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 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 (TTG/GC) and −24 (TT/GG TTG/C/G/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 σ28 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-GGC—AA—G-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

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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% MgSO₄) (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% MgSO₄, 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) HindIII-BamHI 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-32P] 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, BamHI; S, SstI; S, SalI; P, PstI; H, HindIII. Bl. Location of a Bal31 deletion endpoint. The 285-nt genomic nuclease S1 probe for flaO is shown; ftr indicates 5'-32P-labeled ends. Symbols: ——, genomic sequences; •, polynucleotide DNA derived from pUC18.
assays (3). The hybridization temperature for all probes was 55°C. The 285-nucleotide (nt) BamHI-HindIII 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 HindIII end, and the two probes gave the same protected fragment in nuclease S1 assays (21). The probes used to detect transcripts from flaG and flaN in nuclease S1 assays are described in Results. An excess amount of 32P-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-32P]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 EcoRI-HaeIII DNA fragment. EcoRI linkers were

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 Bal31 deletion endpoint. (B) Site of insertion of the 603-bp PstI(a)-PstI(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 flaG and flaN mRNA in pRK2L1-603. *, 5'-32P-labeled ends; □□, location of ftr sequence. (D) Probe for nuclease S1 assays of the level of plasmid-encoded flaG and flaN mRNA in pRK2L1-321. Symbols and abbreviations are as given above.
added to the HaeIII end of this fragment, and after digestion with EcoRI, the resulting polylinker was ligated to the EcoRI site of pRK290 (11) to generate pRK2L1.

The 603-bp Pstl(a)-Pstl(b) fragment (Fig. 1), which contains the flbG and flaN promoters and ftr, extends from the genomic Pstl(a) site 3' from the flaN promoter to a Bal3I deletion endpoint located 54 bp downstream from the flbG transcription start site, to which 6 bp of the polylinker DNA derived from M13mp19 (from HindII to Pstl) had been added. pUC19603 consists of PUC19 with the 603-bp Pstl(a)-Pstl(b) fragment in the Pstl site (flbG promoter is proximal to the BamHI site in the pUC19 polylinker), and M13mp19603 consists of M13mp19 with the 603-bp Pstl fragment in the Pstl site (flbG promoter proximal to the BamHI site in the polylinker). Plasmid pRK2L1-603 (Fig. 2B) was constructed by ligating the 642-bp HindIII-BamHI fragment (Fig. 2C) derived from pUC19603 to pRK2L1 that had been cleaved with HindIII and BamHI.

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 Sstl and HindIII and ligating the purified 434-bp Sstl-HindIII fragment to pRK2L1 cleaved with Sstl and HindIII. pRK2L1-321 was constructed by cloning a 321-bp genomic Sau3A(a)-Sau3A(b) fragment containing the flaN and flbG promoter in the BamHI site of pRK2L1. In this construct, the flbG promoter is proximal to the HindIII site in the polylinker of pRK2L1 (Fig. 2A). The probe used to measure flbG mRNA made by pRK2L1-321 was a 354-bp HindIII-EcoRI fragment (Fig. 2D) 32P labeled at both 5' ends, which contains the 321-bp Sau3A(a)-Sau3A(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 Sau3A(a)-Sau3A(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 Sau3A(a) site in flaN is required for flaN transcription (see Results). pRK2L1-181 was constructed by cleaving pRK2L1-321 with Sstl and HindIII and ligating it to pRK2L1 cleaved with Sstl and HindIII. pRK2L1-181 extends from Sstl(b) to a polylinker-derived HindIII 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 HindIII-EcoRI fragment (Fig. 2D) 32P labeled at both 5' ends.

M13mp18177 was made by cleaving pRK2L1-321 (Fig. 2D) with Sstl, followed by treatment with T4 DNA polymerase in the presence of all four deoxynucleoside triphosphates to remove the overhanging 3' sticky ends of the Sstl site. The plasmid was then digested with HindIII and ligated to M13mp18 that had been cleaved with HindIII and HindIII to generate M13mp18177. M13mp18177, used to determine the nucleotide sequence at the deletion endpoint generated by the T4 DNA polymerase, was cleaved with HindIII and BamHI and ligated to pRK2L1 cleaved with HindIII and BamHI to generate pRK2L1-177. pRK2L1-168 and pRK2L1-150 are deletion derivatives of pRK2L1-181 made by allowing Bal3I nuclease to digest from the Sstl(b) site toward the flbG promoter. The deletion endpoints of pRK2L1-168 and pRK2L1-150 were determined by DNA sequence analysis. The probe used to measure the level of flbG mRNA made from each of these deletion derivatives in nucleosome S1 assays was the 354-bp HindIII-EcoRI fragment 32P labeled at both 5' ends (Fig. 2D).

pUC19285 was constructed by ligating a 285-bp HindIII-BamHI fragment that contains the flaO promoter (21) to pUC19 that had been cleaved with HindIII and BamHI. This plasmid was used as the source of the 285-bp HindIII-BamHI fragment which was 5' end labeled with 32P and used as a probe in nucleosome 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 C325. 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 HindIII and BamHI, 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 nucleosome S1 assay, using in vivo-isolated RNA from plasmid-containing cells and the 642-bp HindIII-BamHI DNA probe (Fig. 2C). Only RNA made from the plasmid-borne flbG and flaN promoters can protect the 5'-32P-labeled ends of the 642-bp BamHI-HindIII probe from nucleosome 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 nucleosome 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 Pstl(a)-Pstl(b) DNA fragment (Fig. 1) that had been cloned in the Pstl site of pRK2L1 (Fig. 2A) to yield pRK2L1-603 (Fig. 2B). The 642-bp HindIII-BamHI fragment from pRK2L1-603, which had been 32P 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 nucleosome 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 CB15pRK2L1-603

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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 substantial 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 of the 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. Consistently 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. 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 HindIII-BamHI probe; B, SC511(pRK2L1-603) RNA, 642-nt HindIII-BamHI probe; C, SC1052(pRK2L1-603) RNA, 642-nt HindIII-BamHI probe; D, SC1132(pRK2L1-603) RNA, 642-nt HindIII-BamHI probe; E and F, 642-nt HindIII-BamHI probe with (lane E) and without (lane F) S1 nuclease.
f1bG (PII)  
5' -TGTGTTCGAGTTGCCGACCGTTGGCTGAGGGAGGC

f1aN (PI)  
5' -TTGCAGGAGACCTCTCGTAATTCCGAGCCGAG

f1aN (PI*)  
5' -CCGCAGCGGCTTGGCGGGCTTGGCAGACCTC

ftr  

Toward f1aN(1)  
SstI(b) -110  
Towards ftrG(11)  
G

FIG. 4. Summary of primer mutagenesis of ftr and the promoters of f1bG and f1aN. 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.

Sequence elements required for regulation of f1bG transcription are contained within a 181-bp SstI-HindIII fragment. To determine whether an active f1bG promoter can be separated from sequences containing the f1aN promoter, the Ssrl(b)-HindIII fragment (Fig. 2D) 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 f1bG transcript (Fig. 8A, lanes B to E) corresponded to the different genetic backgrounds in the same way as described above for pRK2L1-603 (Fig. 3), which contains both f1bG and f1aN. The protection of an 85-nt fragment in nuclease S1 assays using the 354-nt HindIII-EcoRI probe (Fig. 2D) by RNA isolated from CB15(pRK2L1-181) (Fig. 8A, lane B) showed that transcription was initiated within the expected sequence for f1bG. These results suggest that all of the elements needed for regulation of the f1bG promoter are contained within the 181-bp Ssrl(b)-HindIII fragment in pRK2L1-181 and that regulated expression in f1bG does not require the f1aN promoter in cis.

Sequence element ftr is required for transcription of f1bG. To identify nucleotide sequences essential for regulated expression of the f1bG promoter, deletions extending across the 181-bp Ssrl-HindIII fragment in pRK2L1-181 (Fig. 7) were isolated and assayed as described above. Removal of the 4-nt single-stranded 3' end at the Ssrl(b) site of pRK2L1-181 to generate pRK2L1-177 (Fig. 7) resulted in a greatly diminished level of the f1bG 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 f1bG 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 f1bG transcript, which suggests that this sequence can act in cis as an upstream activator of f1bG transcription.

We also examined the effect of the ftr deletion mutations on transcription in flaK mutant strain SC511. Levels of f1bG 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 f1bG, a conclusion consistent with the effect of some f1bG promoter mutations on f1bG expression (Table 2).

Single-base-pair changes were introduced into the ftr sequence to identify specific nucleotides required for activation of f1bG. A G-to-T transversion at -113 from the

TABLE 2. Effect of mutations in the f1bG promoter on levels of f1bG and f1aN transcripts

<table>
<thead>
<tr>
<th>Transcrip</th>
<th>Level with indicated f1bG sequence mutation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>G&lt;sup&gt;G&lt;/sup&gt;</td>
</tr>
<tr>
<td>In vivo</td>
<td>f1bG</td>
</tr>
<tr>
<td>f1aN</td>
<td>++</td>
</tr>
<tr>
<td>In vitro&lt;sup&gt;b&lt;/sup&gt;</td>
<td>f1bG</td>
</tr>
</tbody>
</table>

* 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.

<sup>b</sup> Determined by Ninfa et al. (23) by using <sup>32</sup>P RNA polymerase from <i>E. coli</i>.  

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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 increased level 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 SstI(b) site to the BamHI site in plasmid pRK2L1-603 (Fig. 2C) to generate pRK2L1-603 (Fig. 7). The flaN transcript was still detected from plasmid pRK2L1-603 and increased several-fold 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.

![Graphic](graphic.png)

**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^N^, and recent results show that the _C. crescentus_ flbG and flaN
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 flagellar 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 *figL* (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 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*^c^, which has excellent homology to the Ntr-type sequence at the *flbG* promoter, is the *flbG* promoter (Fig. 4). Since *PI*^c^ 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::iss511*), which overproduced the *flaN* transcript, whereas the *PI*^c^ 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-
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 repressorsensitive 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. 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) Bal3 I deletions of ftr. Position of the 85-nt (flbG) protected fragment is indicated. The DNA probe in each sample was the 354-nt EcoR1-HindIII fragment labeled at both 5' ends (Fig. 2D). An 85-nt fragment instead of an 80-bp fragment was protected by the flbG RNA in this experiment because the 32P-labeled probe was 5 bp longer at the 5' end within the flbG sequence than the 642-bp HindIII-BamHI 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); 1, probe alone; J, probe plus nuclease S1. (b) Site-specific mutations of ftr. The DNA probe in each sample was the 642-bp HindIII-BamHI 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); 1, probe alone; J, probe plus nuclease S1.

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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LITERATURE CITED