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Timing of Flagellar Gene Expression in the *Caulobacter* Cell Cycle Is Determined by a Transcriptional Cascade of Positive Regulatory Genes

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The *Caulobacter crescentus* flagellar (*fla*) genes are organized in a regulatory hierarchy in which genes at each level are required for expression of those at the next lower level. To determine the role of this hierarchy in the timing of *fla* gene expression, we have examined the organization and cell cycle regulation of genes located in the hook gene cluster. As shown here, this cluster is organized into four multicistronic transcription units *flaN*, *flbG*, *flaO*, and *flbF* that contain *fla* genes plus a fifth transcription unit II.1 of unknown function. Transcription unit II.1 is regulated independently of the *fla* gene hierarchy, and it is expressed with a unique pattern of periodicity very late in the cell cycle. The *flaN*, *flbG*, and *flaO* operons are all transcribed periodically, and *flaO*, which is near the top of the hierarchy and required in *trans* for the activation of *flaN* and *flbG* operons, is expressed earlier in the cell cycle than the other two transcription units. We have shown that delaying *flaO* transcription by fusing it to the II.1 promoter also delayed the subsequent expression of the *flbG* operon and the 27- and 25-kDa flagellin genes that are at the bottom of the regulatory hierarchy. Thus, the sequence and timing of *fla* gene expression in the cell cycle are determined in large measure by the positions of these genes in the regulatory hierarchy. These results also suggest that periodic transcription is a general feature of *fla* gene expression in *C. crescentus*.

Molecular dissection of the programs controlling the sequential expression of developmental events is central to an understanding of cell differentiation. We have addressed this problem in the gram-negative bacterium *Caulobacter crescentus* in which the repeated asymmetric division of a mother stalked cell generates new motile swarmer cells by the stage-specific biosynthesis of a series of polar surface structures during the course of each cell cycle.

The major developmental event in formation of the swarmer cell type is flagellum biosynthesis that is initiated at one pole of the dividing stalked cell in mid S phase (DNA synthetic period) and continues during the remainder of the cell cycle. The flagellum begins to rotate shortly before cell division and then segregates with the motile swarmer cell at cell separation. Thus, both the time of flagellum formation and the position of its assembly on the cell surface are under strict developmental control (for reviews, see references 31, 32, and 48). Flagellum biosynthesis is regulated at the level of gene expression in *C. crescentus*, and the experiments described here were initiated to identify components of the developmental program that control the timing of flagellar gene expression.

Formation of the flagellum requires more than 40 flagellar (*fla*, *flb*, and *flg*) genes that are either scattered on the chromosome (6) or located in one of three clusters: the *flaYG* cluster (43), which contains three of the flagellin genes (23, 24); the hook gene cluster, which contains the hook protein gene *flaK* (36); and the *flbON* cluster, which contains basal body genes (9). The *fla* genes are organized in a regulatory hierarchy similar to that proposed previously for *Escherichia coli* by Komeda (14). Genes at each level of the *Caulobacter* hierarchy are required for expression of genes at the next lower level (Fig. 1; also see references 33 and 54), and genetic complementation has indicated that the hierarchy is mediated by a cascade of *trans*-acting regulatory factors (2, 40) with genes at different levels of the hierarchy containing characteristic arrays of promoters and regulatory sequences (27, 32, 35). Genes at the two lowest levels, including *flaN* and *flbG* of the hook gene cluster and the 25- (*flgK*) and 27-kDa (*flgL*) flagellin genes contain highly conserved Ntr/Nif-like promoter sequences at −24 and −12 that are recognized by a specialized RNA polymerase containing σ^{54} (24, 27, 35). In contrast, *flaO*, which is located above these genes in the regulatory cascade, has a promoter with a −10 sequence element similar to that recognized by the σ^{32} heat shock polymerase in *E. coli* (32, 53).

Previous studies of *Caulobacter fla* genes have shown that sequence related 25-, 27-, and 29-kDa flagellins (16, 41), the hook protein (50), and the corresponding mRNAs (17, 24, 37) are synthesized periodically in the cell cycle in the same order that the proteins are assembled in the flagellum. *flaYG* (13) and *flaD* (10) are also discontinuously expressed. Questions raised by these findings are whether periodic expression is a general feature of *fla* gene regulation in these bacteria and, more importantly, whether the organization of these genes in the regulatory hierarchy determines the sequence and timing of *fla* gene expression in the cell cycle or only coordinates the levels of flagellar protein synthesis with flagellum assembly, as proposed previously (1). To address these questions, we have examined the cell cycle control of the *flaN*, *flbG*, and *flaO* operons of the hook gene cluster and the role of *flaO*, which is near the top of the regulatory hierarchy, in the timing of the expression of *flbG*

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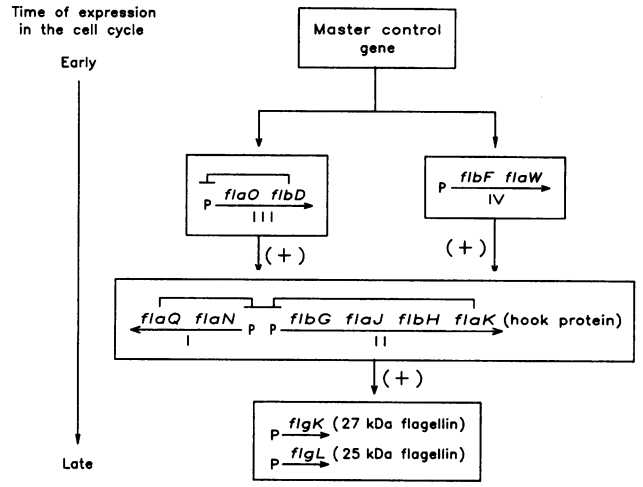

FIG. 1. Abbreviated regulatory hierarchy of *C. crescentus fla* genes. This model depicts the transcriptional control of operons in the hook gene cluster and flagellin genes *flgK* and *flgL* which are discussed in this paper. The bars above the promoters indicate negative regulation. The positions of *fla* genes in the *flaEY* cluster and the basal body cluster are considered in Newton et al. (33) and Xu et al. (54).

and other genes lower in the hierarchy. The results reported here suggest that periodic transcription is a general feature of *fla* gene expression in *C. crescentus* and that the position of these genes in the regulatory hierarchy may be sufficient to determine the sequence and timing of their expression in the cell cycle. We suggest that the transcriptional cascade mediated by the regulatory hierarchy may be initiated by DNA replication or some other cell cycle event.

MATERIALS AND METHODS

Strains and culture conditions. Strain CB15 (ATCC 19089) and the motility mutants of *C. crescentus* used in this study have been described previously (4, 40) and are listed in Table 1. The general procedure for constructing *rec* mutant strains has been described previously (38). Cells were routinely grown at 30°C in peptone-yeast extract (PYE) medium with appropriate antibiotics (40) or in M2 salts medium supplemented with 0.2% glucose for radiolabeling (40). Strain CB15F used in the synchrony experiments is a density variant of strain CB15 that can be synchronized by Ludox density gradient centrifugation (8). *E. coli* strains were grown at 37°C in ML medium (40).

TABLE 1. Bacterial strains

Strain	Genotype	Source or reference
CB15	Wild type	ATCC 19089
CB15F	Wild type, density variant	8
PC5511	*flaO172*::Tn*5*	40
PC5890	*flaO172*::Tn*5 rec-526*	This study
PC5895	*flbD198*::Tn*5 rec-526*	This study
PC5898	*flbF608*::Tn*5 rec-526*	This study
PC5974	*flaO172*::Tn*5*	φ(SC5511) × CB15F
PC5978	*flaO172*::Tn*5*/S385 Tc^r	PC5974(S385)
PC5983	*flaO172*::Tn*5*/S387 Tc^r	PC5974(S387)
PC7812	II.1::omega	This study
SC298	*flaK*::IS*298*	39, 40
SC511	*flaK*::IS*511*	39, 40
SC1032	*flbD198*::Tn*5*	40
SC1132	*flbF608*::Tn*5*	40
SC1140	*lac*::Tn*5*	7

Construction of plasmids. All plasmids used in complementation analysis are derivatives of pRK290 (5) in which *Caulobacter* DNA fragments were inserted at either the *Bgl*II or *Eco*RI site. S385 (see Fig. 3A) (40) was modified as follows. To obtain subclone S386 with a 4-bp addition at the *Hin*dIII site, plasmid S385 DNA was digested with *Hin*dIII and the staggered ends were filled by using Klenow fragments of *E. coli* DNA polymerase I and then religated. To generate the deletion of the *Bam*HI-*Hin*dIII fragment in S387, plasmid S385 DNA was digested with *Bam*HI and *Hin*dIII and ends were filled as described above to make the ends flush and then religated. The deletion truncates the II.1 gene and eliminates the promoter for the *flaO* operon (see Results). In the third variant S388, the omega cassette (42) was inserted at the *Hin*dIII site.

Construction of *lacZ* fusion vectors. Conventional plasmid vectors employing the *lacZ* gene in fusions to detect promoter activity generally carry ColE1-derived replicons. Since these plasmids are not maintained in *C. crescentus*, plasmid vectors with a promoterless *lacZ* gene were constructed for use in *C. crescentus* and other nonenteric bacteria (Fig. 2). The promoterless *lacZ* was obtained from pKM005 (21) by digesting the plasmid with *Sal*I, removing much of the *lacY* sequence by BAL 31 digestion, and then releasing the fragment containing the *lacZ* sequence from the vector by digesting with *Xba*I. The *lacZ* sequence was then ligated to pRK2L4 (26), a derivative of pRK290 (5) which carries multiple polylinker sequences from pUC18; this plasmid was digested with *Hin*dIII, the ends were filled in, and then the plasmid was digested with *Xba*I to generate sites for insertion of the *lacZ* gene fragment. This construction resulted in vector pANZ3 that contains multiple polylinkers, but with a unique *Pst*I cloning site (Fig. 2). The entire 5′ polylinker region extending into the amino-terminal *lacZ* coding sequence (E_a to E_b in Fig. 2) was sequenced to confirm the construction. pANZ4 was constructed by digesting pANZ3 with *Xba*I and religating to obtain a unique *Xba*I cloning site. pANZ4 was partially digested with *Kpn*I, incubated with Klenow fragments and four deoxynucleotides to generate blunt ends, and religated to obtain pANZ5 with unique *Kpn*I and *Xba*I cloning sites. Translational stop codons in two different frames between the cloning sites and the initiation codon of the *lacZ* gene are present in these vectors (21). Since one of these stop codons is in frame with the reading frame of the *lacZ* gene, there will be no in-frame fusions of the inserts to the *lacZ* gene. The promoter fragment and construction of the *flbGp-lacZ* fusion plasmid pANZ405 using pANZ3 have been described previously (33).

Construction of insertion mutant of II.1 unit. The 2.0-kb omega cassette (42) insertion mutant in transcription unit II.1 was constructed by the gene replacement method of Minnich et al. (25). The omega cassette was inserted at the *Bam*HI(d) site of pRK290 subclone S635, which carries the 4.7-kb *Eco*RI fragment (Fig. 3A). The *Eco*RI fragment with the insertion was then recloned in the suicide plasmid pSUP202 (51), and transferred to *C. crescentus*, and the transconjugants were selected for spectinomycin/streptomycin resistance (Spc^r/Str^r). To ensure that recombination had occurred in the hook gene cluster, a Tn*5* transposon linked to this region of the chromosome (3) was used to move the Spc^r/Str^r resistance marker from the original transconjugants into strain CB15 by the selection of Spc^r/Str^r and kanamycin

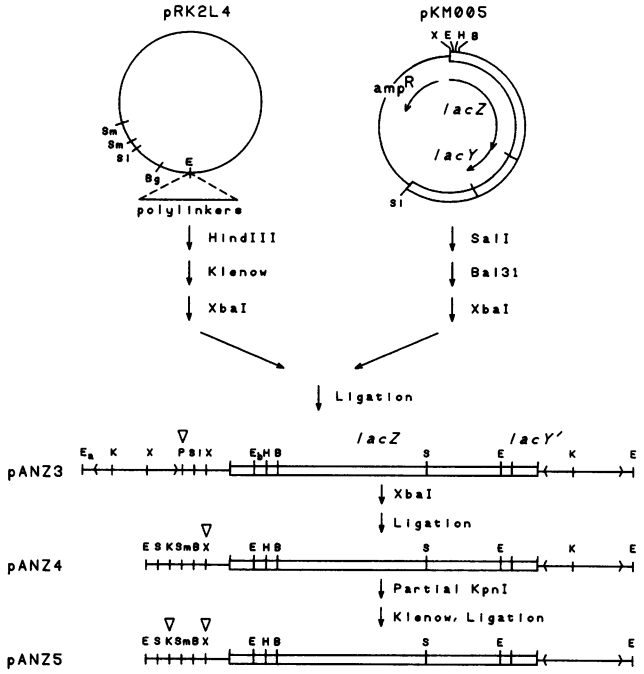

FIG. 2. Construction of *lacZ* transcription fusion vectors. Details are described in Materials and Methods. Arrowheads indicate cloning sites. Restriction site abbreviations: B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; K, *Kpn*I; E, *Eco*RI; Sl, *Sal*I; Sm, *Sma*I; S, *Sst*I; X, *Xba*I. Only relevant sites are shown, and some redundant sites are present within the parentheses.

doubly resistant transductants. The presence of the omega cassette in target DNA sequence was confirmed by Southern analysis of genomic DNA to show that the 4.7-kb *Eco*RI fragment in the wild-type strain was replaced by 6.7-kb *Eco*RI fragment in the mutants (data not shown).

Isolation of total cellular RNA and nuclease S1 analysis. RNA was purified from samples taken from a synchronous culture of *C. crescentus* CB15F by a hot sodium dodecyl sulfate-urea lysis procedure as described previously (37). Thirty micrograms of total cellular RNA from each time point, combined with yeast tRNA to make a total of 200 μg, was used in a nuclease S1 assay (37). Hybridization was at 65°C for 3 h.

Preparation of end-labeled DNA probes. DNA fragments for end labeling were prepared by restriction digestion of cloned DNA carrying the *C. crescentus* hook gene cluster region (37). After electrophoresis on an agarose (ultrapure grade; IBI) or polyacrylamide gel, the desired DNA fragments were excised and electroeluted (20). The purified DNA fragments were treated with alkaline phosphatase (Boehringer Mannheim) at 37°C for 30 min and were precipitated with 2 to 3 volumes of cold ethanol at −20°C after two phenol extractions. Finally, the DNA fragments were 5′ end labeled with [^{32}P]ATP and T4 polynucleotide kinase (22). The single-end-labeled DNA probes were obtained by cleaving the double-end-labeled DNA fragment at an internal site with a restriction enzyme and then repurifying the desired fragment as described above.

Radioimmune assay and quantification. Radioimmune assays for the rates of synthesis of the 25- and 27-kDa flagellins and hook protein were carried out on cultures that had been labeled for 9 min with [^{35}S]methionine (30 μCi/ml; Amersham) as described previously (41, 49, 50). In similar assays, β-galactosidase was immunoprecipitated with a monoclonal antibody (Promega). Synchronous cultures of swarmer cells obtained by Ludox density gradient centrifugation (8) were pulse-labeled at times indicated in Fig. 5 and in Fig. 7, and radioimmune assays were carried out as described above. Procedures used to visualize labeled proteins by autoradiography have been described previously (50). The results of radioimmune assays and S1 nuclease assays were quantified by tracing individual bands on autoradiograms with a Joyce-Loebel recording microdensitometer and determining the areas with a Zeiss MOPIII peak integrator (37).

RESULTS

***fla* gene regulatory hierarchy and plan of experiments.** The hook gene cluster of *C. crescentus* spans approximately 15 kb of genomic DNA (36), and as we show below, the *fla* genes contained in it are organized into four transcription units (Fig. 3A). Genetic studies have shown that genes in the *flaO* (transcription unit III) and *flbF* (transcription unit IV) operons are required in *trans* for the transcription of *flaN* (27, 33) and *flbG* operons (4, 33). This work has also shown that genes in *flaN* and *flbG* operons are required in turn for the expression of the 25-kDa flagellin gene (*flgK*) and 27-kDa flagellin gene (*flgL*). As outlined in Fig. 1 (24, 40), these genes are part of an extensive regulatory hierarchy (33, 54) in which gene activation is controlled by a cascade of transcriptional activators (44, 45).

In the experiments described below, we examined the pattern of *flaO*, *flaN*, and *flbG* transcription in the cell cycle and carried out experiments to determine whether the timing of *fla* gene expression in the cell cycle depends on the organization of these genes in the regulatory cascade (i.e., does the time of expression of genes at the top of the hierarchy determine the time at which genes lower in the hierarchy are expressed?). *flaO* is one of the genes near the top of the regulatory hierarchy, and our approach was to delay the time of its expression in the cell cycle and to measure the effect on *fla* genes lower in the hierarchy. We accomplished this by replacing the *flaO* promoter with the promoter of transcription unit II.1, which is expressed very late in the cell cycle (4). For these studies, it was necessary to define the location of the promoter or other upstream *cis*-acting sequence elements required for *flaO* expression and to characterize transcription unit II.1 in more detail.

Organization and expression of the *flaO* operon. Complementation experiments carried out originally in *rec*$^+$ strains indicated that the *flaO* and *flbD* genes were in separate transcription units (40). Nuclease S1 analyses, however, showed that this region contained only one transcription start site that mapped approximately 80 bp upstream of the *Hin*dIII site (27; Fig. 3). To determine whether this start site identified a promoter required in vivo for transcription of both *flaO* and *flbD*, an omega cassette that contains transcription terminators at both ends (42) was inserted at the *Hin*dIII site of subclone S385 to construct subclone S388 (Fig. 3A) and complementation analysis was carried out in *rec* mutant strains (Table 1). The ability of subclone S385 to complement *flaO172*::Tn*5* or *flbD198*::Tn*5* was completely lost as a result of inserting the omega cassette (Fig. 3A). This result indicated that the *flaO* and *flbD* genes are transcribed from the same, upstream *flaO* promoter (27) and that the genes are in the same operon now designated transcription unit III (Fig. 3A). Insertion of a 4-bp addition at the *Hin*dIII site which would cause a frameshift in the presumptive *flaO*

gene did not, however, interfere with complementation of the *flaO* or *flbD* mutations by S386, suggesting that translation of *flaO* is initiated to the right of the *Hin*dIII site.

We concluded from these results and previous complementation studies (40) that transcription unit III contains *flaO* and *flbD* and, as indicated in Fig. 3A, that *fla* genes in the hook cluster are organized into four transcription units instead of five as originally reported: *flaN* (transcription unit I), *flbG* (transcription unit II), *flaO* (transcription unit III), and *flbF* (transcription unit IV).

Temporal regulation of the *flaN*, *flbG*, and *flaO* operons. Cell cycle regulation of the divergent *flaN* and *flbG* transcription units was examined by nuclease S1 assays of RNA isolated from synchronous cultures of strain CB15F (Materials and Methods). The single-end-labeled DNA fragment a was used to probe for the *flaN* transcript and the single-end-labeled DNA fragment b was used to probe for the *flbG* transcript (Fig. 3B). Consistent with the positions of start sites mapped previously, a partially protected fragment of 415 nucleotides was detected from the *flaN* promoter (27) and fragments of 780, 760, and 730 nucleotides were detected from the *flbG* promoter (4). The *flaN* and *flbG* transcripts accumulated from 130 to 210 min in the cell cycle, with the maximum level present at approximately 165 min (Fig. 4A and B). Thus, the pattern of *flbG* expression was similar to that described by Ohta et al. (37), and it was almost coincident with that measured here for *flaN*, which has not been reported previously (Fig. 3D). Additional evidence for the coordinate regulation of *flaN* and *flbG* are the observations that both transcription units require genes in the *flaO* and *flbF* operons in *trans* for their activation and that genes that negatively autoregulate one of the transcription units also exert negative controls over the other (Fig. 1) (33).

The regulation of *flbG* in the cell cycle was also examined by using transcription fusion plasmid pANZ405 that contains the *flbG* promoter fused to *lacZ* (33). The rate of β-galactosidase synthesis driven by the *flbG* promoter (Fig. 5) displayed a periodicity comparable with that determined by nuclease S1 assay shown in Fig. 4. This result indicates that *flbG* is transcriptionally controlled and that the pattern of gene expression is conferred by the 5′ regulatory sequences, a conclusion supported by site-directed mutagenesis studies (28).

Levels of *flaO* transcript were measured by using the 520-bp *Bam*HI-*Sst*I fragment d as the probe (Fig. 3B). The *flaO* mRNA appeared earlier in the cell cycle than the *flaN* and *flbG* transcripts, with a maximum level detected at approximately 150 min (Fig. 4C). This result was confirmed by experiments in which RNA samples from another set of synchronous cell cultures were assayed with both the *flaO* and *flbG* probes present in the same assay mixtures; consistent with the results shown in Fig. 4, the *flaO* transcript appeared 20 to 30 min earlier than the *flbG* transcript (data not shown). Thus, the sequence in which *flaO* and *flaN* and *flbG* are expressed in the cell cycle corresponds to their positions in the regulatory hierarchy. This result suggested that the organization of *fla* genes in the regulatory cascade may be sufficient to determine the sequence of their expression.

Identification and characterization of transcription unit II.1. The promoter of transcription unit II.1 located between the end of the *flbG* operon and the 5′ end of the *flaO* operon was originally identified on the basis of genetic complementation. Although subclone S1397 contains the 0.67- and 2.3-kb *Bam*HI DNA fragments with the *flaK* gene, it does not complement *flaK144* or *flaK155* because it lacks the *flbG*

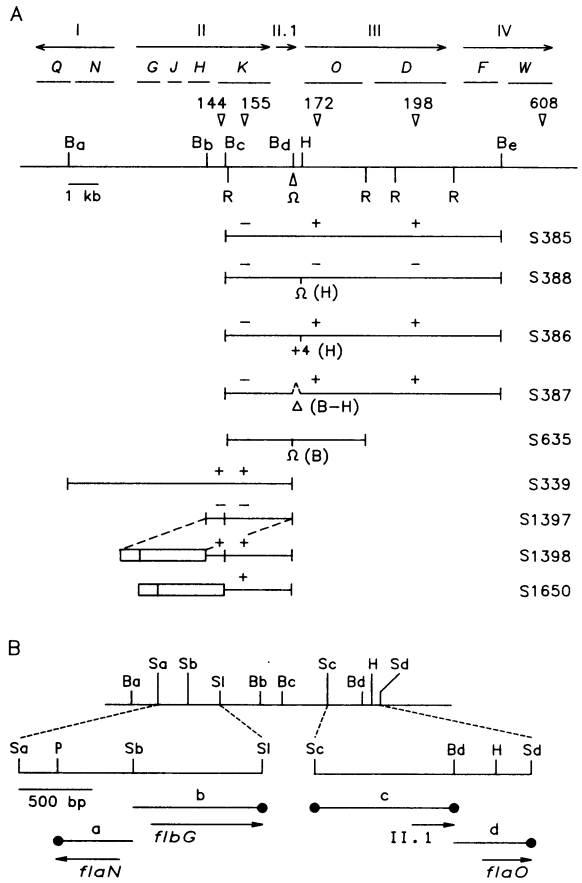

FIG. 3. Definition of transcription units and start sites in hook gene cluster. (A) Restriction map and genetic complementation. Transcription units defined are represented by roman numerals I to IV at the top. Genes *flaQ*, *flaN*, *flbG*, *flaJ*, *flbH*, *flaK*, *flaO*, *flbD*, *flbF*, and *flaW* are indicated by capital letters on the second line. Positions of insertion mutations described in Table 1 are shown by arrowheads. DNA restriction fragments subcloned in pRK290 and their derivatives are shown under the restriction map. Symbols indicate the ability (+ [good motility]) or inability (− [no motility]) of a given subclone to complement a mutation for motility as assayed in swarm plates; Ω, omega cassette; +4, 4-bp insertion; Δ, deletion of the *Bam*HI(d)-*Hin*dIII fragment. (B) Probes for nuclease S1 analysis. The transcription start sites have been determined at the sites indicated in *flaN* and *flaO* (27) and *flbG* (4). The ends labeled with ^{32}P are indicated (●). For restriction site abbreviations, see the legend to Fig. 2, except for *Eco*RI (R) and *Pvu*II (P).

promoter present in the longer subclone S339 (Fig. 3A; 40); the merodiploid strains containing plasmid S1397 were not motile and low levels of flagellins and hook protein were detected by radioimmune assay (Fig. 6A and B, lanes 1 and 2). By contrast, subclone S1398, which contains a tandem repeat of the 0.67- and 2.3-kb *Bam*HI fragments, complemented the same mutations for motility and directed the synthesis of high levels of hook protein and flagellins (Fig. 6A and B, lanes 3 and 4), as did subclone S1650 from which the central 0.67-kb *Bam*HI fragment had been removed (data not shown). These results suggested that the 2.3-kb *Bam*HI fragment contains a promoter in S1398 and S1650 that drives normal transcription of *flaK*. The transcription start site of pII.1 is located 300 bp upstream from the *Bam*HI(d) site (4), and recent findings have shown that transcription from the

II.1 promoter is terminated approximately 71 bp upstream from the *flaO* promoter (29). Nuclease S1 assays have shown that expression of II.1 is initiated very late during the G_2 period and that it continues in the new swarmer cell after cell division (Fig. 4D).

Gene disruption experiments were used to determine whether II.1 is required for flagellum biosynthesis and function. A disruption mutation was made by the insertion of the omega cassette (42) at the *Bam*HI(d) site by a double crossover between the chromosome of strain CB15 and the corresponding cloned DNA fragment in S635 (Fig. 3A; Materials and Methods). Motility of the resulting insertion mutant PC7812 was indistinguishable from the wild-type parent strain, and radioimmune assays showed that the mutant synthesized the hook protein (Fig. 6A, lane 7) and flagellins (Fig. 6B, lane 7) at the levels identical to that of wild-type strain CB15 (Fig. 6A and B, lane 8). We conclude from these results that II.1 is not essential for *fla* gene function.

The effect of mutations in the *flaO* and *flbF* operons on expression from the II.1 promoter was also examined by assaying hook protein synthesis directed by S1398. Tn*5* insertions in *flbD* (*flbD198*::Tn*5*) or in *flbF* (*flbF608*::Tn*5*) did not affect the levels of hook protein synthesis (Fig. 6A, lanes 5 and 6, respectively). This result confirms earlier reports that transcription from II.1 promoter as determined by S1 nuclease assays was not under *flaO* or *flbF* operon control. Synthesis of the 25- and 27-kDa flagellins were not detected in these strains (Fig. 6B, lanes 5 and 6, respectively), however, because flagellin gene expression requires several genes in the *flaN* and *flbG* operons in addition to hook protein gene *flaK* (40, 49) and neither *flaN* or *flbG* is expressed in *flaO* or *flbF* mutants (33). The unusually late timing of promoter pII.1 expression in the cell cycle (Fig. 4D), the independence of its activity from genes in the *fla* gene hierarchy, and its physical location 5′ to the *flaO* operon make the II.1 promoter convenient for the transcription fusion experiments described in the next section.

Effect of delaying *flaO* transcription on *fla* gene expression. If the timing of *fla* gene expression is determined by a gene's position in the regulatory hierarchy, then artificially delaying *flaO* transcription should also delay the activation of genes below it in the hierarchy. To test this hypothesis, *flaO* and *flbD* were fused to the II.1 promoter by removing the *Bam*HI(d)-*Hin*dIII DNA fragment from subclone S385 to generate subclone S387 (Fig. 3A). The DNA fragment removed contains the *flaO* promoter (see above), and the ability of the subclone S387 to complement *flaO* and *flbD* mutations and to restore a fully wild-type phenotype indicated that genes in the *flaO* transcription unit are driven by the II.1 promoter (data not shown).

Plasmid S385 containing the intact *flaO* transcription unit and derivative S387 that contains the II.1 promoter fusion to *flaO* were introduced into a synchronizable strain with the *flaO* mutation *flaO172*::Tn*5*. Products of the *flaO* operon are synthesized in these merodiploid strains only from the plasmid-encoded genes, because the Tn*5* insertion mutation in *flaO* has a polar effect on downstream genes. *flbG* expression in the synchronous cell cultures was determined by measuring the rate of hook protein synthesis by radioimmune assay; the rates of 25- and 27-kDa flagellin synthesis were also determined in the same cell extracts by radioimmune assay. In strain PC5978 carrying S385, the timing of hook protein and flagellin synthesis (Fig. 7A) was identical to that described previously in wild-type cells (50). In strain PC5983 carrying plasmid S387 with *flaO* fused to the II.1 promoter, however, hook protein synthesis was delayed by 40 to 50 min and it continued in the new swarmer cell (Fig. 7b) instead of terminating before cell division, as observed in strain PC5978. This pattern of hook protein synthesis from subclone S387 corresponded closely to that observed for mRNA synthesis from transcription unit II.1 (Fig. 4D).

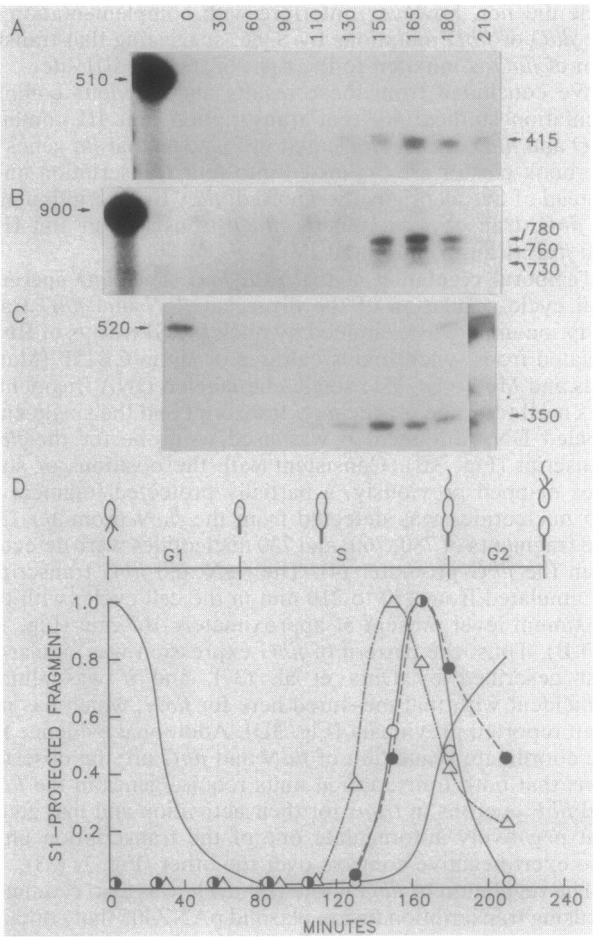

FIG. 4. Sequential expression of *flaO*, *flaN*, and *flbG* in cell cycle. Cultures of strain CB15F were synchronized by density gradient centrifugation, cell samples were taken at the times indicated, and RNA was purified from these aliquots (see Materials and Methods). The RNA preparations were used in nuclease S1 assays with the three DNA probes shown in Fig. 3; *flaN* (A), *flbG* (B), and *flaO* (C). The numbers at the sides of the autoradiograms indicate the fragment sizes in nucleotides. Autoradiograms were quantified as described previously (37), and values were plotted after normalizing the peak amounts to 1 (D). The levels of RNA accumulation from transcription unit II.1 (solid line without symbols), which was quantified by using probe c shown in Fig. 3, is taken from Chen et al. (4). Symbols: ●, *flaN*; ○, *flbG*; △, *flaO*. The stages of the cell cycle were monitored by light microscopy and by incorporation of [^{3}H]guanosine in DNA as described by Osley et al. (41).

Fusion of *flaO* to the II.1 promoter resulted in a comparable delay in the synthesis of the 27- and the 25-kDa flagellins (Fig. 7b). Synthesis of the 27-kDa flagellin also continued in the new swarmer cell, which is never observed in wild-type strains (24, 41). The delayed synthesis of flagellin in this strain would be expected because the *flgK* and *flgL* genes are at the bottom of the regulatory hierarchy and their activation depends on the *flaN* and *flbG* operons,

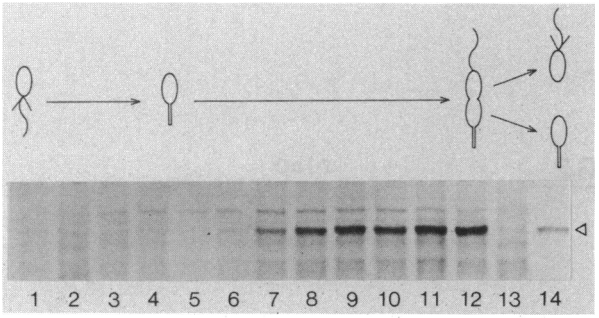

FIG. 5. Periodic cell cycle expression of β-galactosidase from *flbGp-lacZ* transcription fusion plasmid pANZ405. Synchronous swarmer cells of *C. crescentus* CB15F(pANZ405) were pulse-labeled with [^{35}S]methionine for 10 min at intervals during the cell cycle, and the amount of β-galactosidase synthesized was measured by radioimmune assay, using monoclonal anti-β-galactosidase (Promega). The autoradiogram is shown in the lower part of the figure. Lanes (with time in minutes): 1, 20; 2, 40; 3, 60; 4, 80; 5, 95; 6, 110; 7, 120; 8, 130; 9, 140; 10, 150; 11, 160; 12, 175; and 13, 190. Lane 14 represents β-galactosidase made in SC1140 from the induced intact *E. coli lacZ* gene carried on a pRK290 derivative. Cell development and division were monitored by light microscopy and recorded as indicated by cell types shown above the autoradiogram; cell division occurred at approximately 190 min.

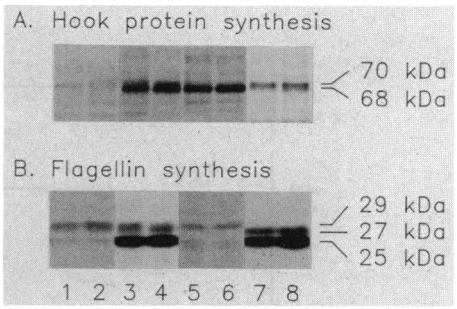

FIG. 6. Flagellar protein synthesis directed by hybrid plasmids S1397 and S1398 and the effect of disrupting transcription unit II.1. Hook protein (A) and flagellin (B) synthesis were assayed by radioimmune assays (Materials and Methods). The 70-kDa protein is the hook protein subunit assembled in the hook structure, and the 68-kDa protein is the unassembled, precursor species (37). Abnormally high levels of the 68-kDa precursor are synthesized in strains that carry extra copies of the functional *flaK* on a plasmid such as S1398 (37). Lanes: 1, SC511(S1397); 2, SC298(S1397); 3, SC511 (S1398); 4, SC298(S1398); 5, SC1032(S1398); 6, SC1132(S1398); 7, PC7812(II.1::omega); 8, wild-type strain CB15.

as well as on *flaO* (Fig. 1). We conclude from these results that the time of *flaO* transcription determines the timing of expression of genes at lower levels of the hierarchy, including *flbG* and the 25- and 27-kDa flagellin genes. Delaying *flaO* expression also resulted in changes in the relative levels and patterns of hook protein and flagellin synthesis that are considered more fully below.

DISCUSSION

An extensive body of work in *C. crescentus* indicates that flagellum biosynthesis is controlled at the level of gene expression. In the experiments reported here, we have examined the timing of *fla* gene expression and the extent to which the organization of these genes in a regulatory cascade can account for the observed temporal patterns. Our results show that *flaO* (transcription unit III) and *flaN* (transcription unit I), which have not been examined previously in synchronous cultures, are periodically expressed (Fig. 4). These studies have also confirmed that *flbG* (transcription unit II) is periodically transcribed (Fig. 5), that it is regulated coordinately with *flaN*, and that *flaN* and *flbG* are turned on after *flaO* in the cell cycle (Fig. 4). Interestingly, recent nucleotide sequence analysis (30) has shown that the first gene in *flaN* operon is homologous to *flgK* of enteric bacteria which codes for HAP1, hook-associated protein 1 (12). Thus, genes in both the *flaN* and *flbG* operons appear to be important for assembly of normal hook structure; e.g., *flaK*, the last gene of the *flbG* operon, encodes the hook protein (37), and mutations in *flaJ* gene result in a polyhook structure (49).

In addition to the *flaN*, *flbG*, and *flaO* operons examined here, recent experiments indicate that *flbF* (transcription unit IV) is also under cell cycle control (45). These results, together with similar reports on the regulation of flagellin genes *flgJ*, *flgK*, *flgL* (24), *flaEY* (13), and *flaD* (10), strongly suggest that periodic transcription is a general feature of *fla* gene regulation in *C. crescentus*.

The observed sequence of *flaO*, *flbG*, and *flaN* expression in the cell cycle (Fig. 4) corresponds to their relative positions in the regulatory hierarchy (Fig. 1); thus, *flaO*, which is near the top of the hierarchy is expressed relatively early in the cell cycle and can in principle determine the time at which *flaN*, *flbG*, and other genes lower in the hierarchy are expressed. This model for cell cycle control is supported by the finding that delaying *flaO* transcription also delayed expression of the *flbG* operon and the 25- and 27-kDa flagellin genes (Fig. 7), which are at the bottom of the regulatory cascade and whose activation depends in turn on *flbG* and *flaN* (Fig. 1). The requirement of a number of genes for proper regulation of *flbG* and transcription units lower in the regulatory hierarchy could be responsible for several additional changes in the observed patterns of hook protein and flagellin synthesis when *flaO* transcription was delayed (Fig. 7). First, the level of hook protein synthesis was higher after cell division in swarmer cells than in predivisional cells. Second, hook protein synthesis was delayed more than flagellin synthesis, which resulted in the almost coincident expression of these genes in the II.1 promoter-*flaO* fusion strain. It is difficult to speculate on reasons for these altered patterns. These results do demonstrate, however, that the regulatory hierarchy is an integral component of the mechanism controlling the timing of *fla* gene expression in *C. crescentus*. The experiments shown also show that the normal timing of all *fla* genes in the *Caulobacter* cell cycle is not required for normal flagellum formation and function (Fig. 7 and complementation results in Fig. 3A).

FlbD, the product of the last gene in the *flaO* operon, has been identified as a regulatory protein that activates *flbG* transcription (44), presumably by binding to a *cis*-acting sequence needed for transcription from the *flbG* promoter. One of these sequence elements, *ftr1*, is located approximately 100 bp upstream from the transcription start site of *flbG*, and site-directed mutagenesis has shown that it is required for activation of *flbG* (28). FlbD is a homolog of the *E. coli* protein NtrC (44, 52) that activates transcription from promoters recognized by the specialized σ^{54} RNA polymerase (for a recent review, see reference 15). In *C. crescentus* this gene is also required for expression of *flaN* and flagellin genes *flgJ* and *flgK* (33, 40). These three genes share with *flbG* the σ^{54} consensus promoter sequences at −12 and −24 and an adjacent *ftr* sequence element (24, 27, 28, 35). Thus it

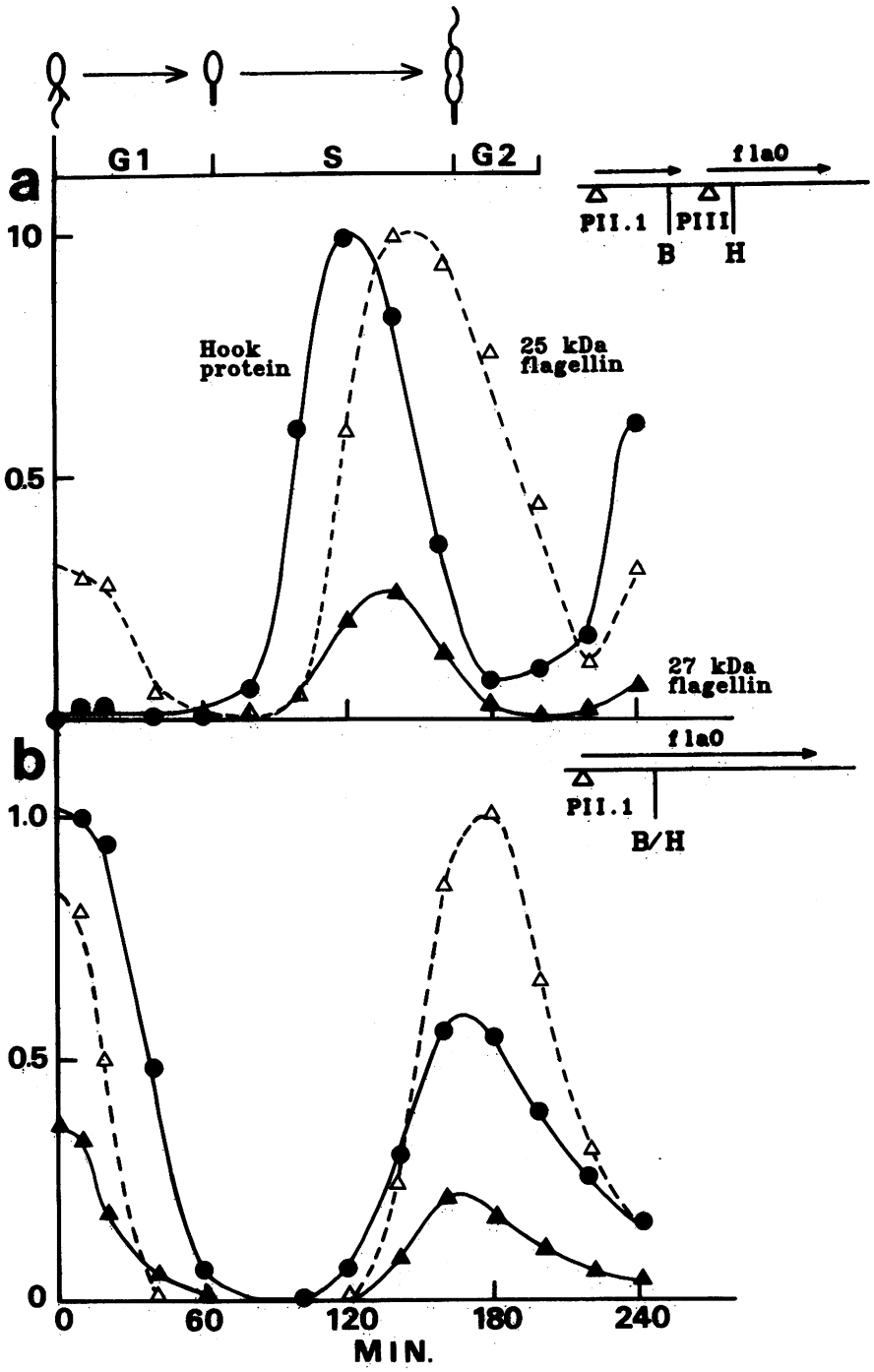

FIG. 7. Effect of delaying *flaO* expression on timing of hook protein and flagellin synthesis. The strains used in both experiments were constructed from the synchronizable parent strain PC5974 that contains *flaO172*::Tn*5* mutation (Table 1); this strain does not synthesize hook protein or the 25- and 27-kDa flagellins (37, 40). The synchrony experiments shown were carried out with motile, merodiploid strains that carried either hybrid plasmid S385 (Fig. 3) that contains the intact II.1 and *flaO* promoters (a) or hybrid plasmid S387 (Fig. 3) in which the *flaO* promoter has been replaced by the II.1 promoter (b). The relevant portion of each plasmid construct is diagrammed in the corresponding panel. Rates of flagellar protein synthesis were determined by radioimmune assays as described in Materials and Methods. The maximum levels of 70-kDa hook protein and 25-kDa flagellin were each adjusted to 1.0, and the level of 27-kDa flagellin was normalized to the level of 25-kDa flagellin.

is easy to visualize how the periodic transcription of the operon containing *flbD* can determine the timed expression of *flbG*, *flaN*, and other transcription units lower in the cascade (Fig. 1).

Although this model offers a framework for understanding the temporal regulation of these *fla* genes, *flbD* is only one of several genes needed for expression of *flbG* and *flaN*. Other genes in the *flaO* and *flbF* operons, (transcription units III

and IV; Fig. 3A), as well as *flbO* and *flaS* (33, 54) are also required. The function of these genes has not been determined, but they could encode the σ^{54} subunit, auxiliary transcription factors, or a protein kinase that modulates FlbD activity. In *E. coli*, NtrC is phosphorylated by NtrB and phospho-NtrC is the transcriptional activator of the σ^{54} promoter $glnAp_2$ (34). FlbD is not phosphorylated by *Salmonella typhimurium* phospho-CheA under conditions when CheY or NtrC is phosphorylated (46) and if FlbD is modified it may be that a specialized protein kinase is required.

We speculate that the regulatory hierarchy in *C. crescentus* functions in two ways to control flagellum biosynthesis. First, it may act to coordinate the level of *fla* gene expression with flagellum assembly, as proposed for the enteric bacteria (reviewed in reference 19). In *C. crescentus*, the level of *fla* gene expression is controlled by regulatory circuits that are negative as well as positive (33, 54). Second, the regulatory cascade would determine the sequence in which the *fla* genes are activated, as illustrated by the regulatory coupling between the timing of *flaO* and *flbG* transcription shown in Fig. 7. Both *flaO* (Fig. 4) and *flbF* (45) are periodically expressed relatively early in the cell cycle, and it has been suggested that the signal required for their transcription would initiate the regulatory cascade and act as the cell cycle clock (37). Genes above *flaO* and *flbF* in the hierarchy have yet to be identified (33).

What is the cell cycle clock? Previous work has shown that the hook protein and flagellins are not synthesized when DNA replication is blocked (50). Thus, one candidate for the cell cycle event that triggers the *fla* gene cascade is DNA replication (discussed in references 31 and 37). A requirement of DNA synthesis for gene expression has also been demonstrated for other procaryotic systems. One of the best studied is bacteriophage T4 in which efficient expression of late genes requires three of the T4 DNA polymerase accessory proteins that apparently act as mobile enhancer-binding proteins (11). Another system where DNA synthesis is implicated is transposon Tn*10* in which transcription of the transposase gene occurs efficiently only from the hemimethylated gene formed after replication (47).

The role of DNA synthesis in gene expression in *C. crescentus* has not been elucidated. Several experiments suggest, however, that replication of the *fla* genes is not sufficient for their expression. The *flbG* promoter was expressed with normal timing in synchronous cells when the operon was carried on a plasmid that replicated throughout most of the cell cycle, even when the plasmid construct also carried the adjacent *flaO* and *flbF* operons (37). The results presented above (Fig. 7) also appear to rule out a timer outside of the regulatory hierarchy whose signal is transient. We base this conclusion on the observation that *flbG* expression can be delayed, presumably by delaying the time when a transcriptional activator(s) is synthesized, until after the time at which the gene is replicated (18) and normally expressed in the cell cycle (Fig. 7). Thus, replicating DNA cannot be required for transcription from the *flbG* promoter.

The *fla* gene hierarchy in *E. coli* and *S. typhimurium* is regulated in response to nutritional conditions. Master control genes at the top of the regulatory hierarchy are transcribed from cyclic AMP/CAP-regulated promoters (for a review, see reference 19). In *C. crescentus*, genes at the top of the hierarchy may be tied instead to a cell cycle signal that initiates the regulatory cascade and sequential expression of *fla* genes. It remains to be seen whether DNA replication acts directly at genes at the top of this cascade.

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