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Escherichia coli σ^{54} RNA Polymerase Recognizes *Caulobacter crescentus flbG* and *flaN* Flagellar Gene Promoters In Vitro

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A set of the periodically regulated flagellar (*fla*) genes of *Caulobacter crescentus* contain conserved promoter sequence elements at −24 and −12 that are very similar to the sequence of the nitrogen assimilation (Ntr) and nitrogen fixation (Nif) promoters of enteric bacteria and *Rhizobium* spp. Transcription from Ntr and Nif promoters requires RNA polymerase containing σ^{54} instead of the usual σ^{70} and, in the case of the Ntr promoters, is activated by the transcription factors NR_I and NR_{II}. We have now demonstrated that the *C. crescentus flbG* and *flaN* promoters, which contain the Ntr/Nif type of consensus sequence, are utilized by purified *Escherichia coli* σ^{54} RNA polymerase ($E\sigma^{54}$) in the presence of NR_I and NR_{II} but not by the purified σ^{70} RNA polymerase ($E\sigma^{70}$) of *E. coli*. Oligonucleotide-generated *flbG* promoter deletions that removed the highly conserved GG dinucleotide at −24 or the GC dinucleotide at −12 or altered the spacing between the −24 and −12 sequence elements prevented utilization of the *flbG* promoter by the *E. coli* $E\sigma^{54}$. Transversions of T to G at positions −26 and −15 also inactivated *flbG* promoter function in the *E. coli* cell-free transcription system, while a transition of G to A at position −16 in the nonconserved spacer region had no effect. The *C. crescentus flaO* and *flbF* promoters, which do not contain the Ntr/Nif-type promoter consensus sequence, were not utilized by either purified $E\sigma^{54}$ or $E\sigma^{70}$ from *E. coli*. Our results help to define the features of the Ntr/Nif-type consensus sequence required for promoter utilization by purified *E. coli* $E\sigma^{54}$ and support the idea that *C. crescentus* may contain a specialized polymerase with similar promoter specificity required for expression of a set of *fla* genes.

In the dimorphic gram-negative bacterium *Caulobacter crescentus*, production of the flagellum is one of several discontinuous cell cycle events that have been shown to occur during precise intervals of swarmer cell and stalked-cell development (for review, see references 14 and 28). Flagellum biosynthesis requires the products of at least 30 flagellar (*fla*, *flb*, *flg*) genes, of which 23 map to the hook, basal body, and *flaEY* gene clusters on the chromosome; the remaining *fla* genes are scattered throughout the genome (13, 17, 35). All of the *fla* genes examined to date are transcriptionally regulated with a periodicity that corresponds generally to the time of product assembly into the flagellum (7, 8, 26, 34). This pattern of gene expression results in part from the organization of the *C. crescentus fla* genes into a regulatory hierarchy in which expression of genes at one level of the hierarchy is required for expression of genes on the next lower level of the hierarchy (7, 28, 34). Unlike *fla* gene regulation in the enteric bacteria, where genes at the top of the regulatory hierarchy are expressed in response to an environmental signal, carbon starvation (23), in *C. crescentus* the regulatory cascade is apparently driven by an internal cell cycle clock that generates the signal to initiate expression independently of environmental stimuli (28).

A number of the *C. crescentus fla* genes have been cloned and their promoters sequenced; none of them contain the canonical −35/−10 promoter sequence of *Escherichia coli* (8, 26, 27). One feature to emerge from those studies is that *flaN* and *flbG* (first gene in the hook operon, which contains the hook protein structural gene *flaK*) of the hook gene cluster and *flgK* (25-kilodalton [kDa] flagellin gene) and *flgL* (27-kDa flagellin gene) of the *flaEY* cluster, which occupy the two lowest levels of the regulatory hierarchy, contain highly conserved promoter sequence elements at −24 and −12 that are very similar but not identical to the consensus sequence of Ntr and Nif gene promoters of the enteric bacteria and *Rhizobium* spp. (25, 27) (see below). In contrast, the *flaO* and *flgJ* promoters, which are located above these genes in the regulatory hierarchy, show no recognizable homology with the −24 and −12 sequence elements (27), while the *flbF* promoter, which is also located higher in the regulatory hierarchy, contains a highly conserved −12 element 29 base pairs (bp) upstream from the putative transcription start site but not a well-conserved −24 element farther upstream (G. Ramakrishnan and A. Newton, unpublished data). Thus, it appears that *fla* genes in *C. crescentus* contain several distinct types of promoters whose structures correlate with the position of the genes in the regulatory hierarchy.

The *flaN*, *flbG*, *flgL*, and *flgK* promoters of *C. crescentus* also contain a highly conserved 19-bp upstream regulatory sequence element, which has been designated II-1 (8, 27), located approximately 100 bp upstream from the site of transcript initiation, and mutagenesis of this II-1 element has shown that it is required for *flbG* transcription in intact cells (D. A. Mullin and A. Newton, manuscript in preparation). This organization of the −100, −24, and −12 elements in the *C. crescentus flaN*, *flbG*, *flgL*, and *flgK* promoters is highly reminiscent of the structure of the globally regulated Ntr and Nif promoters of the enteric bacteria and *Rhizobium* spp. that promote the transcription of genes involved in nitrogen assimilation (reviewed in references 16 and 25). The −24 and −12 elements of the Ntr and Nif promoters reflect their requirement for RNA polymerase containing σ^{54}, product of *rpoN* (*ntrA*, *glnF*), instead of the usual σ^{70}, and purified σ^{54} RNA polymerase from *Salmonella typhimurium* and *E. coli* has been shown to protect this promoter region in footprint-

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TABLE 1. σ^{54} promoters[a]

Promoter	Organism	Sequence[b]
		−24 −12
argTr	*S. typhimurium*	ATGGCATA-N4-CTGCA
dhuA		ATGGCACG-N4-TCGCA
nifL	*K. pneumoniae*	AGGGCGCA-N4-TTGCA
glnA	*E. coli*	TTGGCACA-N4-TCGCT
nifH	*K. pneumoniae*	CTGGTATG-N4-CTGCA
nifE		CTGGAGCG-N4-TTGCA
nifU		CTGGTATC-N4-TTGCT
nifB		CTGGTACA-N4-TTGCA
nifM		CTGGCACA-N4-TTGCA
nifF		CTGGCGCA-N4-TTGCA
nifH	*R. meliloti*	CTGGCACG-N4-TTGCA
"*p2*"		CTGGCACG-N4-TTGCA
nifH	*R. japonicum*	TTGGCACG-N4-TTGCT
nifD		CTGGCATG-N4-TTGCA
flaN (PI)	*C. crescentus*	TTGGCGCC-N4-TTGCG
flbG (PII)		TTGGCCCG-N4-TTGCT
flgK (P25)		TTGGCCCG-N4-TTGCT
flgL (P27)		CTGGCCCG-N4-TTGCG

[a] Promoters known to require σ^{54} RNA polymerase (top two sections) and *C. crescentus* promoters of similar structure (bottom section) are shown. The nucleotide sequences of the Ntr promoters (top section) and the Nif promoters (central section) have been published previously (2–6, 16, 38, 41, 42, and additional references cited and reviewed in reference 16). The sequences of the *C. crescentus* promoters are from references 8 and 27.

[b] Underlined GG and GC dinucleotides are invariant in σ^{54}-dependent promoters. N4 indicates four nonconserved nucleotides.

TABLE 2. Promoters examined in vitro[a]

Template	Promoter	Sequence −24 −12	Activity[b]
pTH8, pAN6	*glnAp2*	TTGGCACA-GATT-TCGCT	+++
pNJ6, pNJ16	*flaN* (PI)	TTGGCGCC-GTCC-TTGCG	+
pNJ5, pNJ15	*flbG* (PII)	TTGGCCCG-ACCG-TTGCT	+
pNJ9	L111	G	−
pNJ10	L5103	—	−
pNJ11	L026	—	−
pNJ12	L086	A	+
pNJ13	L033	G	−
pNJ14	L431	—	−
pNJ7	*flaO* (PIII)	TTTACCTT-GTAC-TGGGT	−
pNJ17	*flbF* (PIV)	GCGGGCCG-CAAA-TCGCT	−

[a] The *glnAp2* sequence is from reference 38, the *flbG* sequence is from reference 8, the *flaN* and *flaO* sequences are from reference 27, and the *flbF* sequence is from Ramakrishnan and Newton (unpublished data). The mutant *flbG* promoters contained in plasmids pNJ9 through pNJ14 were constructed by site-directed mutagenesis (see Materials and Methods). For these promoters, only the changes from the wild-type *flbG* promoter sequence are shown, with deleted bases indicated by a dash.

[b] Qualitative assessment of promoter strength judged by visual examination of autoradiographs of transcription gels. +++, Very strong promoter; +, functional promoter; −, no promoter activity.

ing experiments (22, 24, 31, 32). The Nif and at least some of the Ntr promoters, including the nitrogen-regulated *glnA* promoter (*glnAp2*), contain at about position −100 sequence elements that are functionally equivalent to enhancers (6, 38). These upstream sequences bind transcriptional activators and facilitate transcription from the promoters. In the Nif system, the activator is the product of *nifA*; in the Ntr system, the activator is the product of *glnG* (*ntrC*), NR_I. Expression from the Ntr promoter *glnAp2* in *E. coli* is regulated by a signal transduction pathway in which the protein kinase, NR_{II}, activates transcription by catalyzing the phosphorylation of NR_I, and NR_I-phosphate is the form of NR_I able to stimulate transcription (30). All of the proteins required for expression from the *glnAp2* promoter have been purified from *E. coli*, *S. typhimurium*, and *Klebsiella pneumoniae* (1, 19, 20, 33). The upstream *glnA* sequences (*glnA* enhancer) have been shown to facilitate transcription at low concentrations of NR_I in cell-free transcription systems containing these components (1, 22, 29, 30, 32, 42).

All of the Ntr and Nif promoters contain a GG dinucleotide at positions −24 and −25, a GC dinucleotide at positions −12 and −13, and 10-bp spacing between these two dinucleotides (Table 1). Mutational analysis of the *K. pneumoniae nifH* promoter has indicated that this 10-bp spacing, the GG dinucleotide at −24, and at least the G nucleotide at position −13 are required for promoter function in vivo (3–5, 36). Deletion of the *nifH* upstream sequence element at position −100 abolishes activation by the *nifA* gene product. Similarly, site-directed mutagenesis of the *C. crescentus flbG* promoter has indicated that the conserved −24 and −12 sequence elements, the correct 10-bp spacing between these two elements, and an intact upstream II-1 element at position −100 are all required for *flbG* expression in intact *C. crescentus* cells (D. A. Mullin and A. Newton, manuscript in preparation). Thus, the general architecture of the Nif and Ntr promoters and one set of the *C. crescentus fla* promoters seems to be the same.

In this report we demonstrate that the *C. crescentus flaN* and *flbG* promoters are efficiently utilized by purified *E. coli* σ^{54} RNA polymerase. We used mutant *flbG* promoters to demonstrate that the general features of promoter structure that are invariant in Ntr and Nif promoters and that are required for *flbG* promoter function in intact *C. crescentus* cells are also required in an *E. coli* cell-free transcription system containing $E\sigma^{54}$. Our data thus extend the number of promoters known to be recognized by purified $E\sigma^{54}$ and *E. coli* and provide information on the structural features of promoters required for recognition by this polymerase. Our data also support the idea (27) that *C. crescentus* contains a specialized RNA polymerase of analogous specificity.

MATERIALS AND METHODS

Transcription templates. All transcription templates used in this study are derived from pTE103 (11) (Table 2 and Fig. 1). This plasmid contains the multicloning site from pUC8 positioned upstream from a strong rho-independent transcriptional terminator from bacteriophage T7. The plasmids pAN6 and pTH8 have been described previously (20, 32); these plasmids contain the *E. coli glnAp2* promoter positioned upstream from the T7 terminator so that *glnAp2* transcripts of 484 and 309 nucleotides (nt), respectively, are produced. Plasmid pAN6 contains *glnA* DNA upstream to position −32 and thus lacks the *glnA* enhancer (32); pTH8 contains the entire *glnA* control region upstream to position −625, including the *glnA* enhancer (20).

Plasmid pNJ5. The 650-bp *Hin*dIII-*Eco*RI fragment containing the *C. crescentus flaN* and *flbG* promoters of divergent transcription units I and II of the hook cluster (8, 35) (derived from the replicative form of an M13mp19 recombinant phage; Mullin and Newton, unpublished data) was cloned into *Hin*dIII- and *Eco*RI-digested pTE103 (11) to yield plasmid pNJ5. In pNJ5, the transcriptional start site for the *flbG* operon is positioned 365 bp upstream from the T7 terminator (Fig. 1). The construction was verified by single

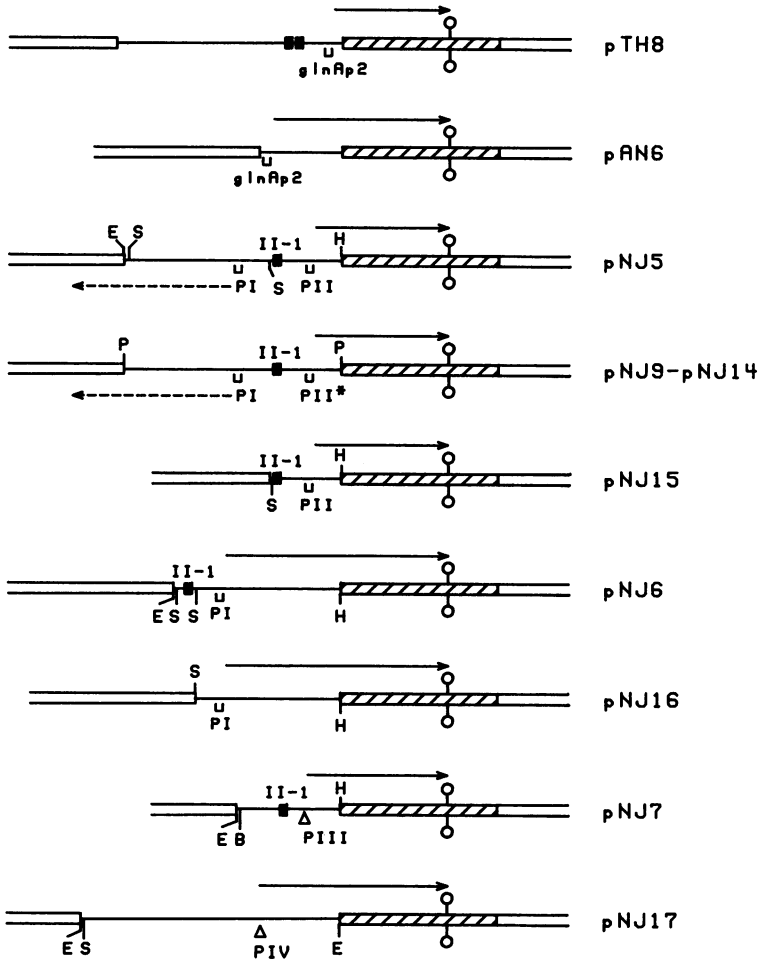

FIG. 1. Transcription templates used in this study. Plasmids containing various *C. crescentus* promoters and the *E. coli glnAp2* promoter are depicted. Vector sequences are drawn as a thick bar, the phage T7 DNA fragment containing the transcriptional terminator is shown as a crosshatched bar, and the inserted promoter-bearing DNA is shown as a thin line. The position of the −24 to −12 consensus sequence is indicated by an open bracket. PI refers to the *flaN* promoter, PII refers to the *flbG* promoter, PIII refers to the *flaO* promoter, and PIV refers to the *flbF* promoter. In pNJ9 through pNJ14, PII* refers to the mutant PII promoters that these plasmids contain. The expected transcript from each promoter is shown by an arrow. The position of the high-affinity NR_I-binding sites in pTH8 and the position of the II-1 sequence element in the *C. crescentus* control regions are depicted by small solid boxes. Only the relevant portions of the plasmids and restriction sites used in these constructions are shown, and the figure is not drawn to scale. E, *Eco*RI; H, *Hin*dIII; S, *Sac*I; P, *Pst*I; B, *Bam*HI.

and double digestions with the enzymes *Fsp*I, *Sac*I, *Rsa*I, *Pvu*II, *Hin*dIII, and *Eco*RI.

Plasmid pNJ15. Plasmid pNJ15 was constructed from pNJ5 by digestion with *Sac*I followed by ligation at low DNA concentration. Deletion of the *Sac*I fragment, which extends from position −116 upstream to the multicloning site of the vector, removes the *flaN* promoter and a portion of the *flaN* operon (Fig. 1). Plasmid pNJ15 thus contains approximately 170 bp of *C. crescentus* DNA, including only the *flbG* promoter and its natural site of transcription initiation.

Plasmid pNJ6. Plasmid pUC605-1, which contains a 605-bp *Pst*I-*Pst*I fragment with the *flaN/flbG* control region (8, 35) inserted into pUC19 (Mullin and Newton, unpublished data), was cleaved with *Hin*dIII and digested with Bal31, after which *Kpn*I linkers were added. The DNA was then cleaved with *Kpn*I and *Pst*I, and Bal31-shortened fragments containing the *flaN* promoter released by the digestion were purified by electroelution. These fragments were then ligated into pUC19 that had been cleaved with *Pst*I and *Kpn*I; among the products of this ligation was pDM101, in which the *Kpn*I linker had been inserted about 130 bp upstream from the *flaN* promoter (the *flbG* promoter had been removed by the Bal31 digestion). Plasmid pNJ6 was then constructed by ligation of the *Hin*dIII-*Eco*RI fragment from pDM101 (containing the *flaN* promoter) into the *Hin*dIII-*Eco*RI sites of pTE103. In pNJ6, the *flaN* transcriptional start site is positioned 637 bp upstream from the T7 terminator.

Plasmid pNJ16. Plasmid pNJ16 was constructed from pNJ6 by digestion with *Sac*I followed by ligation at low DNA concentration. This plasmid is similar to pNJ6 but lacks the *Sac*I fragment extending upstream from approximately position −100 to the multicloning site of the vector that contains the II-1 sequence element. The construction was verified by single and double digestions with the enzymes *Sac*I, *Hin*dIII, *Eco*RI, and *Pvu*II.

Plasmid pNJ7. Plasmid pUC7Kb contains a 7-kilobase (kb) *C. crescentus Bam*HI fragment containing the hook cluster transcription unit III (35) (*flaO* operon) inserted into the

*Bam*HI site of pUC19 (Mullin and Newton, unpublished data). This plasmid was digested with *Hin*dIII and religated under dilute conditions to yield plasmid pNJ8, in which all of the *C. crescentus* insert except for a 286-bp fragment containing the *flaO* promoter was eliminated. The 292-bp *Hin*dIII-*Eco*RI (which contains the 286-bp fragment with the *flaO* promoter) from pNJ8 was then ligated into *Hin*dIII- and *Eco*RI-digested pTE103 to yield pNJ7. In pNJ7, the *flaO* transcriptional start site is positioned 389 bp upstream from the T7 terminator. The construction of pNJ7 was verified by single and double digestions with the enzymes *Rsa*I, *Pvu*II, *Hin*dIII, *Fsp*I, and *Eco*RI.

Plasmids pNJ9 through pNJ14. Other plasmids were constructed from recombinant M13 phage containing the 605-bp *Pst*I fragment with the *flaN/flbG* control region; in these phage, the *flbG* promoter sequence had been altered by site-specific mutagenesis as described in detail elsewhere (D. A. Mullin and A. Newton, manuscript in preparation). The changes in these mutant *flbG* promoters are shown in Table 2. In each case the nucleotide changes were confirmed by sequencing. The replicative form of each mutant phage was isolated and cleaved with *Pst*I, and the 605-bp fragment with the *flaN/flbG* promoter control region was inserted into *Pst*I-digested pTE103. The orientations of the insertions were then checked by single and double digestions with the enzymes *Eco*RI, *Sac*I, and *Hin*dIII. In pNJ9 through pNJ14, the mutant *flbG* promoters are positioned 365 bp upstream from the T7 terminator.

Plasmid pNJ17. Plasmid pGIR112, a derivative of pUC19 that contains a 1.4-kb portion of the *C. crescentus* hook cluster including the *flbF* control region (35), was digested with *Eco*RI, and the 700-bp fragment, containing the *flbF* control region and a portion of the pUC19 multicloning site, was inserted into the *Eco*RI site of pTE103. The orientation of the *flbF* promoter was determined by digestion with *Sac*I, a site for which is located at one end of the insert in the multicloning site derived from pUC19 (G. Ramakrishnan, unpublished data). In pNJ17, the in vivo site of *flbF* transcript initiation (as indicated by S1 nuclease mapping studies; G. Ramakrishnan and A. Newton, unpublished data) is located 540 bp upstream from the T7 transcriptional terminator.

Purified proteins. All proteins used in these experiments were purified in the laboratory of Boris Magasanik and have been described previously (28, 31, 32). The RNA polymerase (σ^{70}) holoenzyme preparation used in the experiment shown in Fig. 2 was purified as described before (20). This RNA polymerase preparation contains trace amounts of σ^{54}-containing holoenzyme.

Transcription assays. The protocol for the transcription assay has been described in detail previously (29, 32). In all experiments shown, supercoiled plasmid templates present at a final concentration of 5 nM were used. The concentration of each template was calculated from the absorbance at 260 nm; protein concentrations were estimated from the absorbance at 260 and 280 nm as described previously (32). NR_{II} was present in all assay mixes at 20 nM. Core RNA polymerase and σ^{54} were present at 100 and 400 nM, respectively, where indicated, except in the experiment shown in Fig. 6, where the concentration of both proteins was varied. NR_I, when present, was used at 370 nM, except where indicated otherwise. RNA polymerase (σ^{70}) holoenzyme was present at 100 nM, where indicated.

Briefly, the transcription assay measures the formation of heparin-resistant transcription complexes formed in the presence of ATP, GTP, and CTP during incubation at 37°C.

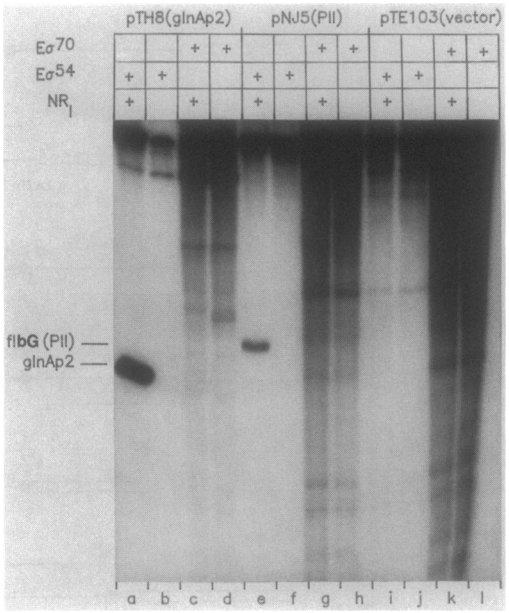

FIG. 2. *C. crescentus flbG* (*flaK* in figure) promoter is utilized by *E. coli* σ^{54} RNA polymerase in the presence of NR_I. Transcription from templates containing the *E. coli glnAp2* promoter (pTH8), *C. crescentus flbG* promoter (PII, pNJ5), and the promoterless vector pTE103 was assayed by using the purified *E. coli* proteins indicated above each lane. All reaction mixtures also contained NR_{II}. The positions of the *glnAp2* and *flbG* (PII) transcripts are indicated.

In the case of the *glnAp2* promoter, such incubation has been shown to result in the production of heparin-resistant ternary elongation complexes that pause at positions requiring the incorporation of UTP (29). For the experiments described here, the assay mixes were incubated for 30 min. A mixture containing heparin and labeled UTP was then added, and the incubation was continued for an additional 10 min at 37°C to allow the formation of full-length labeled transcripts. The reactions were then stopped by the addition of EDTA, and the transcripts were recovered by ethanol precipitation in the presence of tRNA, which serves as carrier, subjected to electrophoresis on urea-acrylamide gels, and detected by autoradiography as described previously (29, 32).

RESULTS

Construction of transcription templates containing *C. crescentus fla* promoters. We constructed a series of plasmids, shown in Fig. 1, that contained *C. crescentus fla* promoters positioned upstream from the strong rho-independent transcriptional terminator found in the vector pTE103. Use of this vector has facilitated the analysis of transcription from supercoiled templates (11, 20, 32). Since the nucleotide sequence and site of transcription initiation in vivo are known for the *fla* promoter fragments, the length of the transcript initiating at the in vivo start site and terminating at the terminator can be predicted for each plasmid. As control templates, we used pTH8 and pAN6 (20, 32), which contain the *E. coli glnAp2* promoter cloned into pTE103. The nucleotide sequence of the promoters contained in these plasmids is shown in Table 2.

Recognition of *flbG* promoter by σ^{54} RNA polymerase. We tested whether the *C. crescentus flbG* (hook operon) promoter could be utilized by purified *E. coli* RNA polymerase

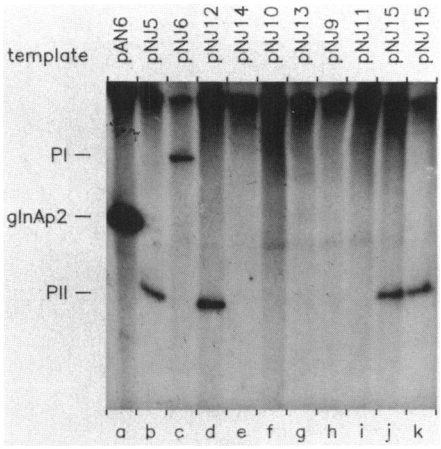

FIG. 3. Effect of *flbG* promoter mutations on promoter utilization by *E. coli* σ^{54} RNA polymerase. Transcription from the template shown above each lane was assayed in reaction mixtures containing NR_I, NR_{II}, and $E\sigma^{54}$. Plasmids pNJ5 and pNJ15 contain the *flbG* (PII) promoter, plasmid pNJ6 contains the *flaN* (PI) promoter, and plasmids pAN6 and pTH8 contain the *E. coli glnAp2* promoter. Plasmids pNJ9 to pNJ14 contain the *flbG* promoter mutations listed in Table 2. The positions of the *flaN* (PI), *flbG* (PII), and *glnAp2* transcripts are indicated.

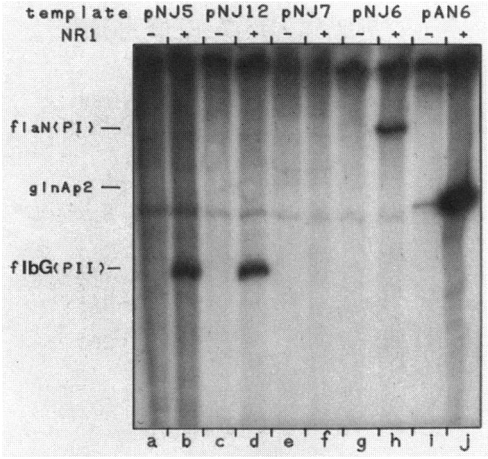

FIG. 4. Comparison of transcription from *C. crescentus flbG*, *flaN*, and *flaO* promoters and *E. coli glnAp2* in the presence and absence of NR_I. All transcription reaction mixes contained NR_{II} and σ^{54} RNA polymerase; NR_I was added as indicated. Templates pNJ5 and pNJ12 contain wild-type and a mutant version of the *flbG* (PII) promoter, respectively (Table 1), pNJ7 contains the *flaO* (PIII) promoter, pNJ6 contains the *flaN* (PI) promoter, and pAN6 contains the *E. coli glnAp2* promoter. The positions of the transcripts from these promoters is indicated.

containing σ^{54}. When supercoiled plasmid pNJ5 was used as the template in the transcription assay reaction mixture containing core RNA polymerase and σ^{54}, a transcript of the size expected from initiation at the *flbG* promoter was produced when NR_I and NR_{II} were present (Fig. 2). This transcript was not observed when NR_I was omitted from the reaction mixture or when σ^{70}-containing RNA polymerase was used instead of the mixture of σ^{54} and core RNA polymerase, whether or not NR_I was present. Thus, transcription from the *C. crescentus flbG* promoter required both the *E. coli* σ^{54} and NR_I. Control experiments showed that the *flbG* transcript was not made in the complete transcription assay with $E\sigma^{54}$ when the parent vector pTE103 was used as the template instead of pNJ5 (Fig. 2).

The *C. crescentus flbG* promoter was utilized less efficiently than the *E. coli glnAp2* promoter by $E\sigma^{54}$ in the presence of NR_I and NR_{II} (Fig. 2, compare lanes a and e). The autoradiogram presented in Fig. 2 minimizes this difference because it is somewhat overexposed. From visual inspection of numerous similar autoradiograms made at different exposures, we estimate that the *C. crescentus flbG* promoter was about 5 to 10% as active as the *E. coli glnAp2* promoter in this assay system. We considered the possibility that the *flbG* promoter might be less active because of either the presence of the *flaN* promoter, which is also present on the pNJ5 template facing in the opposite direction (Fig. 1), the sequence of the *flbG* promoter, or some feature of the *C. crescentus* DNA, such as its high G+C content, which might indirectly affect the ability of σ^{54} RNA polymerase to form a transcription complex. Although we have not completely resolved this question, we have observed that the subclone pNJ15, which also contains the *flbG* promoter but lacks the *flaN* promoter and much of the *C. crescentus* DNA upstream from the *flbG* promoter, was not a better template than pNJ5 in the *E. coli* cell-free transcription system (Fig. 3).

Effect of *flbG* promoter mutations on promoter utilization by σ^{54} RNA polymerase. To verify that the transcript generated from the pNJ5 template was dependent on recognition of the *flbG* promoter, we examined the effect of oligonucleotide-generated mutations in this promoter on the ability of purified *E. coli* σ^{54} RNA polymerase to utilize this promoter. The nucleotide sequences of the mutant *flbG* promoters used are shown in Table 2, and the results of transcription assays with supercoiled plasmid templates containing the *flbG* promoter mutations are shown in Fig. 3. We observed that deletion of the GG dinucleotide at −24 (pNJ10, lane f), the GC dinucleotide at −12 (pNJ14, lane e), or alteration of the 10-bp spacing between these two sequence elements by deletion of the AC dinucleotide at positions −17 and −18 (pNJ11, lane i) eliminated transcription from the *flbG* promoter by purified $E\sigma^{54}$. These results indicate that features of the Ntr/Nif consensus that are highly conserved and required for the utilization of the *flbG* promoter in intact *C. crescentus* cells (D. A. Mullin and A. Newton, manuscript in preparation) are also required for utilization of the *flbG* promoter in the *E. coli* cell-free system containing $E\sigma^{54}$. T to G transversions at position −26 or −15 also appeared to eliminate recognition of the *flbG* promoter by the *E. coli* $E\sigma^{54}$ (Fig. 3; pNJ9, lane h, and pNJ13, lane g), although with longer exposures of similar autoradiograms a very small amount of transcription from two of these mutant promoters could be detected (data not shown). We found that the transition of G to A at position −17, in the nonconserved spacer portion of the promoter had no effect on the ability of *E. coli* σ^{54} RNA polymerase to utilize the *flbG* promoter (Fig. 3, pNJ12, lane d) and that this ability still required NR_I (Fig. 4, lanes c and d).

Utilization of the *flaN* promoter in vitro. When supercoiled plasmid pNJ6 containing the *C. crescentus flaN* promoter was used as the transcription template, *E. coli* σ^{54} RNA polymerase produced a transcript of the size expected from initiation at the *flaN* promoter when both NR_I and NR_{II} were present (Fig. 3 and 4). This transcript was not seen when NR_I was omitted from the reaction mixture (Fig. 4). The *flaN* transcript was not obtained when *E. coli* σ^{70} RNA polymerase was used in place of $E\sigma^{54}$ (data not shown).

In intact *C. crescentus* cells, transcription from the *flbG* and *flaN* promoters requires the 19-bp II-1 element located

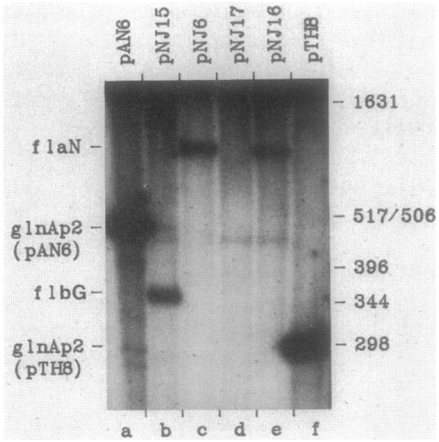

FIG. 5. Utilization of the *flaN* promoter by $E\sigma^{54}$ does not require the II-1 sequence element. Transcription from the templates shown above each lane was assayed in reaction mixtures containing $E\sigma^{54}$, NR_I, and NR_{II}. Template pNJ15 contains the *flbG* promoter, pNJ17 contains the *flbF* promoter, pAN6 and pTH8 contain the *glnAp2* promoter, and pNJ6 and pNJ16 contain the *flaN* promoter. Plasmid pNJ6, but not pNJ16, also contains the upstream II-1 element at position −100. The positions of the transcripts from these promoters is indicated. The size standards, whose positions are indicated on the right side of the autoradiogram, were end-labeled pBR322 *Hin*FI fragments.

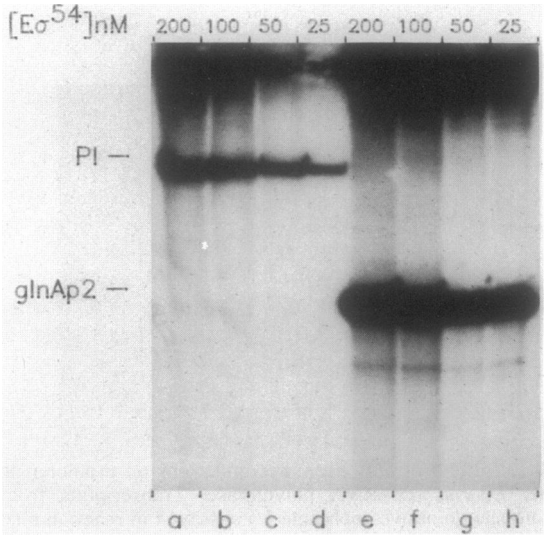

FIG. 6. Effect of varying the concentration of σ^{54} RNA polymerase on the utilization of *glnAp2* and the *flaN* promoter. Transcription from the pAN6 and pNJ6 templates was assayed with the indicated concentration of σ^{54} RNA polymerase. The stated concentration is the nominal core RNA polymerase concentration; σ^{54} was present at four times this concentration. All transcription reaction mixtures contained NR_{II} and NR_I.

at position −100 with respect to both of these divergently transcribed promoters (D. A. Mullin and A. Newton, manuscript in preparation). We observed that the II-1 element was not required for transcription by purified *E. coli* σ^{54} RNA polymerase activated by NR_I and NR_{II}. The *flaN* promoter on template pNJ16, which lacks this element, was effectively utilized by $E\sigma^{54}$ (Fig. 5).

Previous results had indicated that transcription from the *E. coli glnAp2* promoter is facilitated by the presence of the *glnA* enhancer when the concentration of NR_I is limiting (29, 31, 32). We examined the possibility that the II-1 element might facilitate transcription from the *flaN* promoter when lower concentrations of NR_I are present in the transcription reaction mixtures. Titration of the amount of NR_I required to activate transcription from the *flaN* promoter on the pNJ6 and pNJ16 templates revealed that the level of transcription from the *flaN* promoter on these templates was equivalent at limiting NR_I concentrations and that maximal transcription from both templates required about 120 nM NR_I (data not shown). As control templates in this NR_I titration experiment, we examined the amount of NR_I required to activate transcription from the *glnAp2* promoter on templates pAN6 (which lacks the *glnA* enhancer) and pTH8 (which contains the *glnA* enhancer); maximal transcription from pTH8 required 7.5 nM NR_I, and maximal transcription from pAN6 required 60 nM NR_I, in good agreement with previous results (32) (data not shown). Thus, transcription from the *flaN* promoter required more NR_I than did transcription from *glnAp2*, and this transcription was not facilitated by the II-1 sequence element.

Relative efficiencies of *fla* gene promoters. Since the amount of transcription from the *C. crescentus flaN* and *flbG* promoters by σ^{54} RNA polymerase in the presence of NR_I and NR_{II} was clearly less than that observed from the *E. coli glnAp2* promoter, we examined the possibility that these *C. crescentus* promoters had a lower affinity for this RNA polymerase than the *E. coli* promoter. We measured the effect of varying the concentration of σ^{54} RNA polymerase from 25 to 200 nM on the level of transcription observed from the *flaN* promoter and *glnAp2* in the presence of excess NR_I and NR_{II} (Fig. 6). Transcription from *glnAp2* was largely insensitive to variation of the polymerase concentration within the range tested. In contrast, transcription from the *flaN* promoter was noticeably more dependent on the RNA polymerase concentration. These results suggest that the reduced affinity of this polymerase for the *C. crescentus flaN* promoter may be one factor limiting the utilization of this promoter in the *E. coli* cell-free transcription system.

Previous work has established that *E. coli* σ^{54} RNA polymerase can utilize the *glnAp2* promoter on supercoiled and linearized templates in the presence of NR_I and NR_{II} (20, 31, 32). In those studies, it was observed that less transcription occurs from linear templates and that a higher concentration of NR_I is required to fully activate transcription from linear templates (32; A. Ninfa and B. Magasanik, unpublished observations). Thus, the use of linearized templates provides a more stringent assay for promoter utilization in the cell-free transcription system. We compared the ability of $E\sigma^{54}$ (at 100 nM) to utilize the *flbG* and *flaN* promoters on linearized and supercoiled pNJ5, pNJ15, and pNJ6 templates in the presence of 370 nM NR_I and excess NR_{II}; as control templates we used supercoiled and linear pAN6 at the same reaction conditions. We found that the *glnAp2* promoter on linearized pAN6 was utilized, as described previously (32), but that neither the *flaN* promoter nor the *flbG* promoter was utilized on linear templates (data not shown).

Analysis of the *flaO* and *flbF* promoters. In *C. crescentus*, expression from the *flaN* and *flbG* promoters is absolutely dependent on expression of the *flaO* and *flbF* operons of the hook cluster (35). The *flaO* promoter has little homology with the *flaN*, *flbG*, and σ^{54}-dependent promoters of enteric bacteria, nor is this promoter homologous to *E. coli* σ^{70}-dependent promoters or the *C. crescentus trpF* promoter

(40) (Table 1). When pNJ7 was used as the template in the transcription assay, we did not observe transcription from the *flaO* promoter by σ^{54} RNA polymerase in the presence or absence of NR_I (Fig. 4). We also found that this promoter was not utilized by $E\sigma^{70}$, whether or not NR_I was present (data not shown).

In contrast to the *flaO* promoter, nucleotide sequencing of the *flbF* control region has revealed a sequence element upstream from the site of transcription initiation that has some homology to the Ntr/Nif promoter consensus sequence (Ramakrishnan and Newton, unpublished data). As shown in Table 2, this putative promoter was identical to the *E. coli glnAp2* promoter at positions corresponding to the −12 sequence element (TCGCT), and it contained a GG dinucleotide at positions corresponding to −24 and −25 of the Ntr/Nif consensus sequence. Nucleotides at positions corresponding to −27, −26, −23, and −22 of the Ntr/Nif consensus sequence did not conform to the consensus. We examined the ability of this sequence in the *flbF* control region on template pNJ17 to be utilized by σ^{54} RNA polymerase in the presence of NR_I and NR_{II} and found that a transcript of the expected size was not produced (Fig. 5).

DISCUSSION

The results presented in this report show that the *flbG* and *flaN* promoters of *C. crescentus* are utilized by purified *E. coli* σ^{54} RNA polymerase in a defined cell-free transcription system. Transcription from templates containing these promoters gave rise to transcripts of the size expected if transcription were initiated at the same site used in intact cells (8, 27), and the appearance of the *flaN* and *flbG* transcripts was dependent on the presence of both σ^{54} and the Ntr transcription factor NR_I in the transcription reaction mixtures. In the case of the *flbG* promoter, we also demonstrated that specific nucleotides within the promoter sequence are essential for utilization by $E\sigma^{54}$. It has been observed previously that the *K. pneumoniae nifH* and *nifL* promoters are utilized by *E. coli* σ^{54} RNA polymerase in the presence of NR_I and NR_{II} (G. L. Ray and B. Magasanik, personal communication) and by a similar *S. typhimurium* cell-free transcription system (42). Since the only nonvector sequence shared by our smallest *flaN*, *flbG*, and *glnAp2* templates (and by the *nifH* and *nifL* templates used by others) is the Ntr/Nif promoter consensus sequence, we suspect that this sequence is entirely responsible for the transcription from the *flbG* and *flaN* promoters that we observed. The *flaO* promoter, which lacks this Ntr/Nif type consensus sequence, and a sequence in the *flbF* control region that only poorly conforms to this consensus sequence were not utilized by σ^{54} RNA polymerase in the *E. coli* cell-free transcription system.

The *C. crescentus flaN* and *flbG* promoters were utilized less efficiently than the *E. coli glnAp2* promoter in this transcription assay system, as indicated by lower amounts of transcript obtained and the requirement for supercoiled templates and as observed for the *flaN* promoter, the requirement for higher concentrations of NR_I and $E\sigma^{54}$ for maximal transcription. These findings may reflect the fact that the nucleotide sequences of the *C. crescentus flbG* and *flaN* promoters do not perfectly match the *glnAp2* or Ntr/Nif promoter consensus sequence. These factors notwithstanding, the sensitivity and specificity of the *E. coli* cell-free transcription system were sufficient to permit at least a qualitative assessment of the activity of mutant *flbG* promoters, as discussed below.

Mutational analysis of the *K. pneumoniae nifH* promoter has indicated that the GG dinucleotide at positions −25 and −24, the G residue at position −13, and the 10-bp spacing between these two elements are required for promoter function in intact *K. pneumoniae* cells (3–5, 36). Similarly, *flbG* promoter mutations that destroy these features of the promoter eliminate transcription in intact *C. crescentus* cells (D. A. Mullin and A. Newton, manuscript in preparation). We found that destruction of these features of the *flbG* promoter also eliminated promoter function in the *E. coli* cell-free transcription system.

We also examined the effect of transversions of T to G at positions −26 and −15 of the *flbG* promoter sequence on the ability of this promoter to be utilized by *E. coli* $E\sigma^{54}$. Each of these mutations has little effect on expression from the *flbG* promoter in *C. crescentus* (D. A. Mullin and A. Newton, manuscript in preparation), but mutational analysis of the *nifH* promoter has suggested that a T at position −26 plays a role in the activation of transcription from this promoter by NR_I (5). We found that T to G transversions at −26 or −15 essentially eliminated the utilization of the *flbG* promoter by *E. coli* $E\sigma^{54}$. Thus, the effect of these transversions may indicate a slightly different specificity of the transcriptional apparatus that recognizes this type of promoter in *C. crescentus* and *E. coli*.

Nucleotide sequence analysis of *C. crescentus* promoters suggests that this organism contains multiple forms of RNA polymerase responsible for the expression of *fla* genes at different levels of the regulatory hierarchy (15, 27; G. Ramakrishnan and A. Newton, unpublished data). Our data with a heterologous transcription system consisting of *C. crescentus fla* promoters and components of the *E. coli* Ntr genetic switch imply that the promoter specificity of one of these RNA polymerases is nearly identical to that of the *E. coli* σ^{54} RNA polymerase. Similar promoter sequences have been found upstream from the xylene-catabolic genes on the TOL plasmid of *Pseudomonas putida* (9, 21) and the gene encoding the dicarboxylic acid porter (*dctA*) of *Rhizobium meliloti* (39). Thus, it is clear that σ^{54} (or a sigma factor with analogous specificity) is required for the expression of a diverse set of genes in a variety of bacteria.

Most interestingly, the *Caulobacter* σ^{54} type of promoters are under positive control by *fla* genes higher in the regulatory cascade (7, 26, 34) and they require an "enhancer" element located approximately 100 bp upstream from the transcriptional start site for expression in intact cells (D. A. Mullin and A. Newton, manuscript in preparation). These observations suggest that these *C. crescentus* σ^{54}-type promoters may be regulated by a mechanism similar to that functioning at Ntr promoters in *E. coli* (29). It can be argued that the general features of the genetic switch existed prior to the divergence of *E. coli* and *C. crescentus* and that the genetic switch was recruited for different regulatory functions during the evolutionary divergence of these two organisms. In this light it is of interest that in *E. coli*, *fla* promoters contain neither the σ^{54} nor the σ^{70} type of consensus sequence; they contain instead sequence elements homologous to the σ^{28}-dependent promoters of *Bacillus subtilis* (18). A selective advantage may be obtained by dedicating a fraction of the cell's RNA polymerase to functions that require very high levels of transcription for short periods of time, as in the case of flagellum production or, in enteric bacteria, the adaptive response to nitrogen starvation. In the case of flagellum production, *E. coli* has apparently recruited a σ^{28} analog for this purpose, while *C. crescentus* has apparently recruited a σ^{54} analog for one set of the *fla* genes.

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