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Genetic switching in the flagellar gene hierarchy of *Caulobacter* requires negative as well as positive regulation of transcription

(*Caulobacter crescentus*/hook gene cluster/negative autoregulation/*lac* fusion vectors)

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ABSTRACT ***Caulobacter crescentus*** **flagellar (*fla, flb,* or *flg*) genes are periodically expressed in the cell cycle and they are organized in a regulatory hierarchy. We have analyzed the genetic interactions required for *fla* gene expression by determining the effect of mutations in 30 known *fla* genes on transcription from four operons in the hook gene cluster. These results show that the *flaO* (transcription unit III) and *flbF* (transcription unit IV) operons are located at or near the top of the hierarchy. They also reveal an extensive network of negative transcriptional controls that are superimposed on the positive regulatory cascade described previously. The strong negative autoregulation observed for the *flaN* (transcription unit I), *flbG* (transcription unit II), and *flaO* (transcription unit III) promoters provides one possible mechanism for turning off *fla* gene expression at the end of the respective synthetic periods. We suggest that these positive and negative transcriptional interactions are components of genetic switches that determine the sequence in which *fla* genes are turned on and off in the *C. crescentus* cell cycle.**

Cell differentiation in *Caulobacter crescentus* results from the repeated asymmetric division of a stalked cell to produce the parent stalked cell plus a new, motile swarmer cell. The flagellum is the most prominent and best-studied of several polar structures that characterize the newly differentiated swarmer cell. Understanding the biosynthesis of this complex organelle in *C. crescentus* is a challenging problem in morphogenesis and gene regulation, requiring the products of at least 30 flagellar (*fla, flb,* or *flg*) genes and spatial information for targeting the subunits to one of the cell poles. In addition, there is a temporal component of regulation, since flagellum biosynthesis, like other developmental events in *C. crescentus*, is stage specific in the cell cycle (see refs. 1 and 2 for reviews).

The *C. crescentus* genes encoding flagellar subunits that have been examined are periodically expressed in the cell cycle, generally at times of gene product assembly. Thus, the synthesis of the 70-kDa hook protein precedes that of the 27- and 25-kDa flagellins (3–5) and the 29-, 27-, and 25-kDa flagellin gene transcripts appear in the same order that the protein products are assembled into the flagellar filament (6); the *flaD* transcript, which may encode one of the basal body ring subunits, appears earlier in the cell cycle (7).

A major question in *C. crescentus* development is how the complex temporal pattern of periodic *fla* gene expression is programmed in the cell cycle and coordinated with flagellum morphogenesis. A number of results indicate that the *fla* genes are organized in a regulatory hierarchy similar to that described in *Escherichia coli* (8) and that the expression of these genes in *C. crescentus* is controlled by a cascade of positive transcriptional activators (1, 9–12). Recent studies have shown that *flbG* (hook operon; see refs. 5 and 13) and *flaN* (14), two transcription units in the hook gene cluster, are also subject to negative regulation. To assess the extent of negative and positive controls in regulating the *fla* genes we have undertaken a systematic analysis of the effect of mutations in all identified *fla* genes on transcription of the four operons in the hook gene cluster (see Fig. 1*A*). Our results suggest that the positive and negative transcription controls described here may serve two related regulatory functions, one of coordinating the level of *fla* gene expression with flagellum biosynthesis, and the other of acting as genetic switches that determine the sequence in which *fla* genes are turned on and off in the cell cycle.

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MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. *C. crescentus* strain CB15 (ATCC 19089) and the *fla* mutants used in these studies are listed in Table 1 and in the legend to Fig. 1. Cells were grown at 30°C on peptone/yeast extract medium (3).

Nuclease S1 Assays. RNA isolation and nuclease S1 assays were performed as described (9). Hybridizations were at 55°C with the 285-base-pair (bp) *Bam*HI/*Hin*dIII *flaO* probe and at 62°C with the 950-bp *Pvu* II/*Sal* I *flbF* probe. Protected fragments were analyzed by electrophoresis on 5% and 4% polyacrylamide/8 M urea gels for *flaOp* and *flbFp*, respectively.

lacZ **Fusion Vectors.** The promoter fusion vectors were constructed by inserting the promoterless *lacZ* gene from pKM005 (15) into plasmid pRK2L4, a derivative of broad host range plasmid pRK290 (16). The two resulting fusion vectors contain a unique *Pst* I cloning site (pANZ3) and unique *Kpn* I and *Xba* I cloning sites (pANZ5). Details of the construction are discussed elsewhere (N.O., L.-S. Chen, D.M., and A.N., unpublished data).

Construction of *fla* Promoter–*lac* Fusions. flaNp–, flbGp–lac *fusions*. The fragment containing the divergent *flbG* and *flaN* promoters (14) for transcription units I and II, respectively, was obtained from an M13 phage clone that had been generated by BAL-31 digestion of the 3.7-kilobase (kb) *Bam*HI(a) *Bam*HI(b) fragment from the 3′ end (D.M. and A.N., unpublished data). The deletion end point is 1780 bp from the *Bam*HI(a) site and bounded by the *Pst* I site in the polylinker of the phage vector. *Pst* I digestion yielded a 605-bp fragment (Fig. 1*A*), which was cloned in both orientations in pANZ3 to generate pANZ404 (*flaNp–lac*) and pANZ405 (*flbGp–lac*).

flaOp–lac *fusion*. The 285-bp *Bam*HI/*Hin*dIII fragment containing the *flaO* (transcription unit III) promoter (Fig. 1*A*) was first cloned into pUC18 at the *Bam*HI and *Hin*dIII sites to yield pNJ8 (17). pNJ8 was digested with *Hin*dIII, the ends

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were filled in with Klenow, *Xba* I linkers ligated, and then digested at the *Kpn* I site, which was present in the pUC18 DNA, to release the *flaO* promoter-containing fragment. This fragment was ligated into pANZ5 to generate fusion plasmid pANZ406.

flbFp–lac *fusion.* pGIR113 is a pUC18 derivative carrying a 1050-bp *Sac* II/*Sal* I fragment of transcription unit IV (Fig. 1*A*) with the *flbF* promoter between *Kpn* I and *Xba* I sites (G.R. and A.N., unpublished data). The *Kpn* I/*Xba* I fragment was ligated in to pANZ5 to yield the *flbFp–lacZ* fusion pANZ118.

In the *flbGp–lacZ* and *flaOp–lacZ* fusions described above, the promoter fragments were joined to *lacZ* before the translational start sites of respective genes. In the case of the *flaNp* fusion, the two possible reading frames that originate within the fragment (D.M. and A.N., unpublished data) are in-frame with translation stop codons in the vector sequence. Sequence information is not complete for the *flbFp–lacZ* fusion. We have assumed that the plasmid copy number in the strains examined is constant since the strains are generally isogenic except for the *fla* mutation.

RESULTS

Regulation of *flaO* and *flbF* Messenger RNA. Eight of the *fla* genes identified in *C. crescentus* are scattered on the chromosome, while the remaining 23 or so genes map to the hook gene cluster, the *flaEY* cluster, or the basal body gene cluster (see refs. 1 and 18). The hook gene cluster is organized into the *flaN, flbG, flaO,* and *flbF* operons (transcription units I–IV) and transcription unit II.1, whose function is unknown (Fig. 1*A*; refs. 12 and 13; N.O., unpublished data). We have previously mapped the transcription start sites, determined the nucleotide sequences of the promoters (refs. 13 and 14; G.R. and A.N., unpublished data), and shown that these transcription units are part of a trans-acting regulatory cascade in which *flaN* and *flbG* are under positive control by *flaO* and *flbF* (13, 14). The *flaN* and *flbG* operons were also shown to be subject to negative regulation by the hook protein structural gene *flaK* (13, 14). To identify genes that regulate *flaO* and *flbF* expression, we determined the effect of all identified *fla* mutations on the levels of *flaO* and *flbF* messenger RNA using a nuclease S1 assay with the DNA probes shown in Fig. 1*A*.

flaO *expression.* The level of *flaO* expression, as measured by the partially protected 80-base fragment (Fig. 1*A*), was at least 20-fold higher in strains with Tn*5* insertions in *flaO* and *flbD* (Fig. 1*B*, lanes d–i) than in wild-type strain CB15 (lane c). This result implies that *flaO* is subject to strong negative autoregulation. *flaO* is also under negative regulation by genes in the *flbF* transcription unit: Tn*5* insertions in *flbF* (lanes k and l) or a point mutation in *flaW* (lane m) resulted in *flaO* messenger RNA levels ≈5-fold higher than that in strain CB15.

The regulatory effect of genes outside the hook cluster, including the basal body genes *flbN* and *flbO* (19), was also examined. A Tn*5* insertion in *flbN* had no effect on *flaO* expression (Fig. 1*B*, lane n), while mutations in *flbO* and the unlinked *flaS* locus resulted in a 5-fold increased level of *flaO* RNA (lanes o and p). These results showed that, in addition to negative autoregulation, *flaO* expression is also negatively modulated by *flaW*, *flbO*, and *flaS*.

flbF *expression.* *flbF* messenger RNA is present at very low levels in strain CB15, as visualized by the protection of the 540-base fragment (Fig. 1*C*, lane c). The level of messenger was not affected by any of the Tn*5* insertions (lanes d–i) or the point mutation (lane j) in the *flaO* operon or by mutations in the *flaW* gene of the *flbF* operon (lane k). None of the *fla* mutations, including those in the basal body cluster genes and in *flaS*, affected the level of *flbF* RNA (lanes l–n). The regulatory interactions controlling the *flaO* and *flbF* promoters were explored more fully by using transcription fusions, as described below.

Transcription Fusions to *lacZ*. To furnish a direct measure of promoter activity *lacZ*, fusion vectors pANZ3 and pANZ5 were constructed. The promoters and controlling elements of *flaN, flbG, flaO,* and *flbF* were inserted into these vectors to generate plasmids pANZ404, pANZ405, pANZ406, and pANZ118, respectively, and transferred to different *fla* gene mutants of *C. crescentus* by conjugation and selection for the

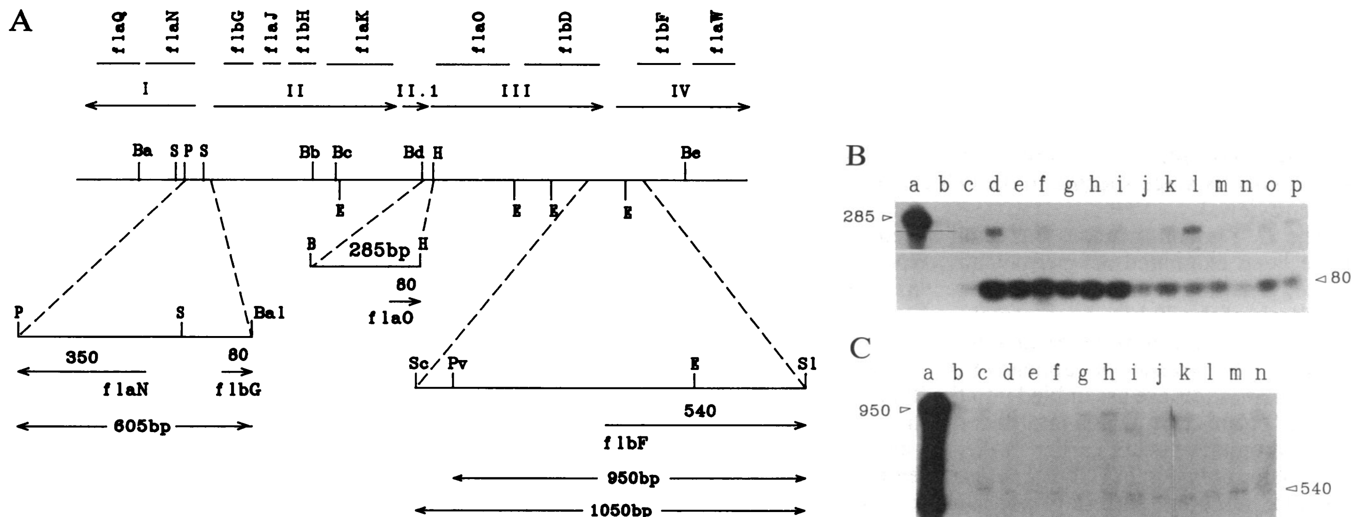

FIG. 1. Restriction map of the hook gene cluster and expression of *flaO* and *flbF* determined by nuclease S1 assay. (*A*) Gene designations are in the top line and arrows below transcription units I–IV indicate the direction of transcription (refs. 12–14; G.R. and A.N., unpublished data). DNA fragments indicated below the map were used as probes in nuclease S1 assays and in the construction of the transcription fusions to *lacZ*. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst* I; Pv, *Pvu* II; S, *Sst* I; Sc, *Sac* II; S1, *Sal* I. Bal represents BAL-31 deletion end point. (*B* and *C*) Nuclease S1 was present in all reactions except in lanes a. One hundred micrograms of tRNA or 100 μg of RNA from the strain indicated was present in the reactions. (*B*) *flaO* (transcription unit III) 285-bp probe. Lanes: a and b, tRNA; c, CB15; d, PC5511 (*flaO172*::Tn*5*); e, PC5520 (*flaO169*::Tn*5*); f, PC5504 (*flaO607*::Tn*5*); g, PC5509 (*flaO188*::Tn*5*); h, PC5510 (*flbD198*::Tn*5*); i, PC5512 (*flbD609*::Tn*5*); j, SC290 (*flaO138*); k, PC5515 (*flbF177*::Tn*5*); l, PC5516 (*flbF608*::Tn*5*); m, SC259 (*flaW120*); n, SC1117 (*flbN174*)::Tn*5*); o, SC1131 (*flbO196*::Tn*5*); p, SC508 (*flaS153*). (*C*) *flbF* (transcription unit IV) 950-bp probe. Lanes: a and b, tRNA; c, CB15; d, PC5511; e, PC5520; f, PC5504; g, PC5509; h, PC5510; i, PC5512; j, SC290; k, SC259; l, SC1117; m, SC1131; n, SC508.

tetracycline-resistance marker of the vector (ref. 12; Table 1). The four fusion plasmids conferred characteristic levels of β-galactosidase activity that were elevated compared to basal levels either in strain CB15 or in strain CB15 carrying the parental vectors (Table 1). The utility of the fusion vectors in assessing promoter regulation was confirmed by showing that *lacZ* expression driven by the *flbG* promoter in plasmid vector pANZ405 was periodic in the cell cycle and identical (data not shown) to that determined previously by direct radioimmunoassay of hook protein (5) and by nuclease S1 assay of *flbG* messenger RNA (9, 13).

Effect of *fla* Mutations on Expression of β-Galactosidase from *fla*–*lacZ* Fusions. The regulation of promoter activity in the four *flap*–*lacZ* fusions was quantified by assaying β-galactosidase activity in strains with mutations in all identified *fla* genes. In the first set of experiments, the effect of point and Tn*5* insertion mutations in the hook cluster was examined (Table 1). Both the *flaN* and *flbG* promoters were up-regulated in strains with mutations in the *flaN* operon (transcription unit I) or the *flbG* operon (transcription unit II), which confirmed the conclusion that the two promoters are negatively autoregulated (13, 14) and showed for the first time that the regulation is at the transcriptional level. In addition, these results confirmed that genes in the *flaO* and *flbF* operons are required for transcription from the *flbG* and *flaN* promoters, as suggested previously by assays of messenger RNA (9, 14).

The use of the *lacZ* fusions also demonstrated that the autoregulation of *flaO* (transcription unit III) and the negative regulation of this operon by genes in the *flbF* operon (transcription unit IV) are transcriptionally controlled. The levels of β-galactosidase activity expressed from the *flaO* promoter were up-regulated 5- to 6-fold in mutants with Tn*5* insertions

Table 1. β-Galactosidase activity of *fla*–*lacZ* fusions in different *fla* mutants

Strain	Ref.	Mutation	Map position	pANZ404 (*flaNp*–*lacZ*)	pANZ405 (*flbGp*–*lacZ*)	pANZ406 (*flaOp*–*lacZ*)	pANZ118 (*flbFp*–*lacZ*)
CB15		Wild type		100	100	100	100
PC8207	*	*flaN128*	I	210	220	90	100
PC5001	12	*flaN311*::Tn*5*	I	100	120	90	105
SC235	18	*flaQ106*	I	230	250	80	100
SC307	12	*flaQ150*	I	300	330	85	80
PC5502	12	*flaQ176*::Tn*5*	I	240	260	80	100
PC5501	12	*flaQ166*::Tn*5*	I	105	110	100	100
PC5506	12	*flbG602*::Tn*5*	II	260	330	80	100
PCM103	5	*flaJ303*	II	240	230	75	100
PC5005	12	*flaJ315*::Tn*5*	II	270	360	90	120
PC5503	14	*flbH612*::Tn*5*	II	285	330	80	120
SC511	12	*flaK155*::IS*511*	II	320	400	80	110
SC290	12	*flaO138*	III	25	0	210	120
PC5511	12	*flaO172*::Tn*5*	III	20	0	670	120
SC1114	12	*flaO607*::Tn*5*	III	20	0	560	130
SC1028	12	*flaO188*::Tn*5*	III	20	0	660	130
PC5510	12	*flbD198*::Tn*5*	III	20	0	570	125
SC1166	12	*flbD609*::Tn*5*	III	10	0	445	110
PC5515	12	*flbF177*::Tn*5*	IV	20	0	220	110
PC5516	12	*flbF608*::Tn*5*	IV	20	0	220	110
PC8205	†	*flaW120*	IV	20	0	190	110
SC1117	7	*flbN174*::Tn*5*	Basal	220	260	100	120
SC252	20	*flaD115*	body	230	230	80	120
SC516	20	*flaB160*	cluster	260	270	70	100
SC243	20	*flaC110*		250	290	80	120
SC1131	7	*flbO196*::Tn*5*		20	0	200	120
SC1121	21	*flaY183*::Tn*5*	*flaEY*	75	90	90	120
SC1062	21	*flaE174*::Tn*5*	cluster	75	80	80	110
SC279	20	*flaF132*		70	70	70	120
SC1065	22	*flbA604*::Tn*5*		75	80	80	110
SC278	20	*flaG131*		80	70	75	120
SC508	20	*flaS153*	‡	20	0	190	110
SC270	20	*flaI126*	‡	120	110	130	120
SC284	20	*flaH135*	‡	95	100	120	110
SC293	20	*flaV140*	‡	120	105	160	120
SC229	20	*flaA104*	‡	100	80	85	100
SC305	20	*flaR148*	‡	100	105	80	110
SC175	20	*flaZ102*	‡	80	80	70	110
SC295	20	*flaP141*	‡	230	260	70	120
PC5236	§	*pleA306*::Tn*5*	‡	250	270	80	110

Activities were normalized to 100 in wild-type strain CB15 where the activities in Miller units (23) were pAN2404, 152; pAN2405, 295; pAN2406, 166; and pAN2118, 69. I–IV refer to transcription units in hook cluster (see Fig. 1*A*).

*SC272 (12) phage × CB15.

†SC259 (12) phage × CB15.

‡Genes in this group are scattered on the chromosome.

§J. M. Sommer and A.N., unpublished data.

in *flaO* or *flbD*, which compares to the ≈20-fold increase found when the messenger RNA levels were measured in the same strains (Fig. 1*B*). We have consistently observed that the deregulated levels of expression from genes subject to negative control were higher when estimated by nuclease S1 assay.

fla genes that map outside of the hook gene cluster can be classified into one of four groups based on their effects on *flaN, flbG, flaO,* and *flbF* expression (Table 1 and see Fig. 2 below): Group a, *flaV* is unique in that its only observed effect is the negative regulation of the *flaO* promoter. Group b, *flbO* (a basal body gene) and *flaS* act positively to regulate the *flbG* and *flaN* promoters and negatively to regulate the *flaO* promoter. These regulatory effects are identical to those described above (Table 1) for mutations in *flbF* and *flaW*. Thus, we have included *flbF* and *flaW* in this group along with *flbO* and *flaS*. Group c, *flaP, pleA* (a pleiotropic gene required for hook protein and flagellin synthesis), and all but one of the basal body cluster genes (*flbN, flaD, flaB, flaC*) act negatively to regulate the *flaN* and *flbG* operons. Group d, the largest group of genes, has no significant effect on expression of the four promoter fusions. These genes, which could lie at the same level or at a level below the hook cluster genes in the regulatory hierarchy, include all genes in the *flaEY* cluster (*flaE, flaY, flaF, flbA, flaG*), as well as flagellin genes *flgK, flgL* (data not shown), and five other *fla* genes (*flaI, flaH, flaA, flaR, flaZ*), which are scattered on the chromosome (cf. Table 1).

An important conclusion from these results is that none of the *fla* genes was required for expression of *flaO* or *flbF*, which places these transcription units at or near the top of the regulatory hierarchy. It is also interesting that *flbF*, unlike *flaO*, was not subject to negative regulation by any of the *fla* genes examined.

DISCUSSION

The developmental program controlling the periodic expression of *fla* genes in *C. crescentus* must at a minimum control the times at which the genes are turned on at the beginning of a synthetic period and turned off at the end of the synthetic period. An important element in programming the expression of these genes is their organization in a regulatory hierarchy (1, 9–11), and the results presented in this study further define the positive transcriptional controls that are required for *fla* gene activation. They also identify an extensive network of negative transcriptional controls, and we argue that some of these regulatory interactions may be responsible for turning off *fla* gene expression, a role demonstrated previously for negative autoregulation in the hook (*flbG*) operon (5).

The results described above (Fig. 1 *B* and *C*; Table 1) and previously have been used to place the *fla* genes at one of three levels (level 2, 3, or 4) in a regulatory hierarchy (Fig. 2). *flaO* and *flbF* were placed near the top of the hierarchy at level 2 because we were unable to identify any *fla* genes required for their expression; *flbO* and *flaS*, which display the same regulatory effects as *flbF* (refs. 11 and 19; Table 1), were grouped with *flbF* at the same level of the hierarchy. We have placed the *flaN* (transcription unit I) and *flbG* (transcription unit II) operons at the next lower level (level 3) because *flaO, flbF, flbO,* and *flaS* are required in trans for their expression (refs. 13 and 14; Table 1). The position of *flgK* and *flgL* at the bottom of the hierarchy is based on the requirement of *flaN, flbG* (14), and a number of other *fla* genes (24) for the expression of these flagellin genes. We speculate that an as yet unidentified master regulatory gene(s) located at level 1 initiates the regulatory cascade in *C. crescentus* by turning on the expression of *flaO* and *flbF* (Fig. 2).

The mechanism controlling the sequential activation of the *fla* gene may be the periodic expression of transcription activators and the use of alternative sigma factors that are specific for genes at different levels of the hierarchy. Genes at the bottom of the hierarchy (*flaN, flbG, flgK, flgL*; refs. 14 and 17) contain nucleotide sequences homologous to Nif/Ntr promoters (25), and mutagenesis studies have shown that these sequences are required for transcription of *flaN* and *flbG* (26). Consistent with the role of promoter specificity

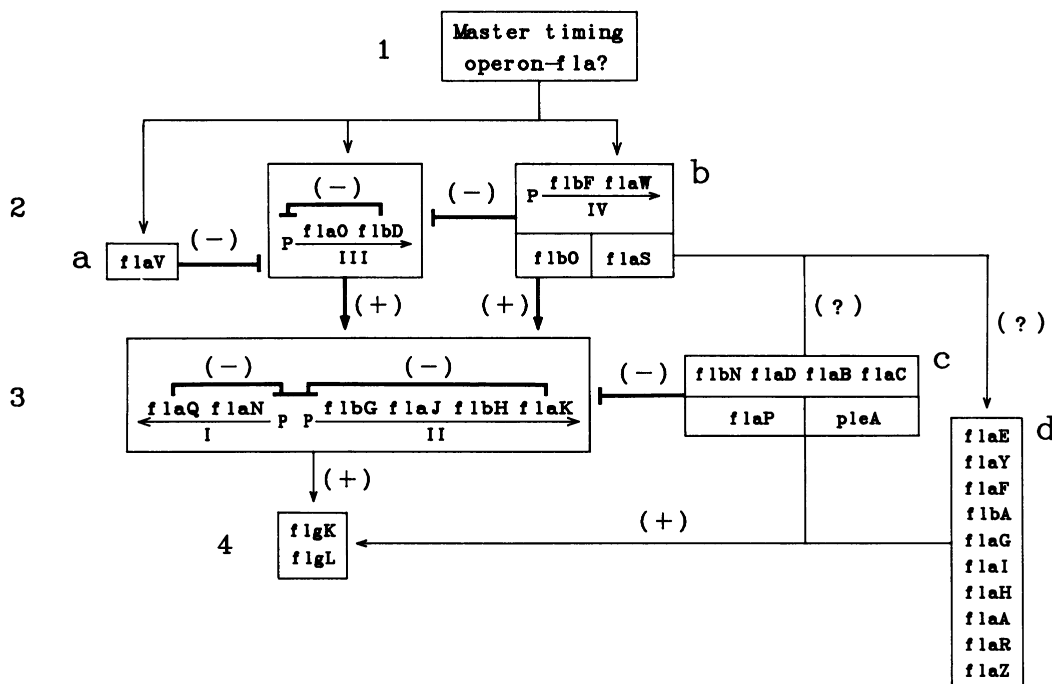

FIG. 2. Model for positive and negative control circuits in the *fla* gene regulatory hierarchy. Genes were placed in the hierarchy as described in the text. Arrows indicate positive regulation and bars on the lines indicate negative regulation of gene expression (see text). Transcriptional regulation of genes defined in this study is indicated by the heavy lines. The dependence of genes in groups c and d on genes in group b is based on the observation that *flaS* is required for the expression of basal body genes *flaC, flaB, flaD, flbN,* as well as *flaY* and *flaE* (L. Shapiro, personal communication).

in regulating *fla* gene expression is the observation that the *flaN* and *flbG* promoters, but not the *flaO* and *flbF* promoters, are recognized *in vitro* by the *E. coli* sigma-54 RNA polymerase (17). In addition, we have identified a conserved *ftr* (flagellar gene transcription regulation) sequence ≈100 bp upstream from the transcription starts of *flaN, flbG, flgK,* and *flgL* (14, 26), which may act as a transcriptional enhancer at levels 3 and 4 of the hierarchy.

Our results also have shown that the positive regulatory cascade in *C. crescentus* is overlaid by an extensive network of negative transcriptional controls, including the negative autoregulation of *flbG, flaN,* and *flaO* (heavy lines in Fig. 2). Although it was known from earlier work that both *flbG* and *flaN* are negatively controlled by genes in the *flbG* operon (13, 14), the present results show that these two promoters are under negative transcriptional control by genes in the *flaN* operon as well (Fig. 2). The negative autoregulation of the *flaO* operon (Table 1) has not been reported previously.

What is the function of negative autoregulation? Genes in the *flbG* operon are required both for the positive control of flagellin genes *flgK* and *flgL* lower in the hierarchy and for the negative regulation of the *flbG* promoter (Fig. 2; ref. 14). These two regulatory functions provide the basic components of a "genetic switch" responsible for the transition from expression of *flbG* to the expression of 25- and 27-kDa flagellin genes (level 3 → level 4). Support for this conclusion is provided by the previous observations (5) that in a *flaJ* polyhook mutant the periods of hook protein synthesis and assembly are extended and the 25- and 27-kDa flagellins are not made. Thus, the mutant apparently lacks regulatory signals needed to turn off expression of *flbG* and to turn on expression of the two flagellin genes (5). One candidate for the gene product mediating these regulatory effects is the 70-kDa hook protein, whose accumulation after completion of hook assembly in wild-type cells could act to inhibit *flaN*/*flbG* expression and to activate the flagellin genes. The role of negative regulation in *C. crescentus fla* gene expression is also discussed by Xu *et al.* in the accompanying paper (27).

An analogous genetic switch may control the transition from *flaO* to *flbG* and *flaN* expression (level 2 → level 3) that occurs earlier in the cell cycle. *flaO* (level 2) is periodically expressed before *flbG* and *flaN* (level 3) (N.O., L.-S. Chen, D.M., and A.N., unpublished data), and we show here that genes transcribed from the *flaO* promoter negatively autoregulate *flaO* and activate the *flbG* and *flaN* promoters (Table 1; ref. 13). Thus, the periodic expression of *flaO* could be turned off by products of the *flaO* and *flbD* genes. If this proves to be the case, it would suggest a general function of negative autoregulatory circuits in turning off *fla* gene expression in the cell cycle once the gene's function in flagellum assembly has been completed.

In addition to negative autoregulation, 10 genes were identified that exert negative transcriptional control over genes in other transcription units. These genes, which could serve to coordinate levels of *fla* gene expression during flagellum assembly, were placed in groups a, b, and c based on their patterns of regulation (Fig. 2). Shapiro and her colleagues have observed some of the same regulatory interactions, and in addition they report that *flbG* is negatively regulated by the basal body genes *flaD* and *flbN* (27). In *E. coli* it has been suggested that the *flaU* gene product acts as a negative regulator to coordinate *fla* gene expression and flagellum assembly (28).

In summary, our results demonstrate that the program of *fla* gene expression in *C. crescentus* is regulated by a complex network of positive and negative transcriptional controls. They also suggest that some of these regulatory interactions are components of genetic switches that control the sequential activation and deactivation of *fla* gene promoters at successive levels of the hierarchy.

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