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# A $\sigma^{54}$ Promoter and Downstream Sequence Elements *ftr2* and *ftr3* Are Required for Regulated Expression of Divergent Transcription Units *flaN* and *flbG* in *Caulobacter crescentus*

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**In this study, we investigated the *cis*-acting sequences required for transcription of the divergent, cell cycle-regulated *flaN* and *flbG* operons of *Caulobacter crescentus*. Previous work showed that transcription of *flbG* in vivo depends on a $\sigma^{54}$ promoter and a sequence element called *ftr1* that is located about 100 bp upstream from the transcription start site (D. A. Mullin and A. Newton, J. Bacteriol. 171:3218–3227, 1989). We now show that regulation of *flaN* transcription in vivo depends on a $\sigma^{54}$ promoter and two *ftr* elements located downstream of the transcription start site at +86 (*ftr2*) and +120 (*ftr3*). Mutations in or between the conserved elements at −24 and −12 in this $\sigma^{54}$ promoter reduced or abolished *flaN* transcription, and one mutation that eliminated *flaN* expression led to an increased level of *flbG* transcript. Mutations in *ftr2* resulted in greatly reduced levels of *flaN* transcript but had no noticeable effect on *flbG* transcript levels. All three mutations constructed in *ftr3* resulted in elevated *flaN* and *flbG* transcript levels. We conclude that *ftr2* is required for positive regulation of *flaN*, whereas *ftr3* appears to play a negative regulatory role in *flaN* and *flbG* expression. To explain the coordinated positive activation and negative autoregulation of these two transcription units and the effect of mutations on gene expression, we propose a model in which the *flaN* and *flbG* promoters interact through alternative DNA looping to form structures that are transcriptionally active or inactive.**

Flagellum biosynthesis in *Caulobacter crescentus* requires more than 50 flagellar (*fla*) genes, and about half of these are organized in transcription units that are located in three major clusters on the chromosome. These *fla* genes are under strict temporal regulation, as indicated by the observation that they are transcribed periodically in the cell cycle at about the time that the gene products assemble to form the flagellum. The precisely ordered sequence of *fla* gene expression during flagellum biosynthesis and the availability of powerful biochemical and genetic tools have made *C. crescentus* an attractive model for studying the mechanism of temporal gene expression during cell differentiation. A central focus of these studies has been to explain, on the molecular level, how genes are turned on and off in a defined sequence during the cell division cycle (for a review, see reference 26).

The role of *trans*-acting genes in the expression of *C. crescentus fla* genes was indicated first by epistasis experiments (3, 31) and later examined in detail by experiments that measured the levels of specific *fla* gene transcripts in different nonmotile mutants (27). These studies suggested that the *C. crescentus fla* genes, like those in *Escherichia coli* (14, 15) and *Salmonella typhimurium* (18), are organized into a regulatory hierarchy in which the expression of *fla* genes at each level depends on the expression of genes above them in the hierarchy (27, 38). Moreover, recent results have shown that the time of *fla* gene expression in the *C. crescentus* cell division cycle is determined by the relative position of a gene in the regulatory hierarchy (4, 29).

Consistent with the above observations, genes at the same level in the hierarchy are transcribed at about the same time and have similar genetic requirements for their expression. Thus, the adjacent and divergent *flaN* and *flbG* (hook) operons are expressed at about the same time in the cell cycle, and their transcription depends on *flbF* and genes in the *flaO* locus, which is now designated the *fliF* operon (5, 23, 30). The *flbF* and *fliF* operons are above *flaN* and *flbG* in the regulatory hierarchy (5, 23) and are transcribed before them in the cell cycle (29, 35). Genes in the *flaN* and *flbG* operons are required in turn for expression of the flagellin genes *flgK* and *flgL*, which are the last genes to be expressed in this transcriptional cascade (22). The part of the transcriptional cascade described by these results is thus *flbF*, *fliF*→*flaN*, *flbG*→*flgK*, *flgL*. The cell cycle signal that initiates the periodic expression of *flbF* and *fliF* has not been identified, but there is evidence that a step in the DNA synthesis pathway is required for the expression of some *fla* genes (7, 37).

The *flaN* and *flbG* transcription start sites were mapped previously, and both genes were shown to contain sequences at −24 and −12 (5, 23) that conform to the consensus sequence for $\sigma^{54}$ promoters in other organisms (for a review, see reference 17); similar promoter sequences were also identified in *flgK* and *flgL* (22). The conclusion that *flbG* is transcribed from a $\sigma^{54}$ promoter was supported by experiments demonstrating that it could be utilized by *E. coli* $\sigma^{54}$ RNA polymerase in vitro (28). Another conserved sequence element of 17 bp, originally referred to as II-1 (5, 23) and subsequently designated *ftr1* (flagellar gene transcription regulation [25]), is located about 100 bp upstream from the transcription start site of each of these genes (22, 25). Site-directed mutagenesis experiments showed that *flbG* expression in vivo required a $\sigma^{54}$ promoter and the *ftr1* sequence element (25).

*ftr1* is situated 101 bp upstream from the *flbG* transcription start site and 78 bp upstream from the *flaN* start site, although *ftr1* is required only for *flbG* expression. Several

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mutations in either *ftr1* or the *flbG* promoter resulted in a large increase in transcription of the divergently transcribed *flaN* promoter, suggesting a negative role for these sequences (25) (see Fig. 1). The presence of the *ftr*-like sequences *ftr2* (+86) and *ftr3* (+120), located downstream from the *flaN* transcription start site, led us to propose originally that the promoters and *ftr* elements of *flaN* and *flbG* interact through protein-mediated DNA looping (25). It was further suggested that these interactions could account for both the positive activation and negative autoregulation of *flaN* and *flbG* (25, 27) and the effects displayed by some of the *ftr1* and promoter mutations observed previously (25). This DNA-looping model is supported by the identification of integration host factor protein (IHF)-binding sites (*ihf*) at −55 (*ihf1*) and −65 (*ihf2*) that lie between the *flbG* promoter and the upstream *ftr1* sequence and the demonstration that *ihf1* is required for *flbG* expression in vivo (10). IHF plays a role in stimulation of transcription from the *nifH* and $glnHp_2$ promoters by its ability to induce a bend in the DNA that brings the $\sigma^{54}$ RNA polymerase bound at the promoter in contact with an activator protein bound at a distant upstream site (6, 12).

To investigate the molecular mechanisms regulating *fla* gene expression in the cell cycle of *C. crescentus*, we have sought to determine the identity, arrangement, and function of additional *cis*-acting sequences that are required for positive and negative regulation of *flaN* and *flbG*. If the promoters and *ftr* elements in these transcription units interact with one another to mediate negative regulation as proposed (25), then some mutations in the promoter and *ftr* elements of *flaN* should destabilize the complex and result in elevated levels of the *flbG* transcript. We have tested this proposition by mutating the *flaN* promoter, *ftr2*, and *ftr3* and assaying the effect on *flaN* and *flbG* expression in vivo. The results presented here show that transcription of *flaN* depends on a $\sigma^{54}$ promoter sequence as well as the downstream sequence element *ftr2* at +85. Consistent with the proposed interaction between these promoters and *ftr* elements, we show that a mutation in the *flaN* promoter results in an elevated level of the *flbG* mRNA and that mutations in *ftr3* result in increased levels of both *flaN* and *flbG* mRNAs. We propose a model to account for the positive and negative regulatory roles of these *cis*-acting sequence elements.

(A preliminary report of this work was presented at the 89th Annual Meeting of the American Society for Microbiology, New Orleans, La. [24].)

## MATERIALS AND METHODS

**Strains and culture conditions.** The bacterial strains, plasmids, and phages used in this work are listed in Table 1. Wild-type *C. crescentus* CB15 (ATCC 19089) and nonmotile mutants were grown in PYE medium (0.2% peptone, 0.1% yeast extract, 0.02% $MgSO_4$) (32). Recombinant pRK2L1-derived plasmids were introduced into *C. crescentus* CB15 by electroporation and grown in PYE broth containing 2 μg of tetracycline per ml. *E. coli* HB101 was used as a host for plasmid transformation (19) and grown in yeast extract-tryptone medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.02% $MgSO_4$) (13) supplemented with ampicillin (50 μg/ml) chloramphenicol (10 μg/ml), kanamycin (50 μg/ml), or tetracycline (10 μg/ml). *E. coli* DH5α was used as a host for bacteriophage M13, and *E. coli* CJ236 was used as a host for bacteriophage M13 prior to oligonucleotide mutagenesis.

**Oligonucleotide mutagenesis.** The DNA of bacteriophage M13mp19603 carries a 603-bp *Pst*I fragment that contains all of the *cis*-acting sequences required for regulated transcription of *flaN* and *flbG* (25). Oligonucleotide primer mutagenesis of sequences in M13mp19603 was performed as described previously (25). Following dideoxynucleotide DNA sequencing (36) to identify phage clones with the desired mutation, the *Bam*HI-*Hin*dIII fragment (Fig. 1C) carrying the mutation was cloned into pRK2L1 and introduced into *C. crescentus* by electroporation. pRK2L1 has been described previously (25) and consists of the multiple restriction site polylinker from pUC18 (39) cloned in pRK290 (8). Following transfer to *C. crescentus*, the relative levels of *flaN* and *flbG* mRNAs expressed from the plasmids were measured by a nuclease S1 protection assay.

TABLE 1. Bacterial strains, plasmids, and bacteriophages

| Strain, plasmid, or phage | Relevant characteristics | Source or reference |
|---|---|---|
| *C. crescentus* | | |
| CB15 | Wild type | 32 |
| SC511 | *flaK511*::IS*511* | 31 |
| *E. coli* | | |
| HB101 | Host for plasmids | 2 |
| DH5α | Host for M13 phage, $Km^r$ | BRL[a] |
| CJ236 | *dut ung* host for M13 phage, $Cm^r$ | 16 |
| Plasmid pRK2L1 | Cloning vector, $Tc^r$ | 25 |
| Bacteriophage M13mp19603 | Template for mutagenesis of *flaN* and *flbG* | 25 |

[a] BRL, Bethesda Research Laboratories Life Sciences, Inc.

**Isolation of *Caulobacter* RNA and nuclease S1 protection assays.** RNA was purified from *C. crescentus* cells as described before (30). DNA restriction fragment probes used in nuclease S1 protection assays were 5′-end-labeled with [γ-$^{32}$P]ATP and T4 polynucleotide kinase (1). The hybridization temperature for all of the probes was 55°C. The DNA probe used to detect *flaN* and *flbG* transcripts expressed from plasmids is the 642-nucleotide (nt) *Bam*HI-*Hin*dIII fragment labeled at both 5′ ends (Fig. 1C). Only *flaN* and *flbG* transcripts made from the plasmid promoters protected the $^{32}$P-labeled ends of the 642-bp *Bam*HI-*Hin*dIII probe from nuclease S1 hydrolysis because the labeled 5′ phosphates are on nucleotides derived from the multiple restriction site polylinker of the plasmid (25). The 1,115-bp *Sty*I fragment (Fig. 1B) labeled at both 5′ ends yielded nuclease S1-protected fragments of approximately 712 and 198 nt, which correspond to the genomic *flaN* and *flbG* transcripts, respectively. The 285-bp *Bam*HI(d)-*Hin*dIII fragment (Fig. 1A), $^{32}$P labeled at both 5′ ends, was used as a probe in nuclease S1 protection assays for the genomic *fliF* transcript. The double-end-labeled probe was used because it is simpler to prepare than the single-end-labeled probe labeled at the *Hin*dIII end, and both probes yielded a protected fragment of 80 nt in nuclease S1 protection assays (23). Excess amounts of $^{32}$P-labeled probe DNA were added to each nuclease S1 assay to ensure that the intensity of the protected fragments reflected the level of the 5′ transcript. The products of the nuclease S1 reactions were denatured by heating to 90°C in a formamide-containing marker dye solution and fractionated by electrophoresis in 4 or 6% polyacrylamide gels that contained 8 M urea.

The sequence of the 642-bp insert in pRK2L1-648 (Fig. 1C) was reported previously (25), and its GenBank accession number is M26955.

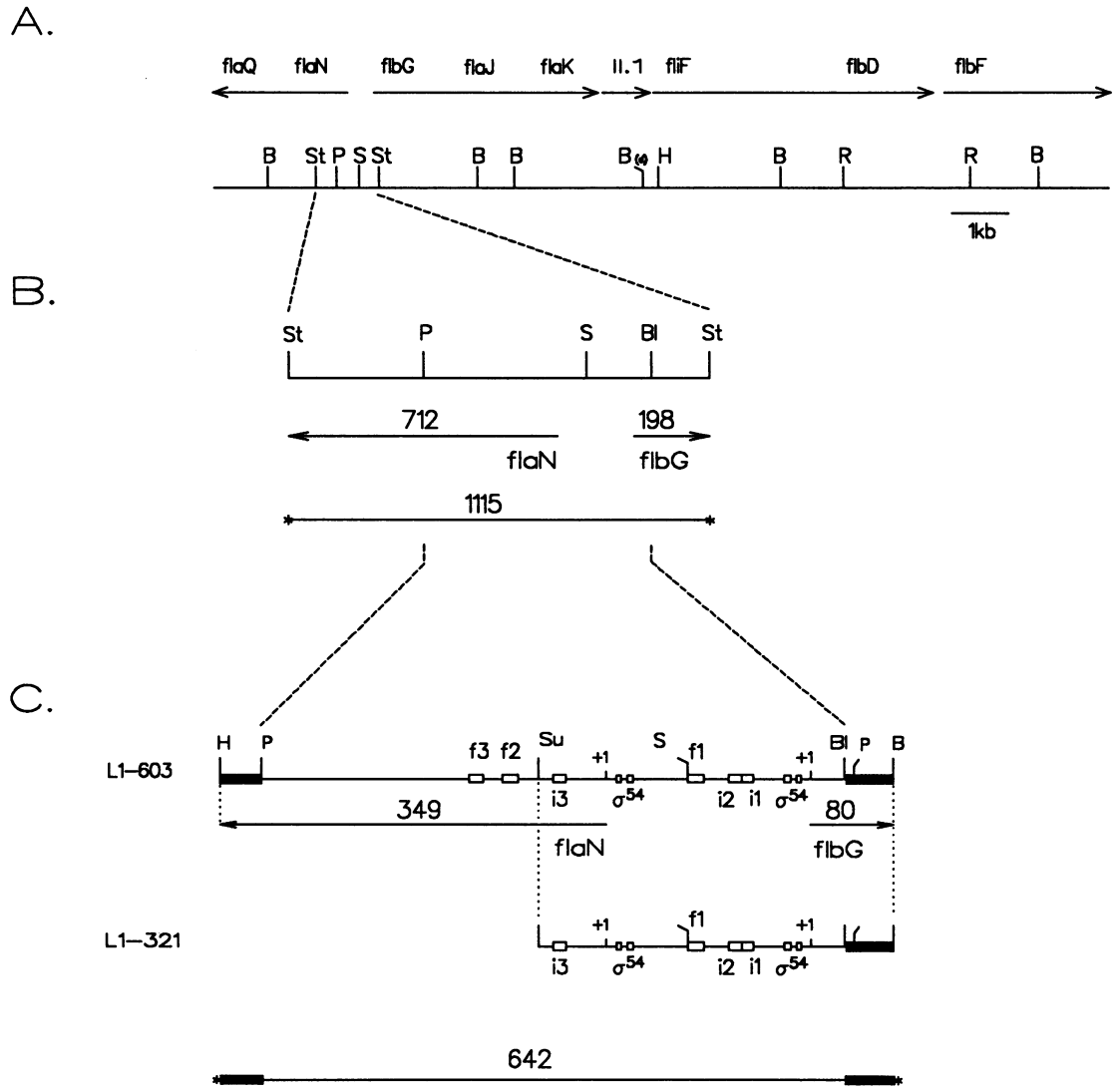


FIG. 1. Genetic and physical map of the hook gene cluster and probes for nuclease S1 protection assays. (A) Transcription units, genes, and restriction map of the hook gene cluster (31). Relevant restriction sites: B, *Bam*HI; $B_{(d)}$, *Bam*HI (d) site; H, *Hin*dIII; P, *Pst*I; R, *Eco*RI; S, *Sst*I; Sl, *Sal*I; Su, *Sau*3AI; St, *Sty*I. Bl marks the location of a *Bal*31 nuclease-generated deletion endpoint; * indicates 5′ $^{32}$P-labeled ends. (B) Probe used to assay genomic *flaN* and *flbG* transcripts. Arrows indicate the origin and direction of transcription and the size of the nuclease S1-protected fragments with the 1,115-kb *Sty*I probe. (C) Plasmid constructs and probe used to detect plasmid-encoded *flaN* and *flbG* transcripts. Thin solid lines are genomic DNA, thick lines represent multiple restriction site polylinker DNA derived from pUC18 (39), and open bars indicate *ftr* (f) and *ihf* (i) sequences. Arrows indicate the origin and direction of transcription, and their length corresponds to the nuclease S1-protected fragments detected with the 642-nt *Bam*HI-*Hin*dIII probe.

## RESULTS

**Organization of the *flaN* and *flbG* regulatory regions and experimental plan.** The *flaN* and *flbG* operons (Fig. 1A) are transcribed at the same time in the cell cycle (5, 29) and have similar genetic requirements for expression (5, 23, 30). Potential regulatory sequences in *flaN* and *flbG* have been identified either by their alignment with *E. coli* regulatory elements or by their similarity to sequence elements previously identified in *C. crescentus* (10, 23, 25). The location and organization of these sequences in the *flaN* and *flbG* operon promoter regions are illustrated in Fig. 1C. Work in our laboratories has demonstrated that a $\sigma^{54}$ promoter and the *ftr1* element at −101 from the transcription start site are required in *cis* for transcription of *flbG* (25), and more recent studies by Gober and Shapiro have shown that a sequence designated *ihf1* at −55 from the *flbG* transcription start site, which matches the consensus binding sequence for *E. coli* IHF, is also required (10). *flaN* contains homologous sequence elements, but their organization within this transcription unit does not mirror that in *flbG*. *flaN* contains a sequence that matches the $\sigma^{54}$ promoter consensus, two *ftr* elements, *ftr2* and *ftr3* at +86 and +120 from the transcription start site, respectively, and an IHF-binding site, *ihf3*, located between the promoter and *ftr2* (Fig. 1C). We report below the effect of mutations in these sequence elements, first on *flaN* expression and second on *flbG* expression.

The 642-bp DNA fragment designated L1-603 (Fig. 1C), which carries the 5′ sequences of *flaN* and *flbG*, has proved

to be a useful model for the study of these promoters because it contains all of the *cis*-acting regulatory elements required for positive and negative regulation exhibited by *flaN* and *flbG* on the chromosome (25). Mutations were introduced in DNA fragment L1-603 by oligonucleotide mutagenesis (Materials and Methods), and the mutant fragments were inserted between the *Bam*HI and *Hin*dIII sites in pRK2L1 (25). The resulting plasmid constructs were transferred to *C. crescentus* by electroporation, and plasmid-encoded *flaN* and *flbG* transcript levels in vivo were determined by using nuclease S1 protection assays with the 642-bp probe (Fig. 1C). This assay permits both the *flaN* and *flbG* transcripts from the plasmid to be detected in the same RNA preparation without interference from chromosomal transcripts (25).

As a control for the level and intactness of RNA in the preparations analyzed in this study, we routinely assayed the relative level of the genomic *fliF* transcript (Materials and Methods) in each RNA preparation at the same time that the *flaN* and *flbG* transcripts were assayed. The relative level of *fliF* transcript was found to be similar in the RNA preparations analyzed in each experiment (data not shown).

**A $\sigma^{54}$ promoter is required for transcription of *flaN* and regulation of *flbG*.** Mutations introduced in a 5′ *flaN* sequence that matches the consensus sequence for $\sigma^{54}$ promoters are summarized in Fig. 2D. Deletion of the conserved GC in the −12 element (mutation 27) or the GC at −19 and −20 (mutation 51) or a change of A to C at −13 (mutation 12) eliminated any detectable *flaN* transcript (Fig. 3a, lanes G, D, and B, respectively). Deletion of the nucleotides at −16 and −17 (mutation 26) or −15 and −16 (mutation 50) in the nonconserved spacer region between the −24 and −12 sequence elements also eliminated expression of *flaN* (Fig. 3a, lanes C and F, respectively). Although this change in spacing between the two conserved sequence elements resulted in no detectable *flaN* transcript, a G to T point mutation at −15 (mutation 52) in the spacer region resulted in a reduced but easily detectable level of the *flaN* transcript (Fig. 3a, lane E). These results demonstrate that nucleotides in the −24 and −12 sequence elements and the spacing between these elements are required for *flaN* transcription, suggesting that this gene, like *flbG* (25), is transcribed from a $\sigma^{54}$ promoter.

Mutations in the nonconserved region between the −24 and −12 elements of the *flaN* promoter also affected the level of the *flbG* transcript. For example, mutations 51 and 52 resulted in a reduced level of *flbG* transcript, while mutation 26 resulted in an increase in the level of the *flbG* transcript (Fig. 3a, lanes D, E, and F, respectively). The increased level of *flbG* transcript resulting from mutation 26 is more apparent in an autoradiogram from a separate experiment, in which the gel with the nuclease S1-protected fragments was exposed to X-ray film for a shorter period of time (Fig. 3b, compare lanes A and B). The effects of *flaN* promoter mutations 51, 52, and 26 on *flbG* expression will be considered later.

***ftr2* is required as a positive regulatory element for expression of *flaN*.** Previous analysis of *ftr1*, which is located 101 bp upstream of *flbG* and 78 bp upstream of *flaN* (Fig. 1C and 2E), showed that it is required for *flbG* expression and negatively regulates *flaN* expression (25). *flaN* contains two *ftr*-like sequence elements downstream from the transcription start site; *ftr2* at +86 and *ftr3* at +120. We originally suggested that one or both of these *ftr* elements are required for *flaN* expression because *flaN* transcripts were not expressed from DNA fragment L1-321, which contains the *flaN* promoter and *ihf3* but lacks *ftr2* and *ftr3* (Fig. 1C) (25). We have examined the role of *ftr2* and *ftr3* in more detail by introducing mutations into these sequence elements and measuring the effect on expression of *flaN* and *flbG*.

As summarized in Fig. 2B, *ftr2* mutations of G to T at +98 (mutation 41), deletion of CG at +99 and +100 (mutation 54), and deletion of CC at +87 and +88 (mutation 55) resulted in a greatly reduced level of the *flaN* transcript (Fig. 4a, lanes C, D, and E, respectively). A point mutation of G to T at +86 (mutation 42) had almost no effect on the level of *flaN* transcript (Fig. 4a, lane F), and a double mutation of C to A at +86 and at +98 resulted in a reduced level of *flaN* mRNA (Fig. 4a, lane B), similar to that resulting from mutation 41, as would be expected from the effects of the individual mutations discussed above. The *ftr2* mutations examined had little effect on the levels of plasmid-encoded *flbG* transcript, however (Fig. 4a).

Our results also indicate that a sequence, *ihf3*, conforming to the binding site consensus sequence ($YAAN_4TTGATW$) for the *E. coli* IHF (10, 20) located between *ftr2* and the *flaN* promoter, is also required for *flaN* expression. We have designated the *ihf* sites in *flbG ihf1* (−53 to −66) and *ihf2* (−65 to −77) (Fig. 2F), and the *ihf* site in *flaN* was designated *ihf3* (+40 to +52) (Fig. 2C). We introduced a T to G mutation at +44 (mutation 44) in *ihf3* (Fig. 2C), and it almost completely abolished detectable levels of the *flaN* transcript but had little effect on the level of *flbG* transcript (Fig. 5, lane B). Although we analyzed only a single mutant here, this result suggests that the IHF-binding site located between the *flaN* promoter and *ftr2* is required for *flaN* expression, just as *ihf1*, which lies between the *flbG* promoter and *ftr1*, is required for *flbG* expression (10). Previous work by Gober and Shapiro showed that mutations in *ihf1* that reduce binding of IHF protein in vitro also reduce transcription of *flbG* in vivo, and they also showed that IHF protein binds in vitro to the sequence identified here as *ihf3* (10). We also isolated a mutant with a 1-bp deletion of the A at −77 (mutation 20) in *ihf2* (Fig. 2F) that resulted in a large decrease in *flaN* and *flbG* transcript levels (Fig. 5, lane C). Although mutation 20 does not alter the consensus IHF-binding sequence, it may exert its effect on expression by changing the spacing between *cis*-acting elements in a way that alters the regulation of both transcription units.

***ftr3* negatively regulates *flaN* and *flbG* expression.** Three mutations were introduced into *ftr3* (Fig. 2A) to determine whether nucleotides in this sequence element play a role in expression of *flaN* and *flbG*. *flaN* and *flbG* transcript levels were increased in strains with the mutations G to T at +132 (mutation 22) (Fig. 4b, lane E), deletion of AA at +128 and +129 (mutation 21) (Fig. 4b, lane D), and C to A at +124 (mutation 201) (Fig. 4b, lane C); mutation 22 resulted in a particularly large increase in the level of the *flaN* transcript (for comparison, lane B shows the derepressed level of plasmid-encoded *flaN* and *flbG* transcripts in a *flaK155* mutant strain, and lane A shows the wild-type level). These findings suggest that the *ftr3* element functions differently from *ftr1* and *ftr2* because it appears to exert exclusively a negative regulatory effect on *flaN* and *flbG* expression.

## DISCUSSION

The *flaN* and *flbG* promoters are subject to a complex and coordinated pattern of regulation. They are periodically expressed at the same time in the cell cycle, and both transcription units are positively regulated by the same set of genes above them in the *fla* gene regulatory hierarchy,

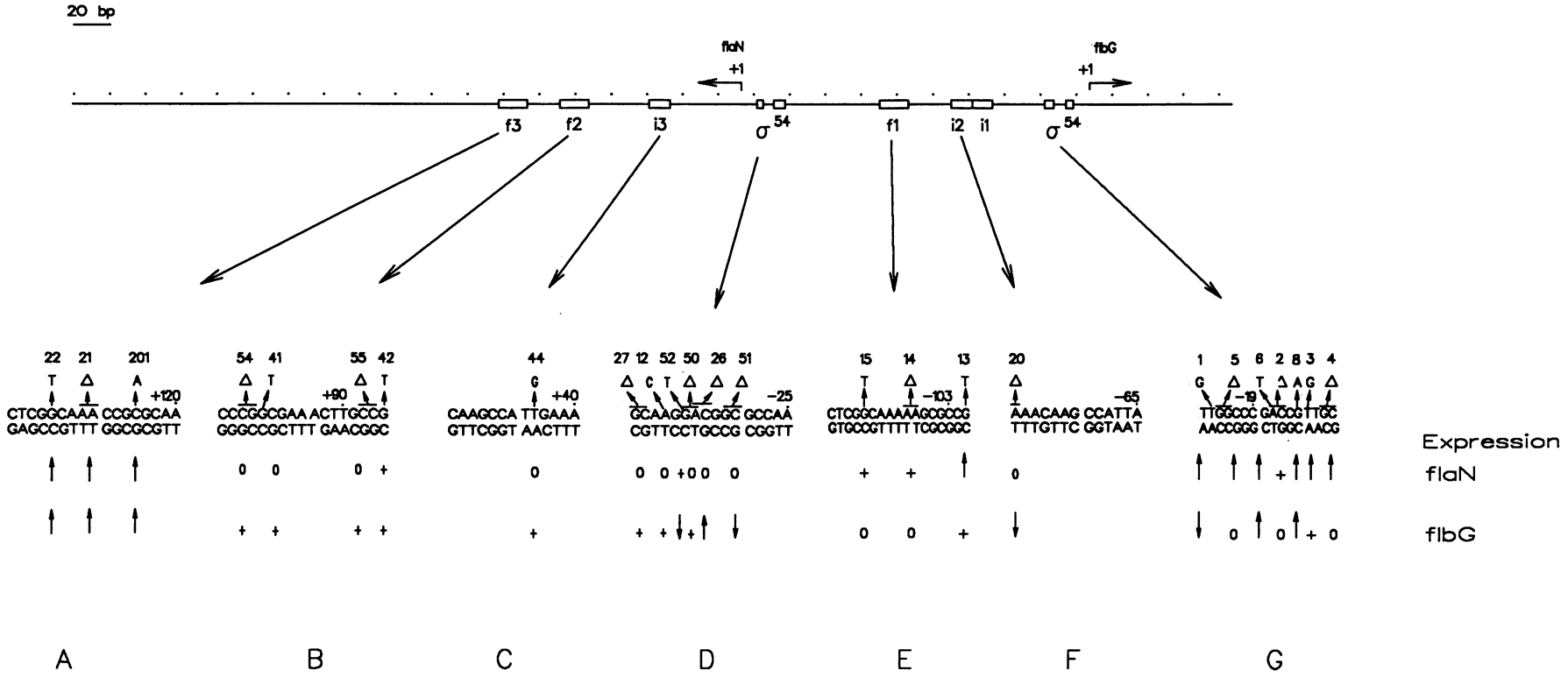


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FIG. 2. Summary of primer mutagenesis. Promoters, *ftr* elements, and *ihf* sequences are labeled as in Fig. 1C and are indicated by open bars. Arrows above the map indicate the origin and direction of transcription. The position and nature of mutations created and analyzed in this work are shown. The mutations shown in the *flbG* promoter and *ftr1* and their effect on *flaN* and *flbG* expression were reported previously (25). The effect of mutations on *flaN* and *flbG* transcript levels is indicated below the sequence: +, transcript made; 0, little or no transcript made; ↑, elevated level of transcript; ↓, reduced but detectable level of transcript.

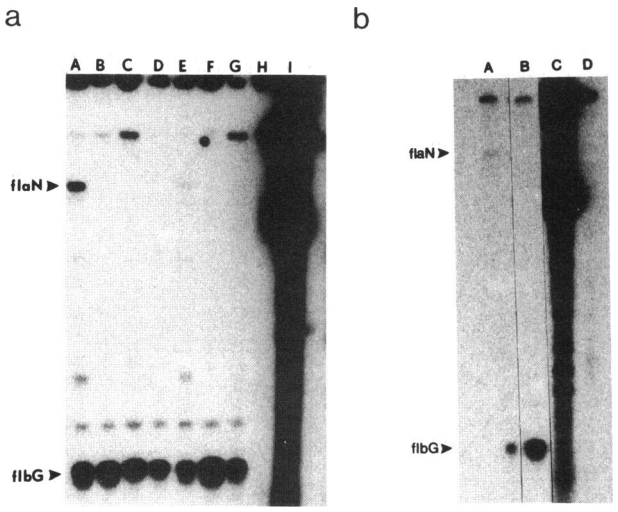


FIG. 3. Nuclease S1 analysis of point mutations in the *flaN* promoter. Total RNA was purified from *C. crescentus* and used in nuclease S1 assays as described in Materials and Methods. The 642-nt probe (Fig. 1C) was used in each reaction, and nuclease S1 was added to the reaction mixes shown in lanes A through H. The positions of the 349-nt (*flaN*) protected fragment and the 80-nt (*flbG*) protected fragment are shown. RNA was isolated from strain CB15 containing wild-type (wt) or mutant pRK2L1-603; plasmid mutations are indicated by number (Fig. 2). (a) Lane A, wt; lane B, mutation 12; lane C, mutation 50; lane D, mutation 51; lane E, mutation 52; lane F, mutation 26; lane G, mutation 27; lanes H and I, yeast tRNA with (H) and without (I) nuclease S1. (b) Reexamination of the effect of mutation 26 with reduced exposure time. Lane A, wt; lane B, mutation 26; lane C, yeast tRNA, no nuclease S1; lane D, yeast tRNA plus nuclease S1.

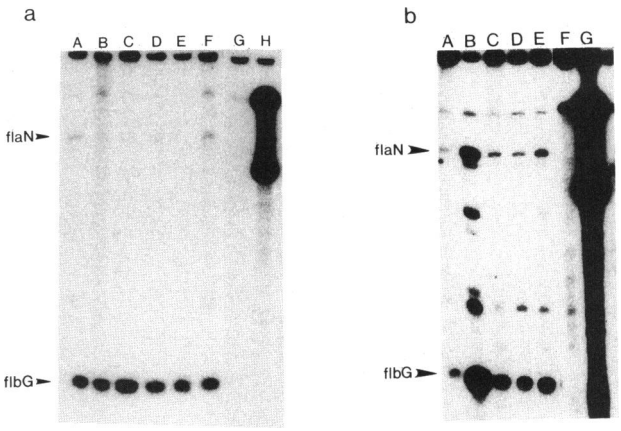


FIG. 4. Nuclease S1 analysis of the effect of *ftr2* and *ftr3* mutations on the level of plasmid-encoded *flbG* and *flaN* mRNAs. Nuclease S1 protection assays were used to characterize *ftr2* and *ftr3* mutations as described in the legend to Fig. 3; nuclease S1 was present in the reaction mixes analyzed in lanes A through G. RNA analyzed was isolated from strain CB15 containing wild-type (wt) or mutant pRK2L1-603; plasmid mutations are indicated by number (Fig. 2). Mutation 411 is a double mutation of G to T at +86 and G to T at +97. (a) *ftr2* mutations. Lane A, wt; lane B, mutation 411; lane C, mutation 41; lane D, mutation 54; lane E, mutation 55; lane F, mutation 42; lanes G and H, yeast tRNA with (G) and without (H) nuclease S1. (b) *ftr3* mutations. Nuclease S1 was present in lanes A through F. Lane A, wt; lane B, strain SC511 (*flaK155*::IS*511*) containing wild-type pRK2L1-603; lane C, mutation 201; lane D, mutation 21; lane E, mutation 22; lanes F and G, yeast tRNA with (F) and without (G) S1 nuclease.

including *flbF* and *flbD*. They are also subject to coordinate negative autoregulation by genes in the *flaN* and *flbG* operons (5, 25, 27). Previous work demonstrated that *flbG* is transcribed from a $\sigma^{54}$ promoter and that the *ftr1* element at −101, which is required for positive control of *flbG*, also negatively regulates *flaN* expression (25). To further elucidate the control of these transcription units, we used site-directed mutagenesis to investigate sequence elements located within *flaN*, including the *flaN* promoter, *ftr2* at +86, and *ftr3* at +120 (Fig. 2). Our results confirm that transcription of *flaN*, like that of *flbG*, depends on a $\sigma^{54}$ promoter and demonstrate that *ftr2* is required for *flaN* expression. In addition, our results suggest that the *flaN* promoter and *ftr3* play a role in negative regulation of *flbG*. Thus, the *ftr* elements in *flaN* and *flbG* appear to play a dual regulatory role; they are required for both the positive and negative regulation of these two transcription units. Our data supporting these conclusions are summarized in Fig. 2.

The observation that certain mutations in either the *flbG* promoter or *ftr1* resulted in not only a decreased level of *flbG* expression but also a greatly increased level of *flaN* expression led us originally to propose a model in which the promoters and *ftr* elements of *flaN* and *flbG* interact through DNA looping to form a transcriptionally active complex that is also sensitive to repression (25). One prediction of the model was that some mutations in the *flaN* promoter should not only abolish *flaN* expression but also result in elevated levels of *flbG* expression. In this study, we have examined six mutations that altered nucleotides in the −24 and −12 elements and spacer region of the *flaN* promoter (Fig. 2).

The observation that all six of these mutations caused a reduction in the levels of *flaN* transcript (Fig. 3) indicates that the −24 and −12 elements and the spacing between them are important elements for *flaN* transcription, as expected for a $\sigma^{54}$ promoter. The elevated level of the *flbG*

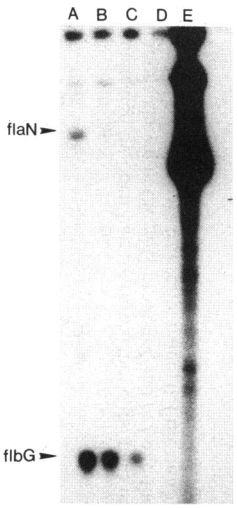


FIG. 5. Nuclease S1 analysis of the effect of *ihf2* and *ihf3* mutations on the level of plasmid-encoded *flbG* and *flaN* mRNAs. Nuclease S1 assays were carried out as described in the legend to Fig. 3. Nuclease S1 was present in lanes A through D. Lane A, wild type; lane B, mutation 44; lane C, mutation 20; lanes D and E, yeast tRNA with (D) and without (E) nuclease S1.

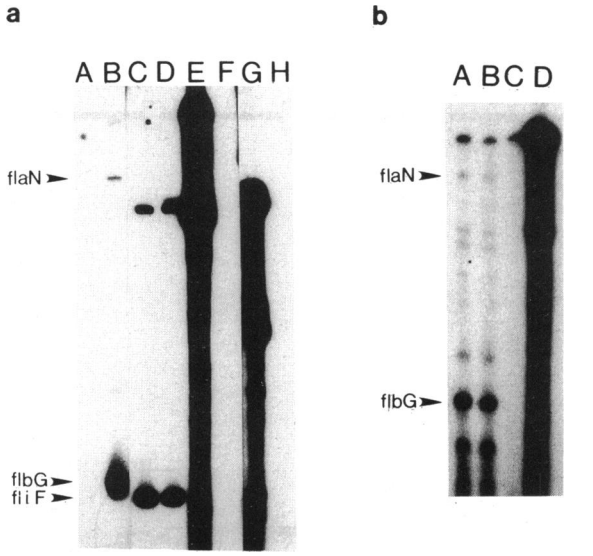


FIG. 6. Effect of pRK2L1-603 on genomic *flaN* and *flbG* transcript levels. Nuclease S1 assays were carried out as described in the legend to Fig. 3. (a) Nuclease S1 was included in the reaction mixes shown in lanes A through D, F, and H. Levels of plasmid-encoded *flaN* and *flbG* transcripts were assayed with the 642-nt *Bam*HI-*Hin*dIII probe (Fig. 1C). The source of RNA in each reaction mix is indicated: lane A, CB15(pRK2L1), lane B, CB15 (pRK2L1-603). Genomic *fliF* transcript was assayed with the 285-nt *Bam*HI(d)-*Hin*dIII fragment $^{32}$P labeled at both 5′ ends, and the source of RNA is indicated: lane C, CB15(pRK2L1); lane D, CB15 (pRK2L1-605); lanes E through H, yeast tRNA with (F and H) and without (E and G) nuclease S1. (b) Genomic *flaN* and *flbG* transcript levels were assayed with the 1,115-bp *Sty*I probe (Fig. 1B). Nuclease S1 was included in the reaction mixes shown in lanes A, B, and C. The source of RNA in each reaction is indicated: lane A, CB15 (pRK2L1); lane B, CB15(pRK2L1-605); lanes C and D, yeast tRNA with (C) and without (D) nuclease S1.

transcript that resulted from *flaN* promoter mutation 26 (see Fig. 3b) supports our model that the *flaN* and *flbG* promoters form part of a complex that is sensitive to repression.

An alternative interpretation of the effect of mutation 26 would be that the $\sigma^{54}$ protein or a transcriptional activator like the FlbD protein is limiting and that mutant *ftr* or $\sigma^{54}$ sequences that do not bind these proteins would result in increased transcription from the *flbG* promoter. To demonstrate that these transcription factors are not titrated in our studies, we examined the levels of plasmid-encoded *flaN* and *flbG* transcripts in a strain carrying the *flaK511* mutation, which is derepressed for transcription from the *flaN* and *flbG* promoters (Fig. 4b, lane B). Quantification by densitometric scanning of the autoradiogram shows that the levels of the *flaN* and *flbG* transcripts are increased about 10-fold over the wild-type level (Fig. 4b, lane A), which suggests that these cells have a high expression potential for *flaN* and *flbG*. We also found that the levels of genomic *flaN* and *flbG* transcripts were unaltered by the presence of pRK2L1-603 (Fig. 6b, compare lanes A and B). As expected, control assays show that plasmid-encoded *flaN* and *flbG* transcripts are detected only in CB15(pRK2L1-603) (Fig. 6a, lane B) and that the *fliF* transcript level is similar in CB15(pRK2L1) and CB15(pRK2L1-603) (Fig. 6a, compare lanes C and D). These results suggest that the regulatory effects on *flaN* and *flbG* expression caused by mutations analyzed in this work are not due to titration of transcription factors.

The *ftr1* sequence is required for activity of the *flbG* promoter (25), and the results presented here show that the downstream *ftr2* element at +86 is required for activity of the divergent *flaN* promoter. The activation of these two $\sigma^{54}$ promoters by distant activator sites may occur by DNA looping, as proposed before (25). This model is supported by our mutagenesis data, which suggest that the IHF-binding site *ihf3* (10) is required for *flaN* expression (Fig. 5) and, as shown previously, *ihf1* is required for expression of *flbG* (10). Thus, transcriptional activation of *flbG* requires an intact promoter, the upstream *ftr1* sequence element (25), and an IHF-binding site (10). The proposed organization of these sequences (Fig. 7) is similar to that demonstrated previously for the sequence elements required for transcription of the NifA-activated *nifH* promoter and from the NtrC phosphate protein-activated $glnHp_2$ promoter, both of which require an IHF-binding site located between the $\sigma^{54}$ promoter and the upstream activator site (6, 12). We propose that the *flaN* transcription unit is organized in a generally similar fashion except that *ftr2* and *ihf3* map downstream from the promoter (Fig. 2).

FlbD protein is the product of the last gene in the *fliF* operon, and *flbD* encodes a $\sigma^{54}$-specific transcriptional factor that stimulates transcription from the *flbG* promoter in *E. coli* (33). Although FlbD protein purified from *E. coli* does not bind tightly to *ftr1* (34), it is possible that protein modification or the direct participation of other proteins is required for its binding to DNA. In addition to *flbD* and *flbF*, genes in the *fliLM* and *flaQR* operons are also required for *flaN* and *flbG* expression in vivo (27), and it will be important to determine which if any of these genes code for products that are directly required for transcription. One *C. crescentus* protein that binds tightly to the *ftr* sequence is RFI (9), but its function in *flaN* and *flbG* expression is not known.

In a report that appeared after our analysis had been completed, Gober and Shapiro investigated the effects of mutations in *ftr2*, *ftr3*, and *ihf3* on *flaN* expression (11), and their results generally agreed with our findings except for the effect of mutations in *ftr3*. They reported that an *ftr3* mutation of CC to AA at +132 and +133 resulted in about a 2.8-fold decrease in level of *flaN* transcript (11), whereas all three of the *ftr3* mutants that we examined resulted in an increase in *flaN* transcript levels (summarized in Fig. 2).

A novel aspect of *flaN* and *flbG* expression is the regulatory coupling between these two operons, as indicated by promoter and *ftr* mutations that result in elevated levels of transcript from one or both of these transcription units (Fig. 2). Not all mutations in the *flbG* promoter and *ftr1* result in increased *flaN* expression, and in a similar fashion, only one of the *flaN* promoter mutations examined resulted in increased *flbG* transcription and none of the *ftr2* mutations altered the level of *flbG* expression. The different effects of mutations in these sequence elements are not surprising given the number of *cis*-acting regulatory sites identified here and the number of possible DNA-protein interactions that are likely to coordinate the expression of these two genes.

DNA looping mediated by the association of proteins bound at several sites along DNA molecules appears to be an important mechanism for both positive and negative regulation of genes (for a review, see reference 21), and we previously proposed a DNA-looping model to account for the effect of mutations on *flaN* and *flbG* expression (25). Our current model, shown in Fig. 7, assumes that the negative autoregulation of transcription requires an interaction between the *flaN* and *flbG* transcription complexes (Fig. 7C) that is mediated in some way by transcription factors which

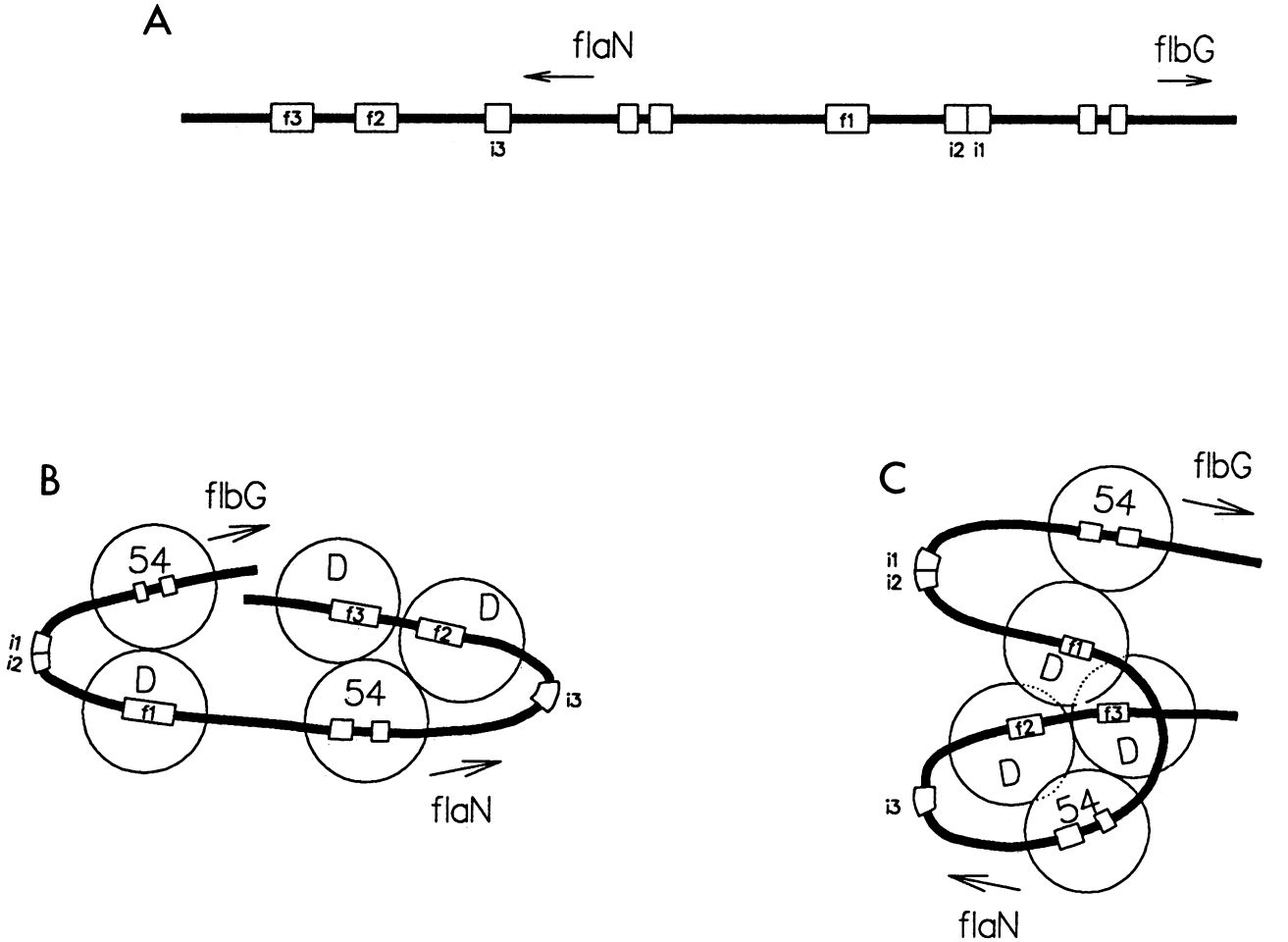


FIG. 7. Model for interaction of *flaN* and *flbG* promoters and *ftr* elements. (A) Arrangement of promoters and *ftr* (f) and *ihf* (i) elements. (B) Bends introduced by binding of IHF protein could facilitate interaction between $\sigma^{54}$ RNA polymerase (circles marked 54) bound to the promoter and activator proteins (circles marked D) bound at *ftr* elements, which leads to active *flaN* and *flbG* transcription. (C) Interaction between the *flaN* and *flbG* transcription complexes mediated by negative regulatory proteins would result in repression of transcription.

remain to be identified. For simplicity, we have assumed that the factor is FlbD, either modified or in association with other proteins. To explain the effects of the mutations observed in this study, we suggest two types of related interactions between these sequence elements. In the first, transcription of *flaN* and *flbG* requires interaction between *ftr1* and the *flbG* promoter and between *ftr2* and the *flaN* promoter, as shown in Fig. 7B. DNA bending mediated by IHF binding to the *ihf* sequences would facilitate the interactions between proteins bound at the *ftr* elements and $\sigma^{54}$ RNA polymerase bound to the *flaN* and *flbG* promoters in a manner analogous to that required for activation of the $\sigma^{54}$ promoters of *nifH* and *glnH* (6, 10, 12).

In the second type of interaction, the two transcription complexes would associate as shown in Fig. 7C to form a repression-sensitive complex which mediates the negative autoregulation of these two genes reported previously (27). Consistent with this part of the model is the observation that the sensitivity of the promoters to negative regulation can be lost as a result of mutations in the *flbG* promoter or *ftr1* (25) and the *flaN* promoter or *ftr3* (Fig. 4b) that might disrupt the proposed interaction between the two transcription complexes, perhaps resulting in a structure like that shown in Fig. 7B (summarized in Fig. 2). The strong negative autoregulation of *flbG* and *flaN* is exerted at the transcriptional level, and mutations in either *flaK*, the hook protein gene, or *flaN* lead to a large increase in transcription from both promoters (27). It is not known, however, which proteins are directly involved in negative regulation of *flaN* and *flbG*. Disruption of the associations discussed above is one but obviously not the only mechanism that could result in the observed regulatory effects.

Why do mutations in *ftr3* and not *ftr2* increase expression of *flaN* and *flbG*? We speculate that (i) either sequence element can interact with the *flbG* transcription complex to mediate negative regulation, (ii) *ftr2* requires an intact *ftr3* element for this interaction, and (iii) only *ftr2* can interact to stimulate transcription from the *flaN* promoter. Thus, in an *ftr2* mutant that fails to express *flaN*, *ftr3* would interact with the *flbG* transcription complex and the level of the *flbG* transcript would not be altered.

Although most mutations in the *flaN* promoter that affect the proposed repressor-sensitive complex are predicted to cause a reduction in its stability, it is possible that some mutations could stabilize the repressed complex. It is possible that mutations 51 and 52 in the *flaN* promoter that resulted in a reduced level of *flbG* transcript exert their effect by altering the ability of *flbG* to escape from repression,

possibly by stabilizing the proposed repression complex. The same argument might also account for the observation that mutation 20 in *ihf2* results in greatly reduced levels of both *flaN* and *flbG*.

In conclusion, our results show that *ftr2* is required for positive regulation of *flaN*, while *ftr3* appears to play a negative regulatory role in *flaN* and *flbG* expression. To explain the coordinated positive activation and negative autoregulation of these two transcription units, we proposed a model in which *flaN* and *flbG* promoters and *ftr* elements interact through alternative DNA looping. The regulated interconversion of looped DNA structures like those shown in Fig. 7B and C could be an integral part of the mechanism for the cell cycle regulation of *flaN* and *flbG* expression, and mutations that result in elevated levels of transcript may mirror the events that normally lead to induction at the correct time in the cell cycle.

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