Negative transcriptional regulation in the *Caulobacter* flagellar hierarchy

(Hong Xu*, Andrew Dingwall*, and Lucille Shapiro†‡)

*Department of Molecular Biology, Albert Einstein College of Medicine, Bronx, NY 10461; and †Department of Microbiology, College of Physicians and Surgeons of Columbia University, New York, NY 10032

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**ABSTRACT** The *Caulobacter crescentus* flagellum is formed at a specific time in the cell cycle and its assembly requires the ordered expression of a large number of genes. These genes are controlled in a positive trans-acting hierarchy that reflects the order of assembly of the flagellum. Using plasmids carrying transcriptional fusions of either a neo or a lux reporter gene to the promoters of three flagellar genes representing different ranks in the hierarchy (the hook operon, a basal body gene *flbN*, and the *flaO* gene), we have measured the level of chimeric gene expression in 13 flagellar mutant backgrounds. Mutants in the hook operon or in basal body genes caused overproduction of both hook operon and basal body gene chimeric mRNAs, suggesting that negative regulation is superimposed on the positive trans-acting control for these events in the flagellar hierarchy. Mutants in the structural genes and in genes involved in flagellar assembly had no effect on *flaO* expression, placing the *flaO* gene near the top of the hierarchy. However, *flaO* expression appears to be under negative control by two regulatory genes *flaS* and *flaW*. Negative control, as a response to the completion of specific steps in the assembly process, may be an important mechanism used by the cell to turn off flagellar gene expression once the gene product is no longer needed.

The generation of asymmetry that yields different progeny cells at cell division is a fundamental problem in development. The bacterium *Caulobacter crescentus* undergoes a programmed cell cycle that yields dissimilar progeny cells at each cell division. An aspect of this cell cycle is the biogenesis of a flagellum at one pole of the presplicidial cell and its subsequent partition to the daughter swimmer cell. This cell type-specific event results from a complex temporal program of flagellar gene expression and subcellular positioning of gene products (1, 2). Genetic studies have identified over 40 genes involved in the formation of the flagellum and the chemotaxis apparatus (3, 4). The products of some of these genes are known, and their location in the flagellar structure is shown schematically in Fig. 1. The flagellum is composed of a basal body (8, 9), a hook (10), and a helical filament (7, 11, 12). The order of expression of the flagellar genes reflects the order of assembly of their products into the flagellar structure (5, 13–20). Mechanisms must exist to ensure the sequential appearance of these gene products and to turn off the expression of individual *fla* genes once their products are no longer needed.

Flagellar gene expression appears to be controlled at the transcriptional level (19–25), and a hierarchy of trans-acting positive control contributes to the ordered transcription of these genes (21–23). To determine whether negative transcriptional controls also act in this regulatory hierarchy to limit the duration of expression to the relevant period of

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fig. 1. Diagram of the *C. crescentus* flagellum, which is composed of a basal body anchored in the membrane, a hook, and a filament. The basal body, containing five rings threaded on a rod, requires the expression of the gene cluster *flbN*, *flaD*, *flaB*, and *flaC* for its biogenesis (5). The hook is composed of a 70-kDa protein encoded by the *flak* gene (6). The filament is composed of 25- and 27.5-kDa flagellins encoded by *flgK* and *flgL*, respectively. A 29-kDa flagellin, encoded by *flgJ*, resides at the junction of the hook and the filament (7). Mutant strains used in these studies are listed under the relevant structures.

assembly, we have constructed hybrid genes in which the promoters of three *fla* genes, each representing a different level of the hierarchy, are fused to either of two promoterless reporter elements: the neo gene encoding neomycin phosphotransferase II (NPT II) or a promoterless lux operon encoding luciferase. These *fla* gene fusions include a regulatory gene (*flaO*), a basal body gene (*flbN*), and an operon that encodes genes used for the biogenesis of the hook. In each case, translation stop signals in front of the promoterless neo or lux genes preclude the formation of protein fusions; measurement of the amount of the reporter gene product should therefore reflect the amount of the chimeric mRNA and hence the activity of the respective *fla* promoter. To define trans-acting effects of *fla* gene products on these promoters, the plasmid-bearing fusions were placed in a

Abbreviation: NPT II, neomycin phosphotransferase II.

†To whom reprint requests should be addressed.
variety of fla− mutant backgrounds, and the level of expression of either the neo gene or the lux operon was determined. We demonstrate here that a network of negative regulation is superimposed on the positive trans-acting control of flagellar biogenesis. Such a network of negative regulation could play an important role in turning off the expression of early flagellar genes when the respective products are no longer needed in the ordered assembly of the flagellar structure.

MATERIALS AND METHODS

Bacterial Strains, Chemicals, and Enzymes. Bacterial strains (26–28) used in this study are listed in Table 1. C. crescentus was grown at 30°C in minimal MBG or rich PYE medium (29). Calf intestine alkaline phosphatase was obtained from Boehringer Mannheim and phage T4 DNA ligase and restriction enzymes were from Boehringer Mannheim or New England Biolabs.

Immunoprecipitation. Aliquots of cells were pulse-labeled with [35S]methionine (1150 mCi/mmol; 1 Ci = 37 GBq) for 10 min. Cells were then lysed and preabsorbed with Staphylococcus aureus cells, and proteins were immunoprecipitated with antisera against NPT II as described (30). Proteins were separated by electrophoresis through SDS/10% polyacrylamide gels. The gels were fixed in 5% methanol/7% acetic acid, enhanced, and subjected to autoradiography.

Assay of Luciferase Activity. C. crescentus cultures grown at 30°C were shifted to 25°C for 30 min. An aliquot of culture was placed in a scintillation vial and n-decyladenylic acid (2% emulsion) was added to a final concentration of 0.002%. Emitted light was measured immediately after a period of 6 sec in a Packard scintillation counter (model 3330). Counts obtained on the 3H channel were converted into quanta emitted by using a known radioactive standard for calibration.

Construction of Plasmids Containing Transcription Reporter Genes. pHX6 (see Fig. 2) and pADN-1 (see Fig. 4) contain fla genes that were cloned from two Tn5-VB32 insertion mutants, AE8006 and AE8002, respectively. The Tn5-VB32 insert contains a promoterless neo gene whose expression is contingent on an upstream accessed promoter (31). AE8006 was shown to contain the Tn5-VB32 insert in the flaK hook structural gene by DNA sequence analysis of the insert junction (H.X., unpublished data). Chromosomal DNA from this mutant was digested with Bgl II and an 8.0-kilobase (kb) Bgl II insert containing 4 kb of chromosomal DNA and 4 kb of the truncated Tn5-VB32 was cloned first into pBR322. A Sac I/Hpa I fragment (see Fig. 2) was further subcloned into pUC19 and then into pRK290 derivative plasmids (32). Strain AE8002 has the Tn5-VB32 insert in the flaN basal body gene (A.D., unpublished data). AE8002 DNA was digested with Bgl II (Tn5-VB32 contains a Bgl II site) and Sac I. DNA fragments were then cloned into pUC19, from which the plasmid pADN-1 was constructed by using a Sac I/Sal I fragment having 800 base pairs (bp) of the 5' end of the flbN gene linked to the neo reporter gene (see Fig. 4). The insert was then cloned into pRK290. The plasmid pADN-6 (see Fig. 3) was constructed by subcloning a 750-bp Sac I/Ava I fragment containing the flbN promoter into pUCD615 (33) that contains a promoterless luciferase operon (see Fig. 3).

The flaO promoter region was fused to either the promotorless neo gene or the promoterless luciferase operon. The plasmid pHX280 contains a 280-bp BamH1/HindIII fragment of the flaO promoter region (34) in front of the nonreporter gene of pKIC7 (ref. 35; see Fig. 2). The plasmid pG642 (see Fig. 3) contains a 2.7-kb BamH1/EcoRI fragment of the flaO region subcloned into pUCD615 (33). Both chimeric fragments were cloned into pRK290 derivative plasmids before conjugation into C. crescentus.

RESULTS

Negative Regulation of the Hook Operon. The hook operon consists of four cotranscribed genes flbG, flaJ, flbH, and flaK (Fig. 2A; ref. 20). The last gene in the operon, flaK, encodes the major 70-kDa hook protein (6, 21), and the other genes in the operon are required for hook biogenesis. The insertion of the transcriptional reporter gene Tn5-VB32 in the flaK gene (Fig. 2A) has allowed us to investigate the trans-acting effects of other genes in the flagellar network on hook operon expression. To do this we cloned the interrupted gene in plasmid pHX6 (Fig. 2A) and introduced this plasmid into strains containing mutations in known fla genes. All of the mutant strains tested showed a loss of part or all of the flagellar structure and reflect lesions at different points of the assembly process (Table 1). In each of these strains, we measured the synthesis of NPT II by immunoprecipitation of labeled cell extracts using anti-NPT II antibody (Fig. 2B). In each strain tested, the highest concentrations of kanamycin that allowed growth was a direct reflection of the relative amount of immunoprecipitated NPT II.

In confirmation of previous results, we show here that the three putative regulatory genes, flaO, flaW (21), and flaS (22, 23), are required for the transcription of the hook operon (Fig. 2A). Mutations in these three genes result in a significantly decreased expression from the hook operon transcription fusion. However, strains with mutations in the basal body genes flaB, flaC, and two alleles of flaD overproduced NPT II, suggesting that the hook operon is under negative control. The hook operon was autoregulated, as indicated here by its overexpression in a flaK mutant strain and previously by Chen et al. (24). Mutants with lesions in flaR, flaZ, flaY, or flaA, all of which make a complete basal body and hook but have a partial or missing filament, have no significant effect on the level of hook operon expression. Apparently, these genes, which are lower in the flagellar hierarchy and only affect the assembly of the filament, do not impact on the expression of the hook operon. Therefore, negative control of the hook operon appears to be exerted by the fla genes at or above the position of the hook operon in the flagellar hierarchy. The hierarchy of genes that results in the assembly of

<table>
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<th>Strain</th>
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<th>Ref.</th>
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The flagellar mutants are listed in descending order of flagellar assembly, so that those at the top of the list lack an entire structure, whereas those near the end of the list lack only a filament or form a complete, but paralyzed, flagellum.
Fig. 2. (A) Schematic representation of the hook operon and the adjacent flaO gene from Ohta et al. (21) and a restriction map of the Tn5-VB32 insertion in the fla gene of AE2006. The direction of transcription is indicated by the wavy line and a triangle indicates the site of the Tn5-VB32 insertion. Tn5-VB32 contains a promoterless neo gene (hatched box) and a complete tet gene (open box). The subclones are shown schematically below the restriction map. pHX6 has 280 bp of the upstream of the hook operon promoter and a portion of the operon fused to a promoterless neo gene. pHX280 has 280 bp derived from flaO linked to a promoterless neo gene. These fusions were cloned into a pRK290 derivative plasmid and mated into C. crescentus CB15N or mutant strains. B, BamH1; Bg, Bgl II; H, HindIII; Hp, Hpa I; R, EcoRI; S, Sac I. (B) Synthesis of NPT II from the hook operon pHX6 fusion in the indicated fla− mutant strains. The autoradiogram shows the 25-kDa NPT II immunoprecipitated with anti-NPT II antibody from cell extracts of [35S]methionine-[1150 mCi/mmol] labeled CB15N (W.T.), SC511 (flaK), SC516 (flaB), SC506 (flaC), SC313 (flaD), SC258 and SC513 (flaD), SC303 (mutC), SC308 (flaS), SC290 (flaO), SC297 (flaW), SC305 (flaR), SC175 (flaZ), SC512 (flaEY), and SC229 (flaA), each carrying pHX6. (C) Synthesis of NPT II from the flaO−neo fusion pHX280 in CB15N and nine fla mutants. Each experiment was repeated multiple times and an internal control of a gene fusion that does not respond to transcriptional control was included in each experiment.

A complex structure might require negative control to shut down the expression of those genes whose products have completed assembly. In this context, it was surprising to find that in a motC mutant background the level of transcription of the hook operon was also increased.

Regulation of the flaO Gene and Its Position in the Flagellar Hierarchy. The genes flaS (22, 23), flaO, and flaW (21, 36) positively control the expression of the hook operon (Fig. 2B). Newton and his co-workers have shown that flaO is near the top of the regulatory hierarchy and that flaO is subject to negative autoregulation (37). flaO and flaW have been mapped in two adjacent operons downstream of the hook operon. However, flaS maps at least 200 kb away from these genes. A flaS deletion strain carrying a flaO fusion to a promoterless neo gene (pHX280) exhibited higher levels of kanamycin resistance than wild-type cells carrying this transcription fusion. Immunoprecipitation of [35S]methionine-labeled cells showed that this increased resistance to kanamycin was due to a higher level of NPT II synthesis in the flaS− genetic background. Therefore, flaS appears to negatively control the expression of flaO. In addition, mutations in flaO and flaW caused a significant increase in NPT II synthesis from the flaO−neo fusion (Fig. 2C), consistent with the results in the accompanying paper (37). However, mutations in the hook structural gene, flaK, or the basal body genes flaC and flaD, had no significant effect on the level of expression from the flaO promoter. These results were confirmed by assaying a transcription fusion of flaO to a lux reporter gene (pJG642) placed in a flaO−, flaK−, flaC−, or flaD− background (Fig. 3). flaR and flaA mutants that have defects in filament assembly showed only a small increase in expression from the flaO promoter (Fig. 2C).

Transcriptional Regulation of a Basal Body Gene. The initiation of transcription of the genes involved in basal body formation occurs prior to the initiation of hook and flagellin gene transcription (19). The basal body gene, flbN, has been shown to be positively controlled by the product of the flaS gene (23). A direct assay of the flbN transcript by nuclease S1 protection assay showed a significant decrease in flbN expression in a flaS− background (A.D., unpublished data). This regulatory effect was also observed with a plasmid-borne flbN gene fused to either a neo (Fig. 4B) or a lux (Fig. 3) reporter gene. Thus, trans-acting control is observed when flbN is in single copy on the chromosome and when it is present on a plasmid at three to five copies per cell.

To determine whether a basal body gene is subject to negative control, we constructed strains of fla− mutants containing the flbN−neo transcription fusion, pADN-1, and measured the synthesis of NPT II by immunoprecipitation with anti-NPT II antibody (Fig. 4). Increased synthesis of NPT II from the flbN promoter was observed when pADN-1 was present in strains with mutations in the basal body genes, flaB, flaC, or flaD (Fig. 4B). The change in level of expression from the flbN promoter was confirmed by using a flbN−lux transcription fusion on plasmid pADN-6 in a flaD− genetic background (Fig. 3B). In addition, we found that a mutation in the hook gene (flaK) increased the level of kanamycin resistance in a strain carrying pADN-1. We have consistently observed an increase in synthesis of NPT II from pADN-1 in flaK− backgrounds, which correlates with the

Fig. 3. Transcription from fla-lux fusions. (A and B) Luciferase activity from flaO-lux (pJG642) and flbN-lux (pADN-6) chimeric plasmids, respectively, in the indicated mutant background. W.T., wild type.
observed increase in kanamycin resistance of these strains (Fig. 4B). In these experiments, the response is smaller than that seen in flaB−, flaC−, and flaD− backgrounds. Nuclease S1 protection assays showed a significant increase in the flaN mRNA in flaK− backgrounds as well as in the basal body mutants flaB−, flaC−, and flaD− (data not shown). These data indicate negative control within the basal body gene cluster and the hook operon.

When pADN-1 was placed in a flaO− mutant background, increased synthesis of NPT II was also observed. However, previous reports (21) have established that flaO is required for flaK expression; hence, the effect of the flaO− mutation may be indirect and result from the lack of hook protein synthesis. Such a pathway underlines the caveat that the regulatory connection between two genes that is deduced from the effect of mutations in one gene on the activity of the other may be direct or indirect.

**DISCUSSION**

The bacterial flagellum is a complex structure that is assembled at the cell surface from the most cell proximal subassembly, the basal body, out to the hook and then the filament (Fig. 1). This example of cellular architecture raises a number of important questions: Does correct assembly depend on ordered expression of each of the structural components, and if so, how is the ordered expression regulated?

Genes that are involved in flagellar biogenesis are turned on sequentially. The temporal sequence is coincident with the order of protein assembly in the flagellum. For example, a gene encoding a product required for basal body formation (flaD) is needed at the early stage of the assembly process and is expressed prior to the flagellin genes (5), whose products are assembled later in the cell cycle (14, 16, 20). The hook structural protein (flaK) is synthesized somewhat later in the cell cycle and continues until just before cell division (21, 24).

The duration of expression of these genes varies, and this may reflect the constraints imposed by the assembly process. Mechanisms must exist to ensure that genes are turned off at the appropriate time.

It has been demonstrated in both _Escherichia coli_ (38, 39) and _C. crescentus_ (22–24) that a cascade of positive transcriptional control contributes to the ordered expression of flagellar components. Komeda (39) has postulated that in _E. coli_ there is a correlation between transcriptional control of flagellar genes and the assembly pathway. Negative control of flagellar gene transcription in _E. coli_ also serves to modulate flagellar biogenesis (39).

The experiments presented in this paper, as well as those by Newton _et al._ (37), demonstrate that the _C. crescentus_ flagellar regulatory network uses both positive and negative control to modulate the level of _fla_ gene transcription. A large number of mutants with defects at different stages of flagellar biogenesis were examined with respect to their effect on the level of transcription of flagellar gene both higher and lower in the regulatory hierarchy. The rationale of these experiments is to measure the trans effect of chromosomal _fla_ mutations on the promoter activity of _fla_ genes in transcriptional neo or lux fusions on plasmids. Our analyses, and those in the accompanying paper (37), revealed that lesions in basal body genes, _flaB_, _flaC_, _flaD_ and _flaN_ result in the overexpression of the hook operon as well the _flbN_ promoter. Similarly, mutations in the hook protein structural gene caused overexpression of the _flbN_ basal body gene and the hook operon. However, mutations in genes for filament assembly, which are lower in the regulatory hierarchy, such as _flaEY_, _flaR_, _flaZ_, and _flaA_, did not affect the level of transcription of the hook operon. A schematic diagram of the flagellar regulatory network with both positive and negative interactions is shown in Fig. 5. We postulate that the cell, upon completion of a portion of the structure, shuts down the synthesis of proteins that are no longer needed. Assembly of the hook and basal body yields a signal that this subassembly is complete—a signal that, directly or indirectly, exerts a negative control, which turns off further transcription of the structural genes. When the assembly of the hook and basal body is complete, as in mutant strains, negative control is lost and the cell continues to synthesize the component proteins. Under normal conditions the same signal, or a modification of that signal, may be used to turn up the level of expression of genes encoding proteins to be assembled next, such as the flagellin and the chemotaxis genes.

Examination of the genes near the top of the hierarchy, _flaS_ and _flaO_ (Fig. 5), showed that both the hook operon and _flbN_ were positively controlled by _flaS_, but that only the hook operon was positively controlled by _flaO_; _flaS_ negatively regulated the transcription of the _flaO_ gene. This may provide a functional balance between the expression of the two genes near the top of the hierarchy. These results, in combination with an earlier result that a low level of hook promoter activity was observed in a _flaK−/flaS−_ double mutant (23),
suggest that the positive regulatory effect of flaS is epistatic to the negative effect of structural gene mutations.

Negative control may place constraints on the duration of transcription of structural genes or their level of expression or both. Thus, the negative control of genes whose products have completed their assembly process may be a mechanism used by the cell to shut off specific flagellar gene expression at the appropriate times in the cell cycle.

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