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Ntr-Like Promoters and Upstream Regulatory Sequence *ftr* Are Required for Transcription of a Developmentally Regulated *Caulobacter crescentus* Flagellar Gene

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The *flbG* (hook operon or transcription unit II) and *flaN* (transcription unit I) operons of *Caulobacter crescentus* have a −12, −24 nucleotide sequence motif that is very similar to those of the Nif and Ntr promoters of enteric bacteria and *Rhizobium* spp. and a conserved *ftr* (flagellar gene transcription regulation) sequence, previously designated II-1 (D. A. Mullin, S. A. Minnich, L.-S. Chen, and A. Newton, J. Mol. Biol. 195:939–943, 1987) at approximately −100. We have used site-directed mutagenesis to examine the role of these sequences in the transcriptional regulation of these periodically expressed flagellar genes. Mutations in the *flbG* promoter that removed the conserved GC at −12, −13, the GG at −24, −25, or an AC base pair at −18, −19 in the nonconserved sequence between the −12, −24 elements completely eliminated detectable transcription. Mutations at other positions resulted in either a slight decrease (position 26), no change (position 15), or an elevated level (position −16 or −19) of the *flbG* transcript. By contrast, most of these *flbG* promoter mutations resulted in greatly elevated levels of transcription from the opposing *flaN* operon. Similar experiments were used to confirm the location of the *flaN* promoter to a −12, −24 Nif and Ntr sequence motif. Deletion of all or part of the *ftr* element or point mutations in the sequence drastically reduced the level of *flbG* transcript and resulted in increased levels of the *flaN* transcript. Thus, the conserved sequences at −12 and −24 in *flbG* and *flaN* are required for transcription of these genes in vivo, and the *ftr* element is required for transcription of *flbG*. This analysis also suggested that the *ftr* sequence and sequences in the *flbG* promoter are required for the negative autoregulation of the *flbG* and *flaN* operons. We speculate that the *flbG* and *flaN* promoters and the *ftr* element interact in some way to mediate the negative control of these divergent transcription units.

Flagellum biosynthesis is the most intensively studied developmental event in the *Caulobacter crescentus* cell cycle because of its well-defined patterns of temporal and spatial regulation (reviewed in reference 22). The *flbG*, *flaN*, and *flaO* transcription units in the hook gene cluster (10, 24) and *flgJ*, *flgK*, and *flgL* units in the *flaEY* cluster (18, 19) are periodically transcribed in the cell cycle at the time of flagellum assembly, and the order of transcription and translation of the hook and flagellin genes parallels the order in which the products of these genes are assembled into the growing flagellum (16, 19, 24, 30). The timing of *fla* gene expression in the *C. crescentus* cell cycle appears to be regulated in part by differential transcription and promoter recognition (9, 10, 19, 21, 24).

To identify the sequence elements that regulate the timing of periodic *fla* gene transcription, we have previously cloned and sequenced promoter regions of the *flbG*, *flaN*, and *flaO* genes, which are in the hook gene cluster (10, 21). Comparison of these sequences with sequences 5′ to the flagellin genes *flgL*, *flgK*, and *flgJ* (19) in the *flaEY* gene cluster revealed that the *flaK*, *flaN*, *flgK*, and *flgL* genes, all of which are at the bottom levels of a proposed *fla* gene regulatory hierarchy (9, 10, 22, 24), have highly conserved sequences at −12 (TTGC) and −24 ([C/T] TGG [C/GC]C) (21). These sequences are very similar to the −12, −24 motif proposed for *Klebsiella pneumoniae* (4) and *Rhizobium* spp. *nifA*-regulated promoters (1) and for the *glnA* gene of *Escherichia coli* (28). The *C. crescentus fla* genes may belong to several promoter classes, since the *flaO* and *flbF* transcription units, which are immediately above the *flbG* and *flaN* genes in the proposed regulatory hierarchy (21, 22), have sequences near the transcription start site with little or no similarity to the −12, −24 promoter motif, and they may contain other classes of promoters (21, 23). Given that the Ntr promoters in *E. coli* and *Salmonella typhimurium* are recognized by a minor σ^{54} RNA polymerase (12, 13), *fla* gene transcription in *C. crescentus* may require multiple, specialized RNA polymerases (22, 23).

A third conserved sequence, *ftr* (flagellar gene transcription regulation), previously designated II-1 (21), contains the consensus sequence C-C-CGGC--AAA--GC-G. This sequence is located at about position −100 from the transcription start sites of *flaK*, *flaN*, *flgL*, and *flgK* (10, 19, 21), and we have proposed that it might be a *cis*-acting element required for the regulation of these *fla* genes (21). Transcription of the four *fla* genes containing the Ntr-like promoters (*flaK*, *flaN*, *flgL*, and *flgK*) requires products of the *flaO* and *flbF* operons in *trans*. This result has suggested that products of *flaO* and *flbF* act directly or indirectly as positive activators of *fla* genes containing the *ftr* element at −100 (10, 24). In addition, the *flbG* and *flaN* operons are under negative control by the hook protein gene *flaK* (10, 21).

In this paper, we present evidence that the conserved promoter elements at −12 and −24 are required for *flbG* expression and conclude that the Ntr-like consensus elements identified in *C. crescentus* are used in vivo as promoters for *fla* gene transcription. We also demonstrate the requirement of a third sequence element *ftr* at −100 for transcription of *flbG* and possibly for autoregulation of *flaN*. We discuss the possibility that the *flbG* and *flaN* promoters

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TABLE 1. Bacterial strains, plasmids, and phages

Strain, plasmid, or phage	Relevant genotype or phenotype	Source or reference
Bacterial strains		
C. crescentus		
CB15	Wild type	27
SC511	*flaK155*::IS*511*	25
SC1052	*flaO172*::Tn*5*	25
SC1132	*flbF608*::Tn*5*	25
E. coli		
HB101		5
JM107	Male	31
CJ236	*dut ung* male Cm^r	15
Plasmids		
pUC19	Ap^r	31
pUC18	Ap^r	31
pUC19603	Ap^r	This work
pUC19285	Ap^r	This work
pRK290	Tc^r	11
pRK2L1	Tc^r	This work
pRK2L1-603	Tc^r	This work
pRK2L1-181	Tc^r	This work
pRK2L1-177	Tc^r	This work
pRK2L1-320	Tc^r	This work
pRK2L1-434	Tc^r	This work
pRK2L1-168	Tc^r	This work
pFK2L1-105	Tc^r	This work
Phages		
M13mp19		31
M13mp18		31
M13mp19603		This work
M13mp18177		This work

and the *ftr* element functionally interact to mediate the regulation of these two divergent transcription units.

MATERIALS AND METHODS

Strains and culture conditions. The bacterial strains, plasmids, and bacteriophages used in this work are listed in Table 1. Recombinant pRK290 (11) plasmids were introduced into *C. crescentus* CB15 or mutants either by conjugation (25) or by electroporation with an Electroporator (Bio-Rad Laboratories, Richmond, Calif.) (S. Minnich and D. Mullin, unpublished data). *C. crescentus* CB15 (ATCC 19089) and mutant derivatives were grown in a peptone-yeast extract medium (0.2% peptone, 0.1% yeast extract, 0.02% $MgSO_4$) (27). *C. crescentus* strains that contained plasmids are grown in peptone-yeast extract broth containing 2 μg of tetracycline per ml. *E. coli* HB101 (5) was used as a host for plasmid transformation and was grown in yeast extract-tryptone medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.02% $MgSO_4$, supplemented with appropriate antibiotics [50 μg of ampicillin or 10 μg of tetracycline per ml]). *E. coli* JM107 (31) was used as a host for phage M13, and *E. coli* CJ236 was used as a host for phage M13 before oligonucleotide mutagenesis (15).

Isolation of *Caulobacter* RNA and nuclease S1 mapping. Cellular RNA was purified from *C. crescentus* cells as described previously (24). As a control for the amount and integrity of RNA in various preparations, the genomic *flaO* transcript (transcription unit III), whose expression is independent of *flbG* and *flaN*, was routinely determined for each RNA preparation by using the 285-base-pair (bp) *Hin*dIII-*Bam*HI fragment (Fig. 1) in the nuclease S1 assay. This probe produced a protected DNA fragment of about 80 bp (data not shown) and was useful for comparing levels of RNA between independent RNA preparations isolated from wild-type cells.

We also isolated RNA from cultures derived from four independently isolated CB15 colonies carrying pRK2L1-603, and the levels of protected fragments corresponding to the *flbG* and *flaN* transcripts appeared to be similar in all preparations (data not shown). This last control suggested that the level of *flbG* and *flaN* transcripts detected in CB15(pRK2L1-603) did not vary widely from preparation to preparation.

DNA restriction fragments from hybrid plasmids were electroeluted from agarose gels, 5′ end labeled with [λ-^{32}P] ATP and T4 polynucleotide kinase, and used in nuclease S1

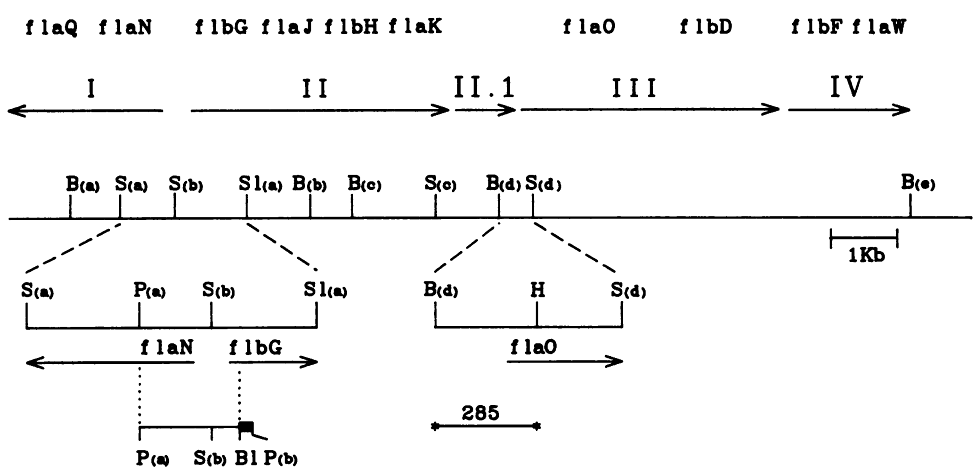

FIG. 1. Physical and genetic map of the hook gene cluster, showing the organization of transcription units, *fla* and *flb* genes, and restriction map of the hook gene cluster (25). Locations of the transcription start sites for *flaN*, *flaK*, and *flaO* are designated I, II, and III, respectively, and have been described previously (10, 21). Transcription unit II.1 is a small transcription unit located between the *flbG* and *flaO* operons (10). Relevant restriction sites: B, *Bam*HI; S, *Sst*I; Sl, *Sal*I; P, *Pst*I; H, *Hin*dIII. Bl, Location of a *Bal*31 deletion endpoint. The 285-nt genomic nuclease S1 probe for *flaO* is shown; *ftr* indicates 5′-^{32}P-labeled ends. Symbols: ——, genomic sequences; ■, polylinker DNA derived from pUC18.

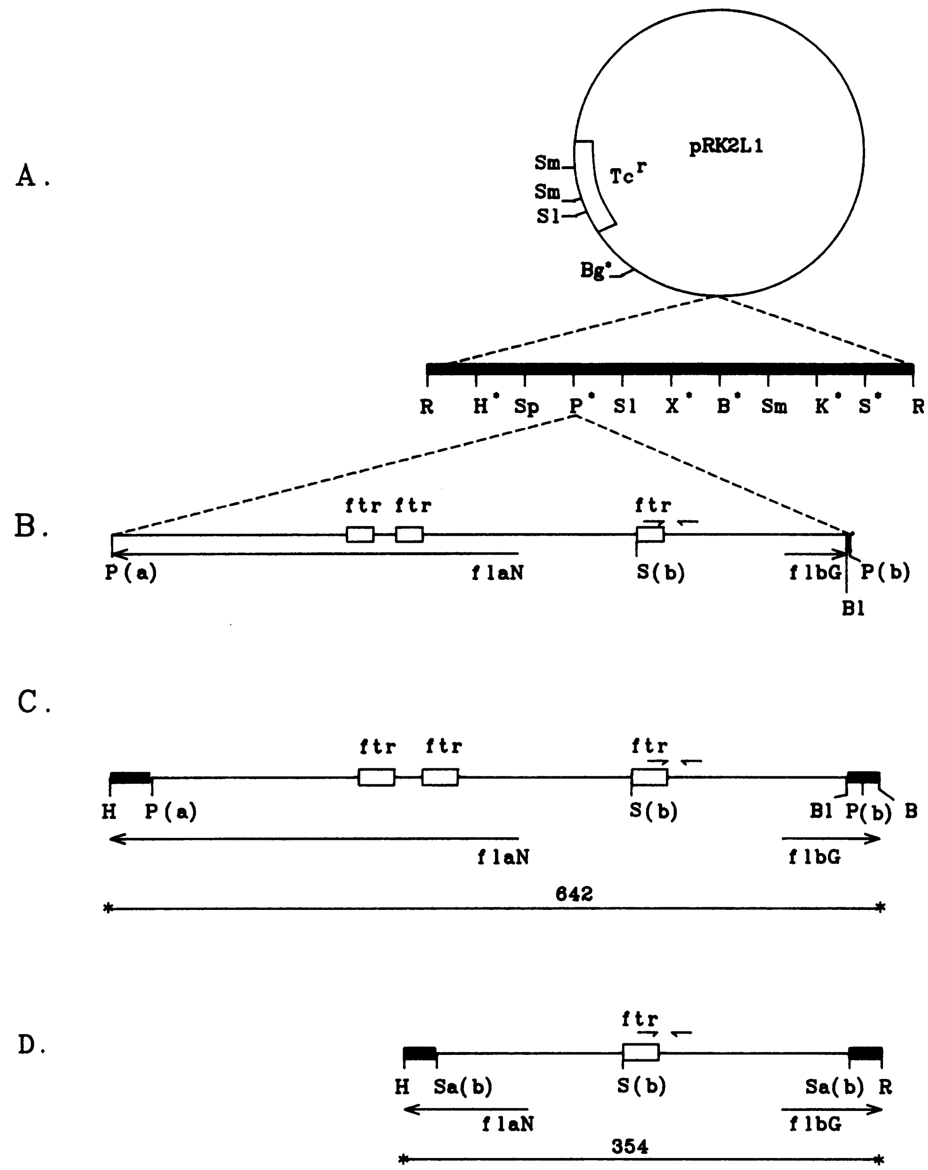

FIG. 2. Physical map of pRK2L1. (A) Map of pRK2L1 showing sequences derived from pRK290 (——) (11) and the polylinker sequence from pUC18 (▬) (31). Restriction sites are as indicated in the legend to Fig. 1; B1, position of a *Bal*31 deletion endpoint. (B) Site of insertion of the 603-bp *Pst*I(a)-*Pst*I(b) fragment (Fig. 1). Shown are the origin and direction of transcription (←, →) and the arms of sequences with dyad symmetry, (⇀, ↽). (C) Probe for nuclease S1 assays of the level of plasmid-encoded *flbG* and *flaN* mRNA in pRK2L1-603. *, 5′-^{32}P-labeled ends; ▭, location of *ftr* sequence. (D) Probe for nuclease S1 assays of the level of plasmid-encoded *flbG* and *flaN* mRNA in pRK2L1-321. Symbols and abbreviations are as given above.

assays (3). The hybridization temperature for all probes was 55°C. The 285-nucleotide (nt) *Bam*HI-*Hin*dIII fragment (Fig. 1) labeled at both 5′ ends was used as a probe for genomic *flaO* 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 the two probes gave the same protected fragment in nuclease S1 assays (21). The probes used to detect transcripts from *flbG* and *flaN* in nuclease S1 assays are described in Results. An excess amounts of ^{32}P-labeled probe DNA was 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 were fractionated in 6% polyacrylamide gels containing 8 M urea.

DNA sequencing. DNA fragments of interest were cloned into M13mp19 replicative-form DNA (31) for dideoxy-nucleotide sequencing (29). Dideoxy sequencing was performed by using [α-^{32}P]dATP as the radiolabel, and 7-deazaguanosine 5′-triphosphate was substituted for guanosine 5′-triphosphate in the sequencing reactions to reduce compression artifacts (2, 20). Plasmids were introduced into *E. coli* by a calcium chloride transformation method (17).

Plasmid construction. Plasmid pRK2L1 (Fig. 2A) was constructed as follows. The multiple restriction site polylinker from pUC18 (31) was purified from a 2% agarose gel as a 64-bp *Eco*RI-*Hae*III DNA fragment. *Eco*RI linkers were added to the *Hae*III end of this fragment, and after digestion with *Eco*RI, the resulting polylinker was ligated to the *Eco*RI site of pRK290 (11) to generate pRK2L1.

The 603-bp *Pst*I(a)-*Pst*I(b) fragment (Fig. 1), which contains the *flbG* and *flaN* promoters and *ftr*, extends from the genomic *Pst*I(a) site 3′ from the *flaN* promoter to a *Bal*31 deletion endpoint located 54 bp downstream from the *flbG* transcription start site, to which 6 bp of the polylinker DNA derived from M13mp19 (from *Hin*cII to *Pst*I) had been added. pUC19603 consists of pUC19 with the 603-bp *Pst*I(a)-*Pst*I(b) fragment in the *Pst*I site (*flbG* promoter is proximal to the *Bam*HI site in the pUC19 polylinker), and M13mp19603 consists of M13mp19 with the 603-bp *Pst*I fragment in the *Pst*I site (*flbG* promoter proximal to the *Bam*HI site in the polylinker). Plasmid pRK2L1-603 (Fig. 2B) was constructed by ligating the 642-bp *Hin*dIII-*Bam*HI fragment (Fig. 2C) derived from pUC19603 to pRK2L1 that had been cleaved with *Hin*dIII and *Bam*HI.

The plasmids described below are derivatives of pRK2L1-603 with various deletions of the 603-bp insert (see Fig. 7). pRK2L1-434 contains the *flaN* promoter but lacks the *flbG* promoter and *ftr* element at −100. This plasmid was constructed by cleaving pRK2L1-603 with *Sst*I and *Hin*dIII and ligating the purified 434-bp *Sst*I-*Hin*dIII fragment to pRK2L1 cleaved with *Sst*I and *Hin*dIII. pRK2L1-321 was constructed by cloning a 321-bp genomic *Sau*3A(a)-*Sau*3A(b) fragment containing the *flaN* and *flbG* promoter in the *Bam*HI site of pRK2L1. In this construct, the *flbG* promoter is proximal to the *Hin*dIII site in the polylinker of pRK2L1 (Fig. 2A). The probe used to measure *flbG* mRNA made by pRK2L1-321 was a 354-bp *Hin*dIII-*Eco*RI fragment (Fig. 2D), ^{32}P labeled at both 5′ ends, that contains the 321-bp *Sau*3A(a)-*Sau*3A(b) fragment flanked by sequences derived from the polylinker cloning site of pRK2L1. Although the *flaK* and *flaN* promoters are present on the 321-bp *Sau*3A(a)-*Sau*3A(b) fragment, only a transcript corresponding to that expected from the *flaK* promoter was detected. It is possible that deletion of one or more of the *ftr* elements or some other sequence that maps 3′ from the *Sau*3A(a) site in *flaN* is required for *flaN* transcription (see Results). pRK2L1-181 was constructed by cleaving pRK2L1-321 with *Sst*I and *Hin*dIII and ligating it to pRK2L1 cleaved with *Sst*I and *Hin*dIII. pRK2L1-181 extends from *Sst*I(b) to a polylinker-derived *Hin*dIII site (Fig. 2C) and contains the *ftr* and the *flbG* promoter, but it lacks the *flaN* promoter. The probe used to measure the level of *flbG* mRNA made by pRK2L1-181 was the 354-bp *Hin*dIII-*Eco*RI fragment (Fig. 2D) ^{32}P labeled at both 5′ ends.

M13mp18177 was made by cleaving pRK2L1-321 (Fig. 2D) with *Sst*I, followed by treatment with T4 DNA polymerase in the presence of all four deoxynucleoside triphosphates to remove the overhanging 3′ sticky ends of the *Sst*I site. The plasmid was then digested with *Hin*dIII and ligated to M13mp18 that had been cleaved with *Hin*dIII and *Hin*cII to generate M13mp18177. M13mp18177, used to determine the nucleotide sequence at the deletion endpoint generated by the T4 DNA polymerase, was cleaved with *Hin*dIII and *Bam*HI and ligated to pRK2L1 cleaved with *Hin*dIII and *Bam*HI to generate pRK2L1-177. pRK2L1-168 and pRK2L1-105 are deletion derivatives of pRK2L1-181 made by allowing *Bal*31 nuclease to digest from the *Sst*I(b) site toward the *flbG* promoter. The deletion endpoints of pRK2L1-168 and pRK2L1-105 were determined by DNA sequence analysis. The probe used to measure the level of *flbG* mRNA made from each of these deletion derivatives in nuclease S1 assays was the 354-bp *Hin*dIII-*Eco*RI fragment ^{32}P labeled at both 5′ ends (Fig. 2D).

pUC19285 was constructed by ligating a 285-bp *Hin*dIII-*Bam*HI fragment that contains the *flaO* promoter (21) to pUC19 that had been cleaved with *Hin*dIII and *Bam*HI. This plasmid was used as the source of the 285-bp *Hin*dIII-*Bam*HI fragment which was 5′ end labeled with ^{32}P and used as a probe in nuclease S1 assays for the 5′ end of the *flaO* transcript.

Oligonucleotide mutagenesis. Single-primer mutagenesis in M13mp19603 was performed essentially as described by Kunkel et al. (15). Mutagenic oligodeoxyribonucleotides were obtained from the DNA synthesis facility in the Department of Molecular Biology, Princeton University, Princeton, N.J. 5′-Terminal phosphates were added to the mutagenic primers by using ATP and T4 polynucleotide kinase. Mutagenic primers were annealed to single-stranded virus M13mp19603 DNA isolated from the phage particles after growth on *E. coli* CJ236. After polymerization with DNA polymerase I Klenow fragment and all four deoxynucleoside triphosphates and ligation with T4 DNA ligase, the resulting DNA molecules were used to transfect *E. coli* JM107. After plaque purification and DNA sequencing to obtain clones with the desired mutation, replicative-form DNA was prepared and cleaved with *Hin*dIII and *Bam*HI, and the 642-bp fragment containing the mutation was cloned into pRK2L1. Initially we introduced recombinant pRK2L1 derivatives into *C. crescentus* by conjugation, but we now routinely use electroporation (Minnich and Mullin, in preparation). After transfer to *C. crescentus*, the relative level of mRNA from the cloned *flbG* and *flaN* promoters was measured in a nuclease S1 assay, using in vivo-isolated RNA from plasmid-containing cells and the 642-bp *Hin*dIII-*Bam*HI DNA probe (Fig. 2C). Only RNA made from the plasmid-borne *flbG* and *flaN* promoters can protect the 5′-^{32}P-labeled ends of the 642-bp *Bam*HI-*Hin*dIII probe from nuclease S1 hydrolysis because the labeled 5′ phosphates in this probe are on nucleotides derived from the plasmid polylinker sequence.

RESULTS

Cloning and expression of the *flbG* and *flaN* promoters. The in vivo transcription start sites of the divergent *flbG* and *flaN* operons (transcription units I and II, respectively; Fig. 1) were determined previously by nuclease S1 mapping (10, 21), and nucleotide sequencing has shown that the 5′ sequences are very similar at −12 and −24 to the Nif and Ntr promoters described in *Rhizobium* spp. and the enteric bacteria (1). *flbG* and *flaN* are coordinately expressed in the cell cycle and have the same genetic requirements for transcription: they are both under positive regulation by genes in the *flaO* and *flbF* operons (transcription units III and IV, respectively; Fig. 1) and under negative regulation by *flaK* (10, 21).

Experiments to determine the requirement of the −12, −24 sequences and the *ftr* sequence at −100 for regulation of the *flbG* and *flaN* promoters were carried out with the 603-bp *Pst*I(a)-*Pst*I(b) DNA fragment (Fig. 1) that had been cloned in the *Pst*I site of pRK2L1 (Fig. 2A) to yield pRK2L1-603 (Fig. 2B). The 642-bp *Hin*dIII-*Bam*HI fragment from pRK2L1-603, which had been ^{32}P labeled at both 5′ ends (Fig. 2C), was used as a probe to estimate in vivo levels of both *flbG* and *flaN* transcripts from the plasmid in the same nuclease S1 assay. Transcripts from the chromosome were not detected in this assay, since the 5′ ends of the probe were derived from sequences in the polylinker of plasmid pRK2L1-603. RNA isolated from strain CB15(pRK2L1-603)

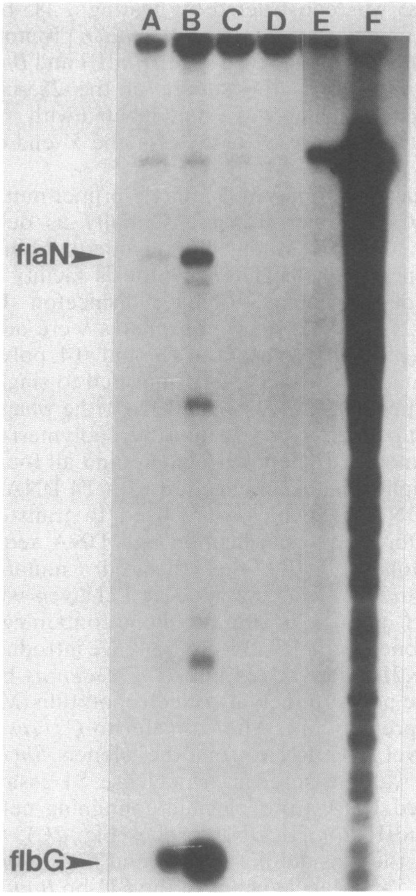

FIG. 3. Nuclease S1 analysis of the level of plasmid-encoded *flbG* and *flaN* mRNA in pRK2L1-603. Total RNA was prepared from *C. crescentus* and used in nuclease S1 assays as described in Materials and Methods. Double-stranded DNA probes labeled at both 5′ ends were present in all of the reactions, and nuclease S1 was present in lanes A to E. RNA (100 μg) was added where indicated. Positions of the 349-nt (*flaN*) and 80-nt (*flbG*) protected fragments are indicated. Lanes: A, CB15(pRK2L1-603) RNA, 642-nt *Hin*dIII-*Bam*HI probe; B, SC511(pRK2L1-603) RNA, 642-nt *Hin*dIII-*Bam*HI probe; C, SC1052(pRK2L1-603) RNA, 642-nt *Hin*dIII-*Bam*HI probe; D, SC1132(pRK2L1-603) RNA, 642-nt *Hin*dIII-*Bam*HI probe; E and F, 642-nt *Hin*dIII-*Bam*HI probe with (lane E) and without (lane F) S1 nuclease.

protected fragments of 349 and 80 nt (Fig. 3, lane A), which are the sizes expected for transcripts from the plasmid-borne *flaN* and *flbG* promoters, respectively (10, 21). Partially protected fragments were not detected when RNA from strain CB15 or CB15 containing the vector pRK2L1 was assayed (data not shown).

To determine whether the *flbG* and *flaN* promoters on plasmid pRK2L1-603 are under the same genetic regulation as are the genomic promoters, the levels of plasmid-encoded *flbG* and *flaN* transcripts were measured in strains with mutations in *flaK*, which is known to negatively regulate transcription units I and II, or in *flaO* or *flbF*, both of which are required in *trans* for expression of *flbG* and *flaN* (10, 21). The levels of the *flbG* and *flaN* transcripts in *flaK* mutant SC511 (Fig. 3, lane B) were about 10-fold higher than in wild-type strain CB15 (lane A), and neither of the transcripts could be detected in *flaO* mutant strain SC1052 (lane C) or *flbF* mutant strain SC1132 (lane D). Therefore, *flbG* and *flaN* on plasmid PRK2L1-603 are subject to the same positive and negative regulation by genes in the hook gene cluster reported previously for the genomic copies of the two promoters (10, 21). The 603-bp insert in pRK2L1-603 was therefore used for mutagenesis to assess the nucleotide sequence requirements for transcriptional regulation of *flbG* and *flaN*.

Mutagenesis of the *flbG* promoter. The nucleotide sequence changes introduced into the *flbG* promoter are summarized in Fig. 4. Plasmids in which the conserved GC at −12, −13 (Fig. 4, #4) or the GG at −24, −25 (Fig. 4, #5) of the *flbG* promoter was deleted did not produce detectable *flbG* mRNA (Fig. 5, lanes E and F); a deletion of the AC at −18, −19 (Fig. 4, #2) in the nonconserved spacer region between the −12 and −24 elements also eliminated detectable levels of the *flbG* transcript (Fig. 5, lane C). Unexpectedly, the levels of *flaN* transcript in *flbG* promoter mutants with the 2-bp deletions at −12, −13 and at −24, −25 (Fig. 5, lanes E and F, respectively) were much higher than the wild-type levels (Fig. 5, lane A), whereas the deletion at −18, −19 had no apparent effect on the level of the *flaN* mRNA (Fig. 5, lane C). Point mutations at −16 and −19 (Fig. 4, #8 and #6, respectively) in the *flbG* promoter resulted in a significant increase in the level of both the *flbG* and *flaN* transcripts (Fig. 5, lanes G and H, respectively). A transition mutation at −15 (Fig. 4, #3) had no apparent effect on the level of the *flbG* mRNA (Fig. 5, lane D), and a transition at −26 (Fig. 4, #1) resulted in a slight decrease in the level of this transcript (Fig. 5, lane B). Both of the latter mutations significantly increased the level of the *flaN* transcript, however (Fig. 5, lanes D and B).

A summary of these results (Table 2) indicates that mutations in the *flbG* promoter that disrupted the highly conserved sequences characteristic of Nif (Ntr) promoters, including the −12 and −24 elements and the spacing between them, resulted in reduced or undetectable levels of the *flbG* transcript. The elevated levels of the *flaN* transcript observed in most of the *flbG* promoter mutants (except in the 2-bp deletion mutant at −18, −19) was not simply a result of lower levels of *flbG* transcription, since mutations at −19 and −16 resulted in higher levels of both the *flbG* and *flaN* transcripts.

Identification of the *flaN* promoter. DNA sequence analysis 5′ from the site of transcription initiation of *flaN* (transcription unit I) revealed two possible promoter sequences, which were designated PI and PI* (21; see legend to Fig. 6). PI is located at the expected position relative to the 5′ end of the mRNA, but the sequence conforms poorly to the Ntr promoter consensus sequence, whereas PI*, which is located 23 bp upstream from PI, matches the Ntr *fla* gene promoter consensus in 11 of 11 nucleotides. The T at position −15 was changed to G in PI and PI* (Fig. 4, #10 and #12, respectively), and the effect on transcription of *flaN* from the plasmid was assayed. The level of the *flaN* transcript was unchanged by mutation 10 in PI (Fig. 5, lane I), whereas mutation 12 in PI* caused a significant reduction in the level of the *flaN* transcript (Fig. 5, lane J). This result indicates that PI* and not PI is the *flaN* promoter sequence. The previous identification of a 5′ *flaN* messenger terminus by nuclease S1 mapping approximately 23 bp downstream from the presumptive transcription start site of promoter PI* suggests that the *flaN* mRNA undergoes a rapid and specific processing at its 5′ end that removes approximately 23 nt. Consistent with this interpretation is the possible formation of a weak stem-and-loop structure in the *flaN* transcript near the site identified as the major 5′ end by in vivo nuclease S1 assay (21). Processing of the *flbG* messenger (transcription

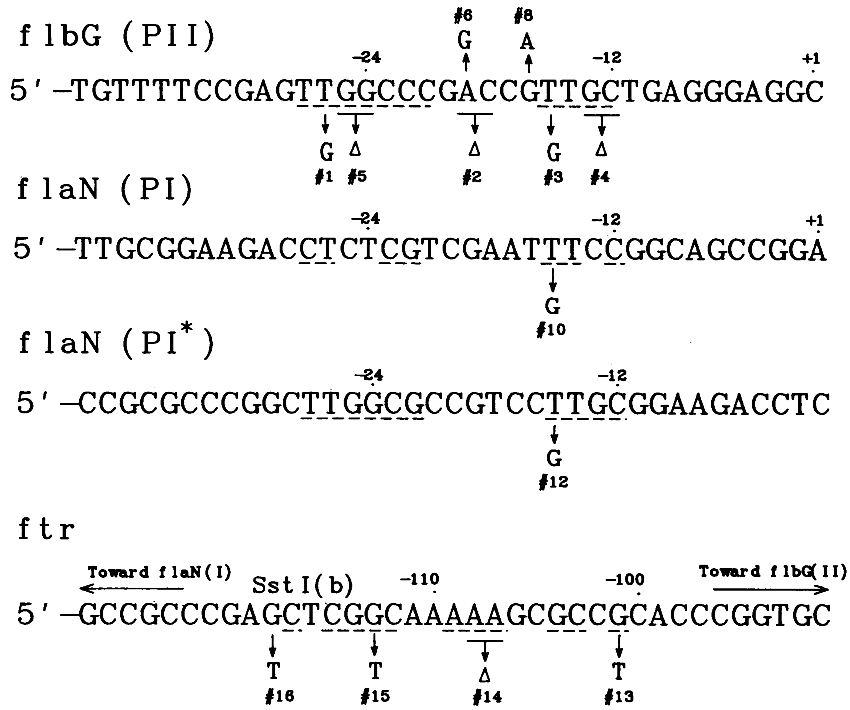

FIG. 4. Summary of primer mutagenesis of *ftr* and the promoters of *flbG* and *flaN*. Symbols: ↓, ↑, position and nature of mutation created and analyzed in this study; – – –, nucleotides that match the consensus sequence for the promoter or *ftr* sequence element.

unit II) has also been observed by nuclease S1 mapping, although the full-length mRNA from *flbG* can be easily detected (10). Additional mutations will be required to confirm the location of the *flaN* promoter and its effect on *fla* gene regulation (see Discussion).

Sequence elements required for regulation of *flbG* transcription are contained within a 181-bp *Sst*I-*Hin*dIII fragment. To determine whether an active *flbG* promoter can be separated from sequences containing the *flaN* promoter, the *Sst*I(b)-*Hin*dIII fragment (Fig. 2D) from pRK2L1-321 was subcloned in pRK2L1 to yield pRK2L1-181 (Fig. 7). When pRK2L1-181 was introduced into strains CB15, SC511, SC1052, and SC1132, the level of plasmid-encoded *flbG* transcript (Fig. 8A, lanes B to E) responded to the different genetic backgrounds in the same way as described above for pRK2L1-603 (Fig. 3), which contains both *flbG* and *flaN*. The protection of an 85-nt fragment in nuclease S1 assays using the 354-nt *Hin*dIII-*Eco*RI probe (Fig. 2D) by RNA isolated from CB15(pRK2L1-181) (Fig. 8a, lane B) showed that transcription was initiated within the expected sequence for *flbG*. These results suggest that all of the elements needed for regulation of the *flbG* promoter are contained within the 181-bp *Sst*I(b)-*Hin*dIII fragment in pRK2L1-181 and that regulated expression in *flbG* does not require the *flaN* promoter in *cis*.

Sequence element *ftr* is required for transcription of *flbG*. To identify nucleotide sequences essential for regulated expression of the *flbG* promoter, deletions extending across the 181-bp *Sst*I-*Hin*dIII fragment in pRK2L1-181 (Fig. 7) were isolated and assayed as described above. Removal of the 4-nt single-stranded 3′ end at the *Sst*I(b) site of pRK2L1-181 to generate pRK2L1-177 (Fig. 7) resulted in a greatly diminished level of the *flbG* transcript relative to that made by pRK2L1-181 (Fig. 8a, compare lanes B and F). The deletion of 13 bp (plasmid pRK2L1-168; Fig. 7) or the entire *ftr* element (plasmid pRK2L1-105; Fig. 7) resulted in barely detectable levels of the *flbG* transcript (Fig. 8a, lanes G and H, respectively). These results demonstrate that removal of all or a small part of the *ftr* element dramatically decreased the level of the plasmid-encoded *flbG* transcript, which suggests that this sequence can act in *cis* as an upstream activator of *flbG* transcription.

We also examined the effect of the *ftr* deletion mutations on transcription in *flaK* mutant strain SC511. Levels of *flbG* transcript made from plasmids pRK2L1-168 and pRK2L1-105 (Fig. 7) in these constructs were elevated in the SC511 background (data not shown). This result argues that a sequence(s) 3′ to the deletion endpoint in plasmid pRK2L1-105 is involved in the autoregulation of *flbG*, a conclusion consistent with the effect of some *flbG* promoter mutations on *flbG* expression (Table 2).

Single-base-pair changes were introduced into the *ftr* sequence to identify specific nucleotides required for activation of *flbG*. A G-to-T transversion at −113 from the

TABLE 2. Effect of mutations in the *flbG* promoter on levels of *flbG* and *flaN* transcripts

Transcript	Level with indicated *flbG* sequence mutation[a]											
	−24				−18				−12			
	T	T/G	<u>GG</u>	CCCG	G	<u>AC</u>	C	G/A	T/G	T	<u>GC</u>	T
In vivo												
flbG		+/−	−		++	−		++	+		−	
flaN		++	++		++	+		++	++		++	
In vitro[b]												
flbG		−	−		ND	−		+	−		−	

[a] Levels of transcripts (judged by visual inspection of autoradiograms from nuclease S1 experiments): +, wild type; +/−, reduced; undetectable; ++, elevated. ND, Not done. Bases removed by deletion are underlined.

[b] Determined by Ninfa et al. (23) by using σ^{54} RNA polymerse from *E. coli*.

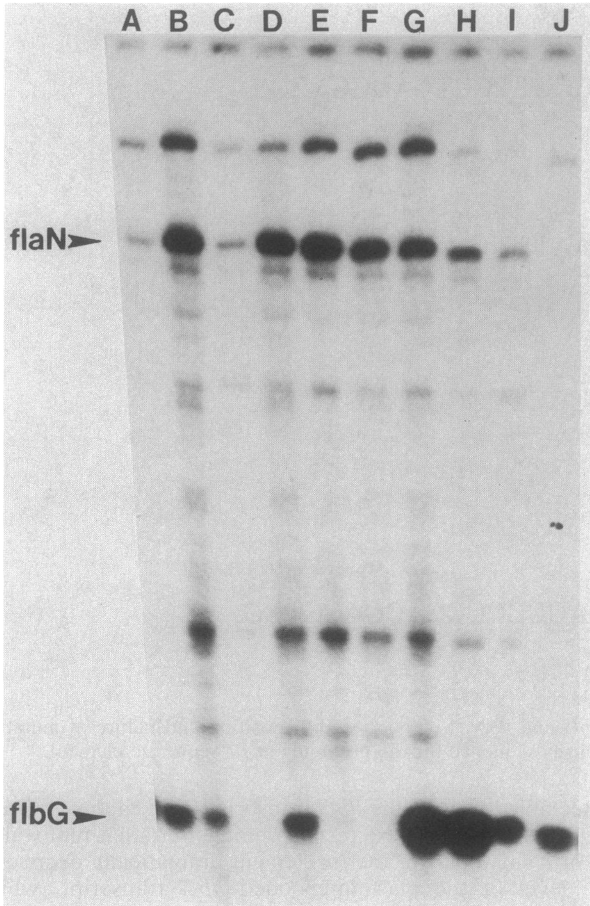

FIG. 5. Nuclease S1 analysis of point mutations in the *flbG* and *flaN* promoters. Nuclease S1 assays were carried out as described in the legend to Fig. 3. Nuclease S1 was present in lanes A to J. The probe in each case was the 642-nt *Bam*HI-*Hin*dIII fragment (Fig. 2C) labeled with ^{32}P at both 5′ ends. Positions of the 349-nt (*flaN*) and the 80-nt (*flbG*) protected fragments are indicated. Mutations present in plasmids are indicated by number (see Fig. 4). Lanes: A, CB15 (pRK2L1-603); B, CB15 (pRK2L1-603), mutation 1; C to J, CB15 pRK2L1-603), mutations 2 to 6, 8, 10, and 12, respectively.

transcription start site of *flbG* (Fig. 4, #15) abolished the *flbG* transcript without affecting the level of the *flaN* transcript (Fig. 8b, lane D); a deletion of the AA dinucleotide at −108, −107 had the same effect (Fig. 4 and Fig. 8b, lane F). Mutations at positions −101 (mutation 13) and −118 (mutation 16) resulted in a somewhat increased level of the *flbG* transcript and a much larger increase in the level of the *flaN* transcript (Fig. 8b, lanes C and E, respectively).

We also examined the effect of deleting the *ftr* sequence element on *flaN* expression by removing DNA from the *Sst*I(b) site to the *Bam*HI site in pRK2L1-603 (Fig. 2C) to generate pRK2L1-434 (Fig. 7). The *flaN* transcript was still detected from plasmid pRK2L1-434 and increased severalfold compared with the level from the parental plasmid (Fig. 8b, lane G). Thus, unlike the case for *flbG*, transcription of *flaN* does not appear to require the *ftr* element at −100. The upstream *ftr* element may be involved in the negative regulation of the *flaN* and the *flbG* operons, as indicated by this result and the effect of *ftr* point mutations #13 and #16 discussed above, as well as in the positive regulation of the *flbG* operon.

```
        10        20        30        40        50
AAGCTTGCATGCCTGCAGGAACTGGTCGGTGGCGCGCTTGATGGCGTCGA
HindIII SphI  PstI(a)
        60        70        80        90       100
TATTGACGCCCACGCCCATGCCGTTGGACAGCAGGTTGGACTGGCTGACC

       110       120       130       140       150
GTCTTGCGGACATAGCCGGTGGTGTTGACGTTGGCGATGTTGTCCGACGT

       160       170       180       190       200
GACGCGCAGACCCGTCTGCGCGGCCATCATGCCGGACGTCGCCGTGCTCA

       210       220       230       240  ftr  250
TGATCGCATTGAGCGACATGGATCAGGCCTCCTTCGGCTCGGCAAACCGC

       260       270       280  ftr  290       300
GCAACGCATGATGCGTCGTTGCCCGGCGAAACTTGCCGGGTCGCGGTGGG

       310       320       330       340       350
GCATGATCCCCGATGATGTGCCAAGCCATTGAAATTCATGGCGATGTGTT
                                        <--------- flaN
       360       370       380       390       400
CCGGCTGCCGGAAATTCGACGAGAGGTCTTCCGCAAGGACGGCGCCAAGC


       410       420       430       440       450
CGGGCGCGGCCCGGCGACGCGGCAACTCCCGTCCCTGAAGGCCGCCCGAG

 ftr   460       470       480       490       500
CTCGGCAAAAAGCGCCGCACCCGGTGCGATTTTTCTTCGTAAACAAGCCA
                  NtrC
       510       520       530       540       550
TTAAAGTCATTGATAACAGAGGTTTAGGTCGTGTTTTCCGAGTTGGCCCG


       560       570       580       590       600
ACCGTTGCTGAGGGAGGCGACGAAGGCGTGTCCGTCATGGAGCGCCGCCA
       flbG  --------->

       610       620       630       640
ACCGAGCAACGACTGCGCATGGACCTGCAGGTCGACTCTAGAGGATCC
                       ↑  PstI(b) SalI  XbaI  BamHI
                     Bal31
```

FIG. 6. Nucleotide sequence of the 603-nt *flbG-flaN* promoter region in pRK2L1-603. The sequence shown includes part of the flanking pUC18-derived multiple-cloning-site polylinker. The *ftr* sequence elements are boxed; →, ←, location of sequence with dyad symmetry. The location of a sequence that is similar to the consensus NtrC binding sites (12, 28) is indicated below the sequence. The G at nucleotide 621 is a deletion end generated by *Bal*31 that was ligated to a cleaved *Hin*cII site in the polylinker of M13mp19603. Double-headed arrows below the sequence indicate locations of the in vivo nuclease S1-determined transcription start sites of *flbG* and *flaN* and indicate the direction of transcription. The conserved −12, −24 elements in PI* are underscored with dots; the corresponding sequences in PI* are underscored with dashes. A graphic representation of this sequence is presented in Fig. 2B.

DISCUSSION

In this paper, we have presented direct genetic evidence that the in vivo expression of *flbG* and *flaN* in *C. crescentus* depends on promoters that are similar if not identical to the Nif and Ntr promoters of enteric bacteria and *Rhizobium* spp. (1, 4). These genes (10, 21), like flagellin genes *flgK* and *flgL*, which share the same highly conserved −12, −24 sequence elements (19), are located in the lowest levels of the *fla* gene regulatory hierarchy (22). Transcription from Ntr promoters in *E. coli* and *S. typhimurium* requires RNA polymerase containing σ^{54} instead of the major σ^{70}, and recent results show that the *C. crescentus flbG* and *flaN*

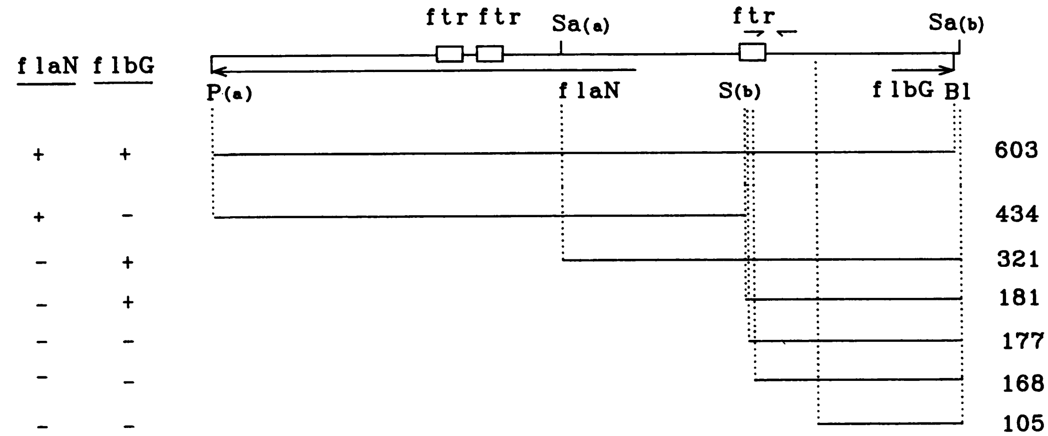

FIG. 7. Summary of deletion analysis of *flbG* and *flaN*. Symbols: ←, →, origin and direction of transcription; ⇀, ↽, location of sequence with dyad symmetry; ▭, location of the *ftr* element. All of the clones are pRK2L1 recombinants, named according to the size (in base pairs) of the inserted DNA fragment (see Materials and Methods for details of plasmid construction). +, Transcript detected in in vivo nuclease S1 assays; −, greatly reduced level or no transcript detected. Restriction sites: Sa, *Sau*3A; other sites are as indicated in the legend to Fig. 1. B1, Location of the *Bal*31 deletion endpoint in the 603-bp insert in pRK2L1-603.

promoters are recognized by purified σ^{54} RNA polymerase from *E. coli* in the presence of the transcription factors NRI and NRII (23). The 5′ regulatory sequences of *flaO*, which is at the next-highest level of the regulatory hierarchy, does not share the Ntr promoter consensus sequence (21), and it is not transcribed in vitro by *E. coli* σ^{54} RNA polymerase (23). These findings have suggested that *C. crescentus* may contain multiple forms of RNA polymerase involved in flagellum development, one of them with a promoter specificity very similar to that of the σ^{54} RNA polymerase (22, 23).

Our results also show that expression of *flbG* requires the presence of an intact regulatory sequence, *ftr*, located approximately 100 bp upstream from the transcription start site. Similar sequences are located at about −100 in *flgK* and *flgL* (19), and it seems likely that these *ftr* elements are also required in *cis* for transcription of these flagellin genes. The *ftr* element located between *flbG* and *flaN* is not required for transcription of *flaN*, however, and levels of the *flbG* and *flaN* transcripts are actually increased in certain point and deletion mutations of *ftr*. Thus, the coordinate, positive regulation of *flbG* and *flaN* cannot be accounted for by this upstream *ftr* sequence alone. There are two homologs of the *ftr* sequence element in the *flaN* operon (Fig. 6), however, and the absence of these downstream elements in plasmid pRK2L1-321 (Fig. 7) could account for our failure to detect the *flaN* transcript in this construct (Fig. 8a). In addition, we have identified a dyad adjacent to the *ftr* site (Fig. 6) with homology to the NtrC (NRI) site required for activation of the Ntr promoter *glnAp2* in *E. coli*, although the arms of the dyad in the *C. crescentus* sequence are more closely spaced than are those of the NtrC site in *E. coli* (28). More extensive site-specific mutagenesis of the *ftr* and adjacent sequences will be necessary to evaluate their roles in *flbG* and *flaN* transcription.

Mutagenesis of the conserved 5′ region of the *K. pneumoniae nifH* promoter has shown that the GG dinucleotide at −24, −25, the G at −13, and the 10-bp spacing between the conserved GG and the GC are essential for the function of this class of promoter in vivo (6–8, 26). Our results indicate that these same sequence elements are required for activity of the *C. crescentus flbG* promoter (Table 2) and very likely for activity of the *flaN* promoter, which has very similar −12 and −24 sequence elements (21). Mutations at other positions in the *flbG* promoter resulted in either a slight decrease (position −26), no change (position −15), or an elevated level (position −16 or −19) of the *flbG* transcript. By contrast, none of the mutant *flbG* templates examined except no. 8, which was changed at position −16 of the nonconserved spacer, was active in vitro with the σ^{54} RNA polymerase from *E. coli* (Table 2; 23). This difference between the in vivo and in vitro results may be explained by a difference in specificity between the *E. coli* σ^{54} enzyme and the *C. crescentus* RNA polymerase that transcribes these promoters. Alternatively, reduced *flbG* promoter activity of some mutants could be masked in the in vivo assay by the coincident derepression of *flbG* transcription, such as that observed for mutations at −16 and −19 and discussed below (Table 2).

Our results suggest that PI*, which has excellent homology to the Ntr-type sequence at the *flbG* promoter, is the *flaN* promoter (Fig. 4). Since PI* is found 33 bp upstream from the 5′ end of the mRNA, as determined by in vivo nuclease S1 mapping, it may be that the *flaN* mRNA is rapidly and specifically processed at its 5′ end. There is also some indication that the *flaN* transcript is processed at additional 3′ sites corresponding to protected fragments of about 293, 233, 125, and 103 nt in length. These fragments were easily detected in strain SC511 (*flaK*::IS*511*), which overproduced the *flaN* transcript, whereas the PI* mutation at −15 that eliminated *flaN* expression also decreased the level of these processed RNA fragments to undetectable levels.

An unexpected consequence of mutations in the *flbG* promoter was their effect on expression from the divergent *flaN* promoter. Except for the 2-bp deletion at −18, −19 in the nonconserved region, all mutations in the *flbG* promoter resulted in a large increase in the level of the *flaN* transcript (Table 2). Transition mutations at −16 and −19 in the nonconserved spacer region resulted in vast overproduction of both the *flbG* and *flaN* transcripts. The *flaK* gene product apparently acts directly or indirectly as a repressor of the *flbG* and *flaN* operons, since mutations in *flaK* also result in high levels of both *flbG* and *flaN* transcripts (10, 21). Thus, the *flbG* promoter mutations mimic the effect of *flaK* mutations on the expression of transcription units I and II.

The mechanism by which *flbG* promoter mutations dere-

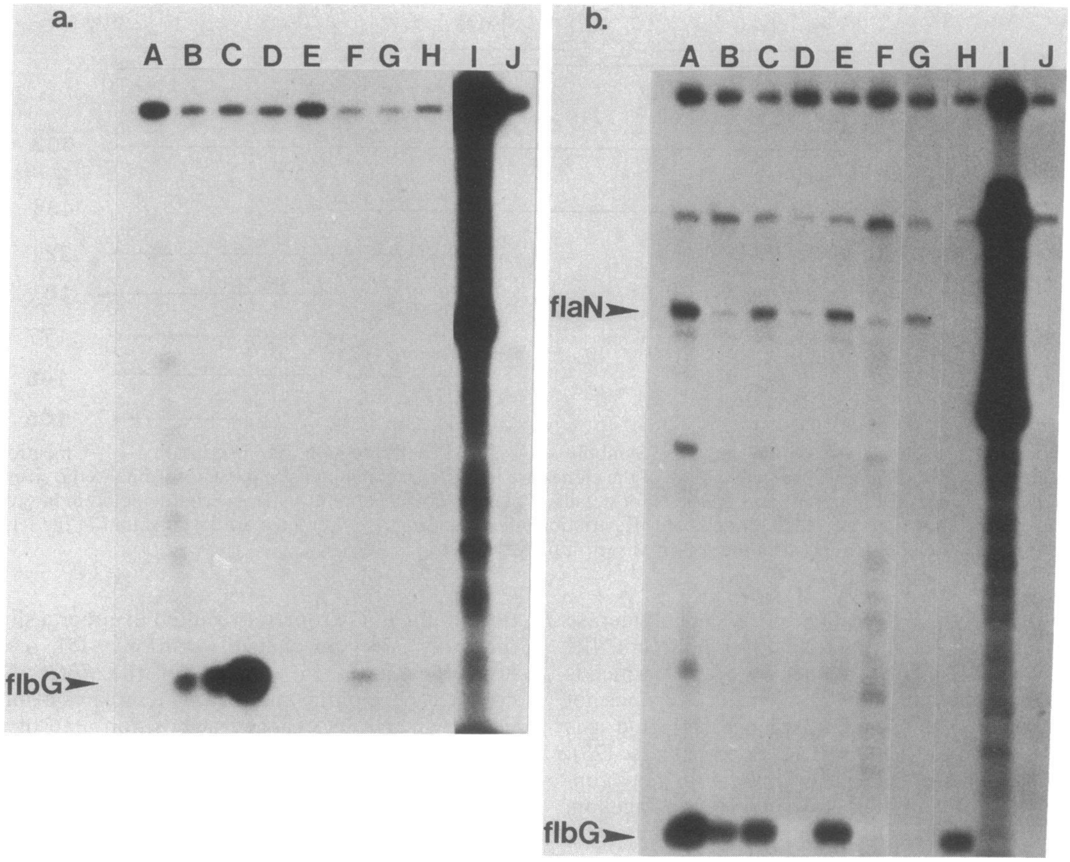

FIG. 8. Effect of mutations in the *ftr* element on transcription of *flbG* and *flaN*. Nuclease S1 assays were carried out as described in the legend to Fig. 3. Nuclease S1 was present in lanes A to H and J. (a) *Bal*31 deletions of *ftr*. Position of the 85-nt (*flbG*) protected fragment is indicated. The DNA probe in each sample was the 354-nt *Eco*RI-*Hin*dIII fragment labeled at both 5′ ends (Fig. 2D). An 85-bp fragment instead of an 80-bp fragment was protected by the *flbG* RNA in this experiment because the ^{32}P-labeled probe was 5 bp longer at the 5′ end within the *flbG* sequence than the 642-bp *Hin*dIII-*Bam*HI fragment used to probe for *flbG* and *flaN* transcripts from pRK2L1-603. Lanes: A to H, RNAs from CB15(pRK2L1-321) (lane A), CB15(pRK2L1-181) (lane B), SC511(pRK2L1-181) (lane C), SC1052(pRK2L1-181) (lane D), SC1132(pRK2L1-181) (lane E), CB15(pRK2L1-177) (lane F), CB15(pRK2L1-168) (lane G), and CB15(pRK2L1-105) (lane H); I, probe alone; J, probe plus nuclease S1. (b) Site-specific mutations of *ftr*. The DNA probe in each sample was the 642-nt *Hin*dIII-*Bam*HI fragment labeled at both 5′ ends (Fig. 2C). Positions of the 349-nt (*flaN*) and 80-nt (*flbG*) protected fragments are indicated. Mutations in the plasmids are indicated by the numbers shown in Fig. 4. Lanes: A to H, RNAs from SC511(pRK2L1-603) (lane A), CB15(pRK2L1-603) (lane B), CB15(pRK2L1-603), mutation 13 (lane C), CB15(pRK2L1-603), mutation 15 (lane D), CB15(pRK2L1-603), mutation 16 (lane E), CB15(pRK2L1-603), mutation 14, (lane F), CB15(pRK2L1-434) (lane G), and CB15(pRK2L1-603), mutation 12 (lane H); I, probe alone; J, probe plus nuclease S1.

press *flbG* and *flaN* expression is not clear, but it could be explained if the two promoters interact in some way to form a complex that is recognized by a repressor. In such a model, destabilizing this complex at one promoter could result in derepression of the other promoter. These two sequences are separated by 177 bp, and one of the ways sequence interaction at this distance could occur is by DNA looping, as described, for example, in the *E. coli gal* operon (14). DNA between the two divergent *C. crescentus* promoters could be looped out in a configuration that brings the sequence together with one or more proteins in a repressor-sensitive complex. Since there is also evidence that the *ftr* element may be involved the negative regulation of *fla* gene transcription (considering the effect of *ftr* mutations at −101 and −118), we speculate that the *flbG* and possibly the *flaN* promoter interact functionally in some way with the central *ftr* element to mediate the negative regulation of these transcription units. One possible organization of these regulatory sequences in two DNA loops is shown in Fig. 9. Alternately, we can imagine that two of the three *ftr* sequences shown in Fig. 6 interact with each other and the *flbG* promoter to mediate the observed regulation of *fla* gene expression.

In summary, conserved sequences at −12 and −24 are

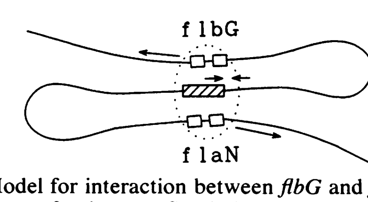

FIG. 9. Model for interaction between *flbG* and *flaN* promoters and the upstream *ftr* element. Symbols: □, −12, −24 promoters of *flbG* and *flaN*; ▨, *ftr* sequence element. Additional *ftr* sequences shown in Fig. 6 are not indicated. Arrows indicate direction of transcription from *flbG* and *flaN*; dotted outline indicates stabilization of the structure by a negative regulatory protein(s) (see Discussion).

required for transcription of *flbG* and *flaN*, and a conserved *ftr* sequence at −100 is also required for transcription of *flbG*. Our results also indicate that sequences in the *flbG* promoter and in the *ftr* element may be involved in the negative autoregulation of *flbG* and *flaN*. The coordinate regulation of these two transcription units could be mediated by the interaction of the *flbG* and *flaN* promoters with the *ftr* sequence element.

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