDKA087 (*fljK-lacZ*) and pDKA089 (*fljL-lacZ*). As judged by the β-galactosidase activity, the *fljK* promoter was expressed in all class II mutants except *rpoN* and *flbD*, which encode transcription factors required for expression of σ^{54}-dependent promoters (1, 5, 38). However, the *fljL* promoter was not expressed in any of the nine class II mutants examined, including *rpoN* and *flbD* (Table 1, class II). These results confirm the previous report of differential regulation of the *fljK* and *fljL* promoters by class II *fla* genes (42).

We also examined the *fljK-lacZ* and *fljL-lacZ* fusions in 9 class III mutant backgrounds and found that both the *fljK* and *fljL* promoters were expressed in all of the mutant strains (Table 1). Since the 25- and 27-kDa flagellins cannot be detected in class III *fla* mutants (16, 36), these results indicate that *fljK* and *fljL* are regulated posttranscriptionally by the class III genes.

The *fljK-lacZ* and *fljL-lacZ* transcription fusions were also expressed in all strains containing a mutation in one of the unclassified genes (Table 1; Fig. 1). The observation that mutations in these genes also make little or no detectable 25- and 27-kDa flagellins indicates that these unclassified genes are also required for posttranscriptional regulation of *fljK* and *fljL*.

Regulation of *fljK* and *fljL* mRNA synthesis in *fla* mutants. We extended the analysis of flagellin gene transcription by using nuclease S1 mapping to assay for *fljK* and *fljL* mRNAs. In these experiments, the end-labeled *Pst*I-*Eco*RI and *Hin*dIII-*Eco*RI DNA probes were the same DNA fragments used to construct the *fljK-lacZ* and *fljL-lacZ* transcriptions fusions, respectively (Table 1; Materials and Methods). In agreement with results of assays using transcription fusions (Table 1), no *fljL* messenger could be detected in the class II *fla* gene mutants (Fig. 2, lanes 2 to 5) but almost wild-type levels of *fljL* messenger were present in the class III *fla* mutants (Fig. 2, lanes 6 to 10). The high steady-state levels of this messenger in the class III strains suggest that the posttranscriptional regu-

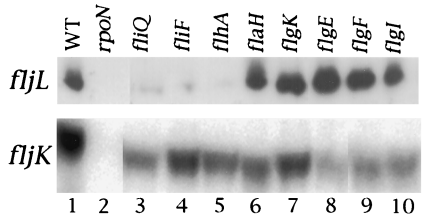


FIG. 2. Nuclease S1 assays of *fljL* and *fljK* transcripts. Total RNA was prepared from *C. crescentus* wild-type (WT) and the *fla* mutant strains indicated and used in nuclease S1 protection assays (Materials and Methods). The 680-bp *Hin*dIII-*Eco*RI and 550-bp *Pst*I-*Eco*RI fragments listed in Table 1 were used to probe for *fljL* and *fljK* transcripts, respectively. Total RNA (100 μg) was annealed to the 5′-end-labeled probe, digested with S1 nuclease, run on a urea polyacrylamide sequencing gel, and visualized by autoradiography. The partially protected products corresponding to the *fljL* and *fljK* transcripts are indicated.

lation of *fljL* is not a result of instability or turnover of the 5′ mRNA.

The *fljK* transcript was present in all class II and class III mutants (Fig. 2, lanes 3 to 10) except the *rpoN* strain (Fig. 2, lane 2). The levels of *fljK* transcript ranged from ca. 10% of the wild-type level in the class III *flgE* mutant to 40 to 50% of the strain CB15 level in the class III *flgK* and class II *fliF* backgrounds (Fig. 2). As suggested below, the reduced levels of 5′ *fljK* transcript detected in the mutant strains may reflect the instability of untranslated *fljK* mRNA.

Regulation of *fljK-lacZ* and *fljL-lacZ* translation fusions. Posttranscriptional regulation of the flagellin genes was examined in greater detail by assaying expression of a *fljK-lacZ* translation fusion in the various *fla* mutant backgrounds (Table 2). The *Pst*I-*Eco*RI fragment of *fljK* that had been used to construct the transcription fusion (Table 1) was fused in frame to the *lacZ* reporter gene in vector pANZ13 (35) (Materials and Methods) to generate plasmid pDKA104. The *fljK-lacZ* translation fusion, which contains the N-terminal 23 amino acids of the 25-kDa flagellin fused to β-galactosidase, was not expressed in any of the class II mutants (Table 2). More significantly, we also failed to detect its expression in the nine class III *fla* mutants examined (Table 2), although *fljK* is transcribed in these strains, as measured using the *fljK-lacZ* transcription fusion (Table 1) and nuclease S1 assays (Fig. 2). These results thus demonstrate that *fljK* is regulated posttranscriptionally. We suggest below that the requirement of all class III *fla* genes for expression of the *fljK-lacZ* translation fusion reflects the posttranscriptional regulation of *fljK* by basal body-hook assembly (see Discussion).

The *fljK-lacZ* translation fusion was expressed at nearly wild-type levels in strains carrying mutations in *flaR*, *flaA*, or *flaG* (Table 2). Failure to observe an effect of mutations in these unclassified genes on *fljK-lacZ* expression could indicate that the genes are directly involved in posttranscriptional regulation and mutations in them uncouple *fljK* expression from the requirement for class III genes or, alternatively, that the unclassified genes function in flagellum biosynthesis after the basal body-hook assembly checkpoint, e.g., during filament assembly. To distinguish between the possibilities, we constructed double mutants between a class II or a class III mutation and each of the three unclassified mutations and measured the effect on expression of the *fljK-lacZ* translation fusion (Table 3). In all cases, the levels of β-galactosidase activity in the double mutants were less than 10% of that in the wild-type *fla*$^+$ strain, which is the regulated phenotype of a class III mutant. Thus *flaR*, *flaA*, and *flaG* must function in flagellum biosynthesis in a step after the posttranscriptional regulation by class III genes, a conclusion supported by the observation that *flaG* is involved either in a flagellin processing pathway or assembly of the flagellar filament (16, 40).

To examine *fljL* expression, the *Hin*dIII-*Eco*RI fragment of *fljL* (Table 1) (Materials and Methods) was fused in frame to the *lacZ* reporter gene in vector pANZ13 (35). The resulting plasmid, pDKA106, encodes the N-terminal 19 amino acids of the 27-kDa flagellin protein fused to β-galactosidase. The *fljL-lacZ* translation fusion was not expressed in the class II *fla* mutant backgrounds (Table 2) where *fljL* is not transcribed (Table 1), but unlike the *fljK-lacZ* translation fusion, it was expressed in all class III and unclassified mutants (Table 2). Given the absence of 27-kDa flagellin protein by radioimmune assay in these strains (16, 36), these results suggest that *fljL* is regulated posttranslationally or, alternatively, by a posttranscriptional mechanism involving a 3′ *fljL* sequence(s) that is not present in the *fljL-lacZ* fusion construct (see Discussion).

Stability of the FljK′-′LacZ fusion protein. One mechanism that could account for the posttranscriptional regulation of *fljK* observed in the *fljK-lacZ* fusion (Table 2) is protein turnover, assuming that sequences required for protein degradation were located in the N terminus of FljK. We examined this possibility by comparing the stabilities of a hybrid FljK′-′LacZ protein in the wild-type strain and a *flaH* mutant. The *flaH* mutant SC284 was used because *fljK* is transcribed at a high level in this strain (Table 1), but the *fljK-lacZ* translation fusion is regulated and expressed at only 9% of the wild-type level (Table 2).

Exponentially growing cultures of strains CB15 and SC284/pDKA104 were pulse-labeled with [^{35}S]methionine for 7 min, and samples were taken at intervals during a 60-min chase period (Fig. 3) under conditions shown to stop further incorporation of label (Materials and Methods). Labeled FljK′-′LacZ protein and hook protein were assayed by immunoprecipitation from the same cell extracts. Labeled FljK′-′LacZ hybrid protein measured in the *flaH* mutant by immunoprecipitation at 0 min after the pulse was ca. 7% of that in wild-type strain, which is approximately the same level measured by β-galactosidase assays (Table 2) and suggests that the fusion protein is stable. In addition, the FljK′-′LacZ fusion protein and the hook protein, which served as an internal control, were stable during the 60-min chase period in the *flaH* and wild-type strains (Fig. 3). These results rule out rapid protein turnover as

TABLE 3. Posttranscriptional regulation of *fljK* in double mutants

Mutant	Mutation	Class	β-Galactosidase (*fljK-lacZ*)[a]
Single	*flhA177*::Tn*5*	II	0.06
	flbG602::Tn*5*	III	0.03
	flaG131	Unclassified	1.10
	flaR148	Unclassified	0.85
	flaA104	Unclassified	0.89
Double	*flhA177*::Tn*5*+*flaG131*	II + unclassified	0.12
	flhA177::Tn*5*+*flaR148*	II + unclassified	0.05
	flhA177::Tn*5*+*flaA104*	II + unclassified	0.06
	flbG602::Tn*5*+*flaG131*	III + unclassified	0.04
	flbG602::Tn*5*+*flaR148*	III + unclassified	0.05
	flbG602::Tn*5*+*flaA104*	III + unclassified	0.03

[a] β-Galactosidase expression was measured from plasmid pDKA104 containing the *fljK-lacZ* translation fusion in strains containing the *fla* mutations indicated. Levels of β-galactosidase are normalized to 1.0 for the *fla*$^+$ wild-type strain.

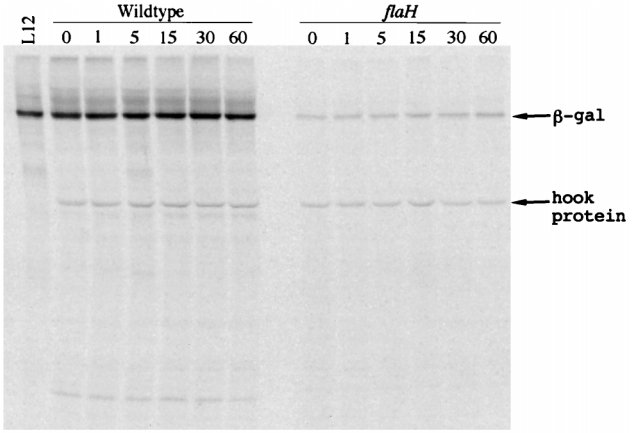


FIG. 3. Stability of the FljK′-′LacZ fusion protein. Exponentially growing cultures of CB15 (wild type) and SC284 (*flaH*) were pulse-labeled with 20 μCi of [^{35}S]methionine per ml for 7 min and chased for the times (minutes) indicated (Materials and Methods). The samples were then lysed and immunoprecipitated with anti β-galactosidase (β-gal) and anti-FlgE (hook) antibodies simultaneously. After extensive washes, the samples were run on a 10% polyacrylamide gel and exposed to film. *E. coli* MC1061, carrying a plasmid (pLac12) containing the intact *lacZ* gene (35), was pulsed and immunoprecipitated with anti-β-galactosidase antibody and is shown in the first lane (L12).

an explanation for the posttranscriptional regulation of *fljK*, as assayed using the *fljK-lacZ* translation fusion (Table 2).

Effect of translation on *fljK* messenger levels. Although *fljK* is transcribed at nearly wild-type levels in class III mutants (Table 1), the reduced levels of genomic *fljK* transcript in these strains (Fig. 2) indicate that the mRNA is less stable than in the wild-type strain. As one explanation for this result, we considered the possibility that the 25-kDa flagellin transcript is unstable when not translated, which could result if *fljK* were regulated at the level of translation by class III genes. To determine the effect of translation on *fljK* messenger levels, we compared the *fljK-lacZ* translation fusion (plasmid pDKA104) to the same translation fusion containing a translation terminator after the third codon of the *fljK* ORF (plasmid pDKA110 [Fig. 4A and Materials and Methods]). In the wild-type *fla*$^+$ strain, the mutant *fljK-lacZ* translation fusion in plasmid pDKA110 expressed little β-galactosidase activity compared to the parent plasmid pDKA104 (Fig. 4C). To determine levels of *fljK-lacZ* mRNA, S1 nuclease assays were carried out with DNA probes designed to detect *fljK* RNA transcribed exclusively from the plasmids (Materials and Methods). The level of

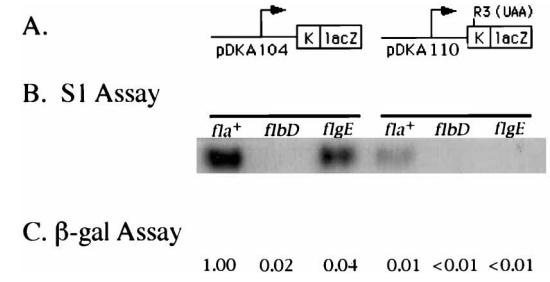
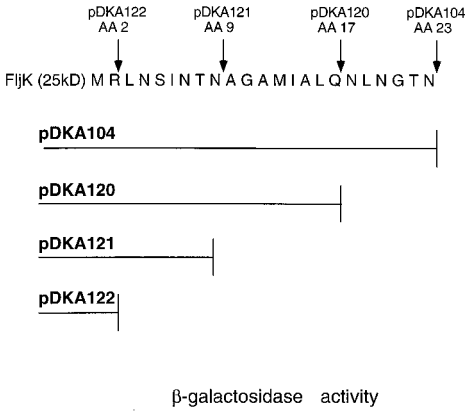


FIG. 4. Effect of premature translation termination on *fljK* mRNA stability. (A) Diagram of translation fusion constructs carried in pDKA104 (Table 2) and pDKA110, which is identical to pDKA104 except that it contains the termination codon UAA inserted after the third codon of the *fljK* ORF. (B) Nuclease S1 assays were carried out on the wild-type and *fla* strains indicated, using 5′-end-labeled probes specific for plasmid-derived RNA (Materials and Methods). (C) β-Galactosidase (β-gal) activities of the strains assayed in panel B.



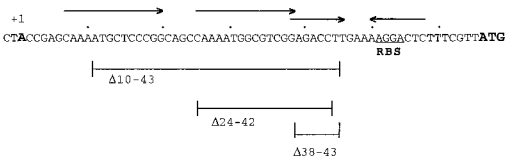
plasmids	β-galactosidase activity: *fla*$^+$	*fliQ*	*flgE*	*flgF*	*flgI*	*flgG*
pDKA104	1.00 (2730)	0.09	0.04	0.09	0.10	0.03
pDKA120	1.00 (2425)	0.07	0.05	0.15	0.15	0.08
pDKA121	1.00 (591)	0.09	0.10	0.12	0.13	nd
pDKA122	1.00 (573)	0.28	0.16	0.23	0.21	0.14

FIG. 5. Effects of 3′ deletions in the *fljK* ORF on regulation of the *fljK-lacZ* translation fusion. A series of *fljK* fragments having identical 5′ ends but different 3′ ends were generated by site-directed mutagenesis and used to construct the *fljK-lacZ* translation fusions. The locations of the 3′ ends are indicated on the predicted amino acid sequence of FljK shown at the top. β-Galactosidase activities of *fla* mutants carrying the translation fusions are shown at the bottom. In all cases, activities were normalized to 1.0 for the wild-type (*fla*$^+$) strain CB15; the activities in Miller units (23) are shown in parentheses.

fljK transcript from plasmid pDKA110 with the UAA termination codon was approximately 10-fold less than that from parental plasmid pDKA104 in the *fla*$^+$ background (Fig. 4B). We also constructed and assayed transcription fusions to *lacZ*, using the same *Pst*I-*Eco*RI fragments of *fljK* contained in the translation fusions (plasmids pDKA104 and pDKA110) to verify that transcription of the *fljK* fragment with the nonsense mutation (plasmid pDKA110) was not adversely effected (data not shown).

A similar effect of premature chain termination on the levels of *fljK-lacZ* messenger was observed in a class III *flgE* (hook protein gene) mutant. The level of *fljK* mRNA from parental plasmid pDKA104 was reduced substantially compared to the *fla*$^+$ strain, and the level of *fljK* mRNA transcribed from plasmid pDKA110 with the translation terminator codon was reduced to an undetectable level in the same *flgE* strain (Fig. 4B). These data are consistent with the rapid turnover of 5′ mRNA from the *fljK-lacZ* fusion when it is not translated. No *fljK* messenger was detected from either plasmid in the class II *flbD* mutant in which the σ^{54}-dependent *fljK* promoter cannot be expressed (Table 1).

Effect of 3′ deletions in the *fljK* ORF on regulation of the *fljK-lacZ* translation fusion. The 550-bp *Pst*I-*Eco*RI DNA fragment used in the *fljK-lacZ* translation fusion includes the 5′ UTR of *fljK* and extends to codon 23 of the gene. To identify sequences within this DNA fragment required for posttranscriptional regulation of *fljK* by the class III *fla* genes (Table 2), we first constructed a set of 3′ deletions in the *Pst*I-*Eco*RI insert at sites corresponding to *fljK* codons 17, 9, and 2 (Fig. 5). These fragments were then fused in frame to *lacZ* (Fig. 5) and assayed in the wild-type *fla*$^+$ strain and in class II and III *fla* mutants. A translation fusion containing only the first nine



β-galactosidase activity

	Protein Fusions				Transcription Fusions		
Deletions	*fla*$^+$	*fliQ*	*flgE*	*flhA*	*fla*$^+$	*fliQ*	*flgE*
none	3113	343	156	187	2059	3810	2162
Δ38-43	1796	nd	69	177	5527	7450	3747
Δ24-42	7169	6136	2128	nd	6483	7929	3660
Δ10-43	6632	7315	4000	nd	5070	4997	4428

FIG. 6. Internal deletions of the *fljK* 5′ UTR. The nucleotide sequence of the *fljK* 5′ UTR is at the top, with the transcription start site (+1) at the left and the translation initiation ATG codon in boldface at the right. The ribosome binding site (RBS) is underlined. Direct and inverted repeats in the UTR discussed in the text are indicated above the sequence. The extents of three internal deletions generated by site-directed mutagenesis within the 380-bp *Pst*I-*Eco*RI fragment are shown below the sequence (see text). β-Galactosidase activities were determined in strains containing either translation fusions (labeled as protein fusions) or transcription fusions of the internal *fljK* deletions to *lacZ*. Translation fusions with the deletion constructs correspond to plasmids pDKA109 (Δ38-43), pDKA183 (Δ24-42), and pDKA175 (Δ10-42), while the transcription fusion with the same deletions correspond to plasmids pDKA185 (Δ38-43), pDKA186 (Δ24-42), and pDKA187 (Δ10-42). Constructs without a deletion contained the intact 5′ *fljK* fragment also in a translation and transcription fusions to *lacZ*.

codons of the *fljK* ORF (pDKA121 [Fig. 5]) was fully regulated in the class II (*fliQ*) and III (*flgE*, *flgF*, *flgI*, and *flgG*) *fla* mutants. When the *fljK* sequence between codons 2 and 9 was deleted, the resulting construct displayed a partial loss of regulation in the mutant backgrounds, with β-galactosidase activities measured for plasmid pDKA122 in the class III mutant backgrounds ranging from 14 to 23% of that in the *fla*$^+$ strain. Thus, the deleted sequence between codons 2 and 9 only partially accounts for the posttranscriptional regulation of *fljK.*

We also noted that the first 17 codons of *fljK* were required for a high level of β-galactosidase activity (Fig. 5). Translation fusions at codon 2 (pDKA122) or 9 (pDKA121) resulted an almost fourfold reduction in activity compared to those in pDKA120 and pDKA104, which suggests that a sequence between codons 9 and 17 is required for efficient translation.

Posttranscriptional regulation of *fljK* requires an intact 5′ UTR sequence. To identify other sequences involved in the posttranscriptional regulation of *fljK*, we examined the 67-bp 5′ UTR of *fljK*. Three deletions were introduced into the *Pst*I-*Eco*RI fragment upstream of the *fljK* ribosome binding site by site-directed mutagenesis, and the resulting DNA fragments (Fig. 6) were fused in frame to *lacZ* at the site corresponding to amino acid 17 of the *fljK* coding sequence (Fig. 5). Expression of *lacZ* in the translation fusions was assayed in the class II *fliQ* and *flhA* mutants and the class III *flgE* mutant.

The 6-bp deletion from +38 to +43 (Δ38-43) disrupts one arm of a dyad overlapping the *fljK* ribosome binding site (Fig. 6). Although this inverted repeat is a potential site of translation regulation, the *fljK-lacZ* translation fusion construct containing the Δ38-43 deletion was fully regulated in the *flgE* and *flhA* mutants. The only detectable effect of the deletion was a somewhat lower β-galactosidase activity in the *fla*$^+$ background. The two translation fusions with the next longer deletions, extending from +24 to +42 (Δ24-42) and from +10 to +43 (Δ10-43), lost essentially all regulation in response to the class II and III gene mutations and displayed β-galactosidase activities in the *fliQ* and *flgE* mutants comparable in most cases to those in the wild-type, *fla*$^+$ strain (Fig. 6). These results suggest that a sequence bounded by the 5′ ends of deletions Δ24-42 and Δ38-43, i.e., between nt +24 and +38, is necessary for the posttranscriptional regulation of *fljK*. The latter deletions (Δ24-42 and Δ10-43) disrupt an imperfect 14-bp direct repeat sequence in the UTR located just 5′ of the inverted repeat.

As a control for these experiments, we constructed transcription fusions of the same 5′ UTR mutant fragments of *fljK* to *lacZ* and assayed for β-galactosidase. For reasons that are not clear, transcription from the *fljK* promoter was two- to threefold higher in all of the 5′ deletion constructs (Fig. 6). It should be noted, however, that the increase levels of transcription were comparable, at least in the wild-type, *fla*$^+$ and the *fliQ* backgrounds, whether translation regulation was lost (Δ24-42 and Δ10-43) or not (Δ38-43).

DISCUSSION

Flagellar gene expression and flagellum biosynthesis are coordinated in *C. crescentus* by two assembly checkpoints: completion of the M/S ring-switch complex and completion of the basal body-hook structure (39). The first of these checkpoints is required for transcription of the class III *fla* genes (31, 39, 46) and the class IV gene *fljL* (21). In the experiments discussed below, we investigated the regulation of the flagellin genes using transcription and translation fusions of *fljK* and *fljL* to the *lacZ* reporter gene. We interpret the results of these experiments to show that the second assembly checkpoint, completion of basal body-hook structure, is responsible for the posttranscriptional regulation of *fljK* expression. Although these studies were focused on *fljK* regulation, we showed that *fljL* is also regulated posttranscriptionally, and the results suggest that expression of *fljK* and expression of *fljL* are controlled by different mechanisms. Thus, transcriptional regulation of class IV *fla* gene expression within the *C. crescentus* hierarchy is overlaid by levels of posttranscriptional control.

Posttranscriptional regulation of *fljK.* Only the transcription factors encoded by class II genes *rpoN* and *flbD* are required for transcription of *fljK* (Table 1 and reference 42). The observation that the *fljK-lacZ* transcription fusion was expressed in the nine class III mutants examined (Table 1) is consistent with this conclusion. It is known, however, that neither the 25- or the 27-kDa flagellin is made in these strains (15, 36), and our results now demonstrate that the *fljK-lacZ* translation fusion is not expressed in any of the class III mutants (Table 2). We conclude that the novel requirement of multiple class III genes for expression of the *fljK-lacZ* translation fusion reflects the role of the assembled basal body-hook structure in the posttranscriptional regulation of *fljK*, rather than the independent regulatory function of individual class III genes. Similar arguments have been considered in discussions of assembly checkpoint control of flagellum biosynthesis, initially in *E. coli* (17) and subsequently in *C. crescentus* (39). The observations that a *fljK-lacZ* translation fusion is not expressed in class II *fla* mutant strains (Table 2 and reference 21) is presumably explained by the requirement of class II *fla* genes for class III gene expression.

Results of pulse-chase experiments suggest that the posttranscriptional regulation of *fljK* is not at the level of protein turnover. The stability of the FljK′-′LacZ fusion protein was the same in the wild-type strain and a *flaH* mutant, which exhibits posttranscriptional regulation (Fig. 3). These findings do not eliminate the existence of a subpopulation of the fusion

protein that is degraded too rapidly to be detected in the pulse-chase experiment. However, the identification of a sequence element in the 5′ UTR required for this regulation (Fig. 6) supports the conclusion that a posttranslational mechanism is not involved.

If *fljK* is regulated at the translational level, mRNA turnover or processing could be involved. In one case, the overall stability of the *fljK* transcript could be directly regulated in such a way that the *fljK* mRNA would be rapidly degraded when the basal body-hook structure is not assembled. Several findings argue against this model. First, if regulation affected messenger stability exclusively, we would not expect to detect *fljK* mRNA in class III mutants. Nuclease S1 assays indicate, however, that there is up to 40% of wild-type levels the 5′ *fljK* mRNA in these strains (Fig. 2). Second, a messenger stability model might predict that nonsense mutations in the *fljK* ORF should not affect mRNA stability. However, the level of mRNA was severely reduced when a chain termination codon was introduced in the *fljK* ORF at the third codon, both in the *fla*$^+$ strain and in the regulated *flgE* mutant (Fig. 4C). These results suggest that the reduced levels of messenger in these strains is a consequence of the failure of the mRNA to be translated, as observed previously in systems known to be regulated at the level of translation (44). Also, the detection of a single species of partially protected probe in the nuclease S1 assays of wild-type and class III mutants (Fig. 2 and 4) argues against the possibility that processing at the 5′ end of the *fljK* mRNA plays a regulatory role, although processing at the 3′ end of the *fljK* mRNA would not be detected in these experiments.

Studies in other systems have shown that translation can be regulated either positively or negatively. If the mRNA forms a secondary structure that occludes the ribosome binding site, a negative regulator binding to the RNA could stabilize the structure and inhibit translation, as described for the negative control of the bacteriophage R17 replicase gene by its coat protein (6). Alternatively, a regulator binding upstream of such a stable secondary structure could disrupt it and allow initiation of translation, as described for the positive regulatory function of the Com protein in stimulating translation of the *mom* mRNA in bacteriophage Mu (13, 45). Although there is an inverted repeat in the 5′ UTR of *fljK* that might assume a secondary structure occluding the proposed ribosome binding site, deleting one arm of the dyad had no detectable effect on *fljK* regulation (Δ38-48 [Fig. 6]).

The next-longer deletions in the 5′ UTR of *fljK* also removed either one arm (Δ24-42) or both arms (Δ10-43) of an imperfect direct repeat, and both of the latter two deletions resulted in the loss of *fljK* regulation in class II and class III mutants (Fig. 6). This result implicates the direct repeat sequence located 5′ of the ribosome binding site in the posttranscriptional regulation of *fljK*, possibly acting as the site of RNA-protein interaction, and is consistent with a translational model for the regulation of *fljK*. The effect of deletions within the *fljK* ORF suggests that a sequence between codons 2 and 9 may also contribute to posttranscriptional regulation of this flagellin gene (Fig. 5).

Posttranscriptional regulation of *fljL*. The posttranscriptional regulation of *fljL* is apparently by a different mechanism. Both the *fljL-lacZ* transcription fusion and *fljL-lacZ* translation fusion were expressed at nearly wild-type levels in all class III *fla* mutants (Tables 1 and 2). However, the failure of these strains to make detectable 27-kDa flagellin by radioimmune assay (16, 36) raises the possibility that *fljL* is regulated posttranslationally; e.g., the protein could be rapidly degraded when it is not assembled into the flagellar filament. Alternatively, a sequence at the 3′ end of *fljL* required for the posttranscriptional regulation may be missing from the *fljL-lacZ* translation fusion used in these studies. As noted above, the high steady-state levels of 5′ *fljL* mRNA in class III *fla* mutants (Fig. 2) make it unlikely that the 27-kDa flagellin gene is regulated posttranscriptional by mRNA turnover.

Organization of the *fla* gene hierarchy. The two assembly checkpoints in *C. crescentus* function at different levels of control (39). Completion of the M/S ring-switch complex regulates transcription of class III genes, while the results presented here suggest that completion of the basal body-hook structure is required for the posttranscriptional regulation of the class IV flagellin *fljK* gene. The *fla* gene hierarchy of *E. coli* and *Salmonella typhimurium* contains one assembly checkpoint, completion of the completed basal body-hook, which functions at the transcriptional level. The assembled basal body-hook provides for the export of the anti-σ^{28} factor FlgM outside the cell and allows transcription of the σ^{28}-dependent flagellin gene and other late *fla* genes to proceed (14, 19). Further analysis of the analogous assembly checkpoint in *C. crescentus* will be required to determine if it functions in a similar manner, for example, to remove a hypothetical negative regulator of *fljK* expression.

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