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# Regulation of the *Caulobacter crescentus rpoN* gene and function of the purified $\sigma^{54}$ in flagellar gene transcription

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**Abstract** The sequential transcription of flagellar (*fla*) genes in the *Caulobacter crescentus* cell cycle is controlled by the organization of these genes in a regulatory hierarchy of four levels (I-IV). Level III and level IV genes at the bottom of the hierarchy are dependent on level II genes and are transcribed late in the cell cycle from $\sigma^{54}$-dependent promoters. To study the regulation of genes at levels III and IV, we have isolated and sequenced the *rpoN* gene in order to analyze its expression, purified the *rpoN* gene product, and examined the role of the RpoN protein in initiation of transcription from $\sigma^{54}$-dependent promoters. We report here epistasis experiments that show *rpoN* is required for transcription of level III genes, but that the expression of the *rpoN* gene itself is not dependent on any of the *fla* genes examined; these results place *rpoN* at level II near the top of the hierarchy. Consistent with this conclusion were nuclease S1 assays that mapped the *rpoN* transcription start site and identified a sequence centered at –24, GTTA/TACCA/TT, which is similar to the core consensus sequence of the level IIB *fliF*, *fliL*, and *fliQ* promoters. We purified the full-length *rpoN* gene product to near homogeneity and demonstrated that the RpoN protein is required for transcription from the well-characterized $\sigma^{54}$-dependent *glnAp2* promoter of *Escherichia coli* and specifically recognizes the level III *flbG* gene promoter of *C. crescentus*. These last results confirm that *rpoN* encodes the *C. crescentus* $\sigma^{54}$ factor and opens the way for the biochemical analysis of transcriptional regulation of level III and IV *fla* genes.

**Key words** *rpoN* regulation · RpoN purification in vitro transcription

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## Introduction

Asymmetric cell division in *Caulobacter crescentus* produces two morphologically distinct progeny: a sessile stalked cell and a motile swarmer cell. Flagellum biosynthesis in the incipient swarmer cell depends on the sequential transcriptional activation and/or repression of the flagellar (*fla*) genes. The *fla* genes have been organized into four levels (I-IV) of a regulatory hierarchy (see Fig. 1) in which transcription of operons at the lower levels depends on transcription of operons above them (Newton et al. 1989; Xu et al. 1989; Ohta et al. 1991). The order in which the flagellar components are pro-

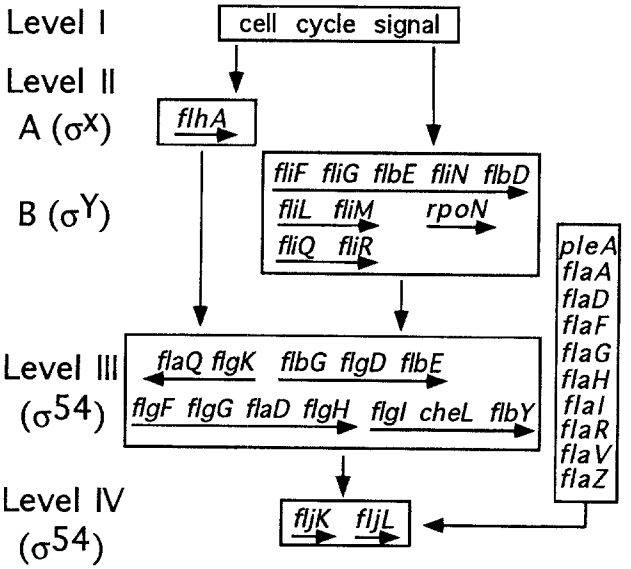


**Fig. 1** Abbreviated regulatory hierarchy of *Caulobacter crescentus fla* genes. The *fla* genes are assigned to levels I-IV. In this model, genes at the lower levels require expression of genes above them for transcription. The sigma factors thought to be involved in transcription of genes at each level are indicated (Benson et al. 1994a). Genes at the right of the Figure beginning with *pleA* have not been placed in the hierarchy, but are known to be required for production of the level IV flagellins

duced generally reflects their order of assembly. Level I genes have yet to be identified, but they are thought to respond directly to cell cycle signals. Level II genes include the level IIA *flhA* gene, which encodes a protein that may be required for translocation of flagellar components (Ramakrishnan et al. 1991; Sanders et al. 1992), as well as the level IIB genes, which encode the M-ring and switch proteins (Dingwall et al. 1992b; Ramakrishnan et al. 1994), and the transcription factor FlbD (Ramakrishnan and Newton 1990). Level III genes encode the basal body proteins and hook protein (Ohta et al. 1982; Dingwall et al. 1992a) and level IV genes make the subunits of the flagellar filament (Minnich and Newton 1987).

One type of *fla* gene regulation may involve the use by RNA polymerase of secondary σ subunits that recognize specialized *fla* gene promoters. The level IIB *fliF* (VanWay et al. 1993), *fliL* (Stephens and Shapiro 1993), and *fliQ* (Dingwall et al. 1992b) promoters share a promoter consensus that is thought to be recognized by a novel sigma factor, designated $\sigma^Y$ (Benson et al. 1994a), also referred to as $\sigma^R$ (Brun and Shapiro 1992). The *flhA* gene is expressed earlier in the cell cycle than the level IIB operons and from a different promoter class (level IIA; Ramakrishnan et al. 1991; Sanders et al. 1992) that may be recognized by another sigma factor, designated $\sigma^X$. Work from this and other laboratories (Minnich and Newton 1987; Mullin et al. 1987; Ninfa et al. 1989; Dingwall et al. 1990; 1992a; Mullin and Newton 1993) has shown that genes at levels III and IV are transcribed from promoters recognized by the specialized $\sigma^{54}$ (RpoN) RNA polymerase (Kustu et al. 1989). The conclusion that level III *flgK* and *flbG* promoters are transcribed from $\sigma^{54}$-dependent promoters is supported by the results of site-directed mutagenesis (Mullin and Newton 1989) and in vitro transcription using core RNA polymerase and $\sigma^{54}$ ($E\sigma^{54}$) from *Escherichia coli* (Ninfa et al. 1989).

Transcription initiation by $E\sigma^{54}$ in *Salmonella typhimurium* and other bacteria depends on interactions with specific transcriptional activators that typically bind to essential DNA sequences ca 100 nucleotides upstream of the transcriptional start site (Kustu et al. 1989). Genetic analysis of the *C. crescentus fla* genes has identified analogous *cis*-acting sequences termed *ftr* (*f*lagellar *t*ranscriptional *r*egulator) that are required for transcription of the $\sigma^{54}$-dependent genes (Mullin et al. 1987; Mullin and Newton 1989; 1993; Gober and Shapiro 1992). Ramakrishnan and Newton (1990) reported that *flbD*, a level IIB gene, is required for expression of genes at levels III and IV and encodes a homologue of the response regulator NtrC, a protein that activates transcription from $\sigma^{54}$-dependent promoters in other bacteria (Kustu et al. 1989). Recent work has demonstrated that FlbD is an *ftr*-specific DNA-binding protein and that FlbD binding to *ftr* sequence elements is required to activate transcription in vitro from the level III *flbG* promoter by $E\sigma^{54}$ (Benson et al. 1994a). Alignment of putative *ftr* sequences together with in vivo and in vitro transcription analysis now suggests that FlbD is a global activator of transcription from all identified level III and IV $\sigma^{54}$-dependent *fla* gene promoters (Benson et al. 1994b; Wu et al., submitted).

Reconstitution of level III *fla* gene transcription in vitro has so far depended on the use of $E\sigma^{54}$ from *E. coli*. In order to develop a transcription system comprised of *C. crescentus* proteins, we have cloned the *C. crescentus rpoN* gene and begun a characterization of the activity of its gene product. We show here that purified *C. crescentus* RpoN protein recognizes the well-characterized *E. coli glnAp2* promoter, as well as the $\sigma^{54}$-dependent *flbG* promoter of *C. crescentus*. Results from in vitro transcription experiments confirm that the *rpoN* gene identified in this bacterium does in fact encode $\sigma^{54}$. Mapping of the *rpoN* transcription start site by S1 nuclease analysis permitted us to identify a 5′ nucleotide sequence similar to a conserved core consensus sequence present in level IIB promoters. We also show that *rpoN* transcription does not require any of the *fla* genes examined. These genetic and biochemical results are consistent with the assignment of *rpoN* to level II in the *fla* gene hierarchy.

## Materials and methods

### Strains and culture conditions

*C. crescentus* strains used were all derived from strain CB15 (ATCC 19089). PC strains were isolated or constructed in this laboratory and SC strains were isolated by Johnson and Ely (1979). Recombinant, pRK2L1-derived plasmids were introduced into *C. crescentus* by conjugation (Ohta et al. 1984). *C. crescentus* strains were routinely grown in M2 medium or PYE medium supplemented, where indicated, with kanamycin (150 μg/ml in M2 and 50 μg/ml in PYE) or tetracycline (2 μg/ml) as described (Ohta et al. 1984). *E. coli* strains were grown in ML medium supplemented with ampicillin (50 μg/ml) or tetracycline (15 μg/ml).

### PCR amplification and cloning

Degenerate deoxyoligonucleotides used as primers corresponded to two stretches of amino acids found to be identical in all $\sigma^{54}$ proteins examined and were synthesized at the Princeton SynSeq facility. Third position degeneracy was minimized by exploiting the GC bias of the *C. crescentus* genome. The sequences used were 5′ GGG GAT CCY TCV GCR TAC TTV GCV AC 3′ and 5′ CGG AAT TCG ARC TSA AGT AYT TCT TC 3′ (R is A or G, S is C or G, V is G, C, or A, and Y is C or T). To facilitate cloning of the PCR product, the 5′ termini of the primers contained *Bam*HI and *Eco*RI sites, respectively. PCR reactions were performed on a thermal reactor (Ericomp) with a program of 35 cycles of 95°C for 30 s, 50°C for 1 min. A 221 nucleotide product was produced and cloned into pBluescript (Stratagene) to yield pDKA004.

A *C. crescentus* genomic library in bacteriophage λL47.1 (Ohta et al. 1982) was probed using the purified PCR product that had been labeled with α-[$^{32}$P]ATP by nick translation (Maniatis et al. 1982). One hybridizing clone was analyzed with restriction endonucleases and the 3.2 kb *Hin*dIII hybridizing fragment was cloned into pBluescript to give pDKA010.

### Mapping the *rpoN* gene

Restriction endonuclease digestion conditions were those recommended by the enzyme vendors. The chromosomal location of *rpoN* was mapped by Southern analysis (Southern 1975) of *C. crescentus* genomic DNA digested with *Dra*I, or *Ase*I, and separated by pulsed-field gel electrophoresis (PFGE; Ely and Gerardot 1988). The PCR product was used as a probe. The hybridizing fragments were 305 kb and 250 kb long, respectively, consistent with a gene located at approximately 6 o'clock on the chromosome (data not shown). The only existing flagellar mutant which mapped to this region was *flbU610*::Tn*5* in SC1055 (Ely and Ely 1989) and the insertion was subsequently mapped to within the *rpoN* coding sequence by Southern analysis. Subcloning was performed according to established protocols.

### Complementation analysis

Plasmids used for complementation were obtained by subcloning the *Eco*RV-*Sma*I, *Eco*RV-*Xho*I, or *Xho*I-*Sma*I fragments of pDKA010 into the mobilizable vector pRK2L1 (Mullin and Newton 1989). These constructs were mated into *rpoN* mutant PC5794 by triparental crosses (Ohta et al. 1984). Complementation of the *rpoN* motility phenotype was judged by the size of the resulting swarm in media supplemented with 0.3% agar and by observing the behavior of individual cells by light microscopy.

### Nucleotide sequencing

DNA sequencing was performed in this laboratory on single-strand template using Sequenase 2.0 (United States Biochemical) or by Lark Sequencing Technologies; 7-deaza-dGTP was used in place of dGTP. Sequence analysis was carried out using the Genetics Computer Group (GCG) package.

### Construction of transcriptional fusions

The 378 bp *Bam*HI$_a$-*Pst*I$_a$ fragment of pDKA010, which contains the *rpoN* promoter, was first cloned into pGEM3Z (Promega) to give plasmid pDKA019 and then moved into pBluescript (Stratagene) as a *Hin*dIII-*Eco*RI fragment to place a *Kpn*I site at the 5′ end and an *Xba*I site at the 3′ end of the fragment. The resulting *Kpn*I-*Xba*I fragment was then cloned into the promoter fusion vector pANZ5 (Ohta et al. 1991) to obtain plasmid pANZ442 with an *rpoN*p-*lacZ* fusion.

### S1 nuclease protection assays

RNA was isolated from CB15 as previously reported (Ohta et al. 1985). S1 assays were carried out essentially according to Berk and Sharp (1977). 5′ end-labeled restriction fragments [*Bam*HI$_a$-*Pst*I$_a$ or the *Eco*RI-*Pst*I$_a$ (Fig. 2D) of pDKA019] were hybridized to 100 μg of total cellular RNA at 60°C. After treatment with S1 nuclease, the resistant DNA fragments were electrophoresed through a polyacrylamide gel and visualized by autoradiography. The sequencing ladder was generated by using a primer designed to have the same 5′ end as the *Pst*I site of the protected fragment: 5′ GTT GCG GGG TGA TGA CGA GG 3′.

### Overexpression and purification of the *rpoN* product

In order to overexpress the gene product, the intact *rpoN* gene was fused to a T7 promoter in pRSETC (Kroll et al. 1993) as follows. An *Nde*I site was introduced at nucleotide 358 of the sequence shown in Fig. 3 by site-directed mutagenesis using oligonucleotide 5′ AGG GCG CAT ATG GCG CTC AG 3′. This changes the putative translation initiation codon from TTG to ATG, and creates a unique *Nde*I site. The *Nde*I-*Hin*dIII fragment was subsequently subcloned in pRSETC (Kroll et al. 1993), creating pDKA053, which placed the new ATG immediately downstream of the ribosome binding site (RBS) of the vector. Plasmid pDKA053 was transformed into *E. coli* strain E509[BL21(DE3)pLysS] (Tabor and Richardson 1985).

The RpoN protein was purified from the soluble fraction of the cells harvested after T7 RNA polymerase was induced with 50 μM IPTG for 1 h. The purification steps included precipitation with polyethyleneimine followed by column chromatography on heparin agarose, Sephacryl S200, and concentration on a DEAE column. Fractions containing the *rpoN* product were identified by SDS-PAGE throughout the procedure. Finally, the fractions containing the *rpoN* product were dialyzed against storage buffer (20 mM TRIS-Cl, pH 7.0, 50% glycerol, 0.1 mM EDTA, 1 mM DTT, 50 mM KCl) and stored at −80°C. The identity of the purified protein as the product of the *rpoN* gene was confirmed by N-terminal protein sequencing (Princeton SynSeq facility).

### In vitro transcription assays

The procedure for the transcription assay has been described in detail previously (Ninfa et al. 1987). Templates were supercoiled plasmids and were present at 5 nM in all experiments. NtrB and NtrC were included where indicated at 100 nM and 160 nM, respectively. *E. coli* core RNA polymerase was used at 100 nM in all experiments. *E. coli* $\sigma^{54}$ and *C. crescentus* $\sigma^{54}$ were included at 400 nM unless otherwise noted. FlbD was used at 1 μM (Benson et al. 1994a). The transcripts were separated by electrophoresis on urea-acrylamide gels and detected by autoradiography.

### Nucleotide sequence accession number

The *rpoN* sequence, including the sequence shown in Fig. 3, has been assigned GenBank accession number L11003.

## Results

### Cloning and nucleotide sequence of the *C. crescentus rpoN* gene

The λ clone L47.1Cc8397 containing the *rpoN* gene was identified in a genomic library of *C. crescentus* DNA (Ohta et al. 1982) using a 221 nucleotide probe generated by PCR amplification from chromosomal DNA, as described in Materials and methods (Fig. 2A). A single 3.2 kb *Hin*dIII fragment from this clone hybridized to the labeled PCR product and DNA sequencing indicated that this fragment contained the entire *rpoN* sequence.

We used genetic complementation of the nonmotile phenotype of an *rpoN* mutant by subcloned DNA fragments to localize the *rpoN* gene within a 2.0 kb *Eco*RV-*Sma*I fragment (Fig. 2B). The Tn*5* mutation used in these studies was originally described by Ely and Ely (1989) as *flbU610*::Tn*5*. We physically mapped this Tn*5* insertion, which is now referred to as *rpoN610*::Tn*5*, within the *Pst*I$_b$-*Pst*I$_c$ fragment of *rpoN* (see Fig. 2B; Materials and methods) and constructed the isogenic *rpoN* strain PC5794 in the wild-type CB15 background.

The DNA nucleotide sequence of the 2.0 kb DNA insert in pDKA015 was determined on both strands as described (Materials and methods). Analysis of the previously unpublished 5′ portion of this sequence, which

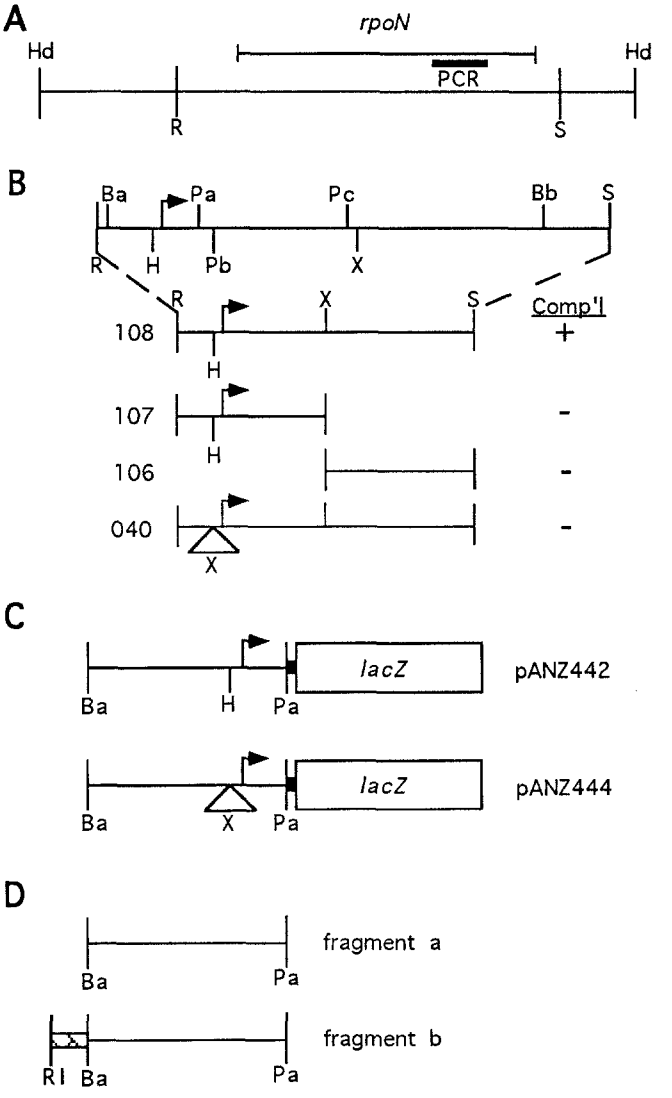


**Fig. 2A–D** Structure of the *C. crescentus rpoN* gene and its 5′ regulatory and promoter region. **A** Restriction map of the *rpoN* region contained in pDKA010 indicating location of DNA fragment (PCR) isolated by PCR amplification. **B** Detailed restriction map of the *rpoN* gene and complementation of *rpoN*::Tn*5*. Plasmids indicated correspond to pNOR106, pNOR107, pNOR108, and pDKA040. **C** Schematic of the *rpoN* promoter *lacZ* fusions. The 378 bp $BamHI_a$-$PstI_a$ fragment was placed upstream of *lacZ* in the mobilizable vector pANZ5 (Ohta et al. 1991). A 6 nucleotide *Xho*I linker was inserted at the *Hpa*I site to generate pANZ444. **D** Restriction fragments used for S1 nuclease analysis. Both fragments derive from pDKA019. The *hatched* sequences between *RI* and $B_a$ is derived from vector sequences. (*R*, *Eco*RV; *RI*, *Eco*RI; *B*, *Bam*HI; *Hd*, *Hin*dIII; *H*, *Hpa*I; *P*, *Pst*I, *S*, *Sma*I; *X*, *Xho*I)

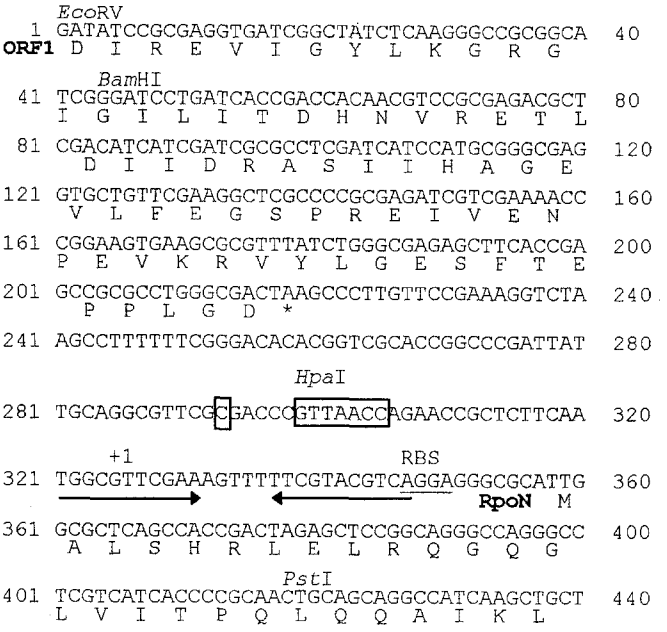


**Fig. 3** Nucleotide sequence of the *C. crescentus rpoN* 5′ regulatory and promoter region. The partial sequence of the 2.0 kb *rpoN* complementing clone pDKA015 is shown starting at the *Eco*RV site. The translated sequences of the partial ORF1 and RpoN are indicated below the nucleotide sequence. Relevant restriction sites are indicated above their recognition sequences. The proposed ribosome binding site preceding the TTG start codon is *underlined* and the probable transcription initiation site is also indicated (+1). Nucleotides conserved in level IIB promoters (see Fig. 5) are *boxed*. An inverted repeat is indicated by the *arrows*

extends 440 bp from the *Eco*RV site (Fig. 3), showed that it contains a partial open reading frame, ORF1, extending from the *Eco*RV site to nucleotide 220. The translated sequence of this ORF is highly similar in amino acid sequence to the C-terminus of the ORF which is typically located upstream from *rpoN* in other bacteria (data not shown; Kullik et al. 1991). The sequence 3′ to ORF1 contains a possible Rho-independent transcription terminator and the probable *rpoN* translation start codon TTG at nucleotide 357. The start codon is preceded by a *C. crescentus* RBS (Kambaty and Ely 1992), AGGA, 9 bases upstream. An inverted repeat partially overlaps this RBS and the proposed transcription start site (Fig. 3; see below).

The ORF corresponding to *rpoN* extends to the UGA termination codon at nucleotide 1849 (data not shown; GenBank accession number L11003) and encodes a predicted protein of 497 residues with a molecular mass of 55,167 Da. While this work was in progress, Brun and Shapiro (1992) reported the cloning of *rpoN* from *C. crescentus* using a similar approach to the one described in this paper, and our translated RpoN sequence (data not shown) is identical to the one published by these authors.

## Regulation of *rpoN* transcription and the requirement of RpoN for *fla* gene expression

Genes at the two lowest levels of the *C. crescentus fla* gene hierarchy (levels III and IV) are transcribed from $\sigma^{54}$-dependent promoters (Chen et al. 1986; Minnich and Newton 1987; Mullin et al. 1987; Ninfa et al. 1989; Dingwall et al. 1990; 1992a; Mullin and Newton 1993). Recent work indicates that *rpoN* is required for expression of several level III and IV genes (Brun and Shapiro 1992; Wingrove et al. 1993) and our results confirm this conclusion. When assayed in transcriptional fusions to

**Table 1** Effect of *rpoN* mutation on the expression of *fla-lacZ* fusions. All values are in Miller units. (*ND* not determined)

| Strains | Plasmids | | | | |
|---|---|---|---|---|---|
| | pANZ404 (*flgK*p) | pANZ405 (*flbG*p) | pANZ406 (*fliF*p) | pANZ118 (*flhA*p) | pANZ4 |
| CB15 (wild type) | 146 | 225 | 194 | 60 | 16 |
| PC5794 (*rpoN*::Tn5) | 29 | 5 | 146 | 51 | ND |

**Table 2** β-Galactosidase activity of *rpoN*p-*lacZ* fusion in different *fla* gene mutants

| Strain[a] | Mutation | Level[b] | β-Galactosidase activity[c] |
|---|---|---|---|
| CB15 | | | 150 |
| CB15/(pANZ5) | | | 15 |
| CB15/(pANZ444) | | | 12 |
| PC5794 | *rpoN*: :Tn5 | II | 427 |
| PC5510 | *flbD*: :Tn5 | II | 120 |
| PC5515 | *flbF*: :Tn5 | II | 136 |
| PC8843 | *fliM*: :Tn5 | II | 130 |
| SC508 | *fliQ153* | II | 114 |
| PC8257 | *flaJ303* | III | 135 |
| SC511 | *flgE155* | III | 152 |
| SC243 | *flgF110* | III | 146 |
| SC295 | *flgI141* | III | 125 |
| PC5236 | *pleA*: :Tn5 | Unclassified | 128 |
| SC229 | *flaA104* | Unclassified | 141 |
| SC252 | *flaD115* | Unclassified | 130 |
| SC279 | *flaF132* | Unclassified | 118 |
| SC278 | *flaG131* | Unclassified | 115 |
| SC284 | *flaH135* | Unclassified | 124 |
| SC270 | *flaI126* | Unclassified | 114 |
| SC305 | *flaR148* | Unclassified | 113 |
| SC293 | *flaV140* | Unclassified | 130 |
| SC3975 | *flaZ102* | Unclassified | 121 |

[a] All strains except wild-type CB15 strains as noted, contain the *rpoN*p-*lacZ* fusion plasmid pANZ442
[b] See Fig. 1 for the location of genes in the *fla* gene hierarchy
[c] All activities are in Miller units (Miller 1972)

*lacZ*, neither the level III *flgK* nor *flbG* promoter was expressed in the *rpoN* mutant background (Table 1). Expression from level IIA *flhA* and level IIB *fliF* promoters, which do not contain the conserved elements of $\sigma^{54}$-dependent promoters, was unaffected in the *rpoN*::Tn*5* strain PC5794 (Table 1), however, as has been demonstrated previously for the level IIB *fliL* and *fliQ* promoters (Brun and Shapiro 1992). Thus *rpoN* is required for expression of $\sigma^{54}$-dependent genes at level III, as expected, but not for expression of level II genes.

We also examined whether *fla* genes at various levels of the hierarchy are required for *rpoN* expression. As judged by the levels of *lacZ* expression from *rpoN*p-*lacZ* fusion plasmid pANZ442 (Fig. 2C), mutations in level II transcription units *fliF*, *fliL*, *fliQ*, and *flhA* had little or no effect on *rpoN*p-*lacZ* expression. The level of transcription from the *rpoN* promoter was increased threefold in *rpoN* mutant strain PC5794 (Table 2). Thus, like the level IIB genes, *rpoN* is negatively autoregulated. Unlike *fliF*, *fliL*, and *fliQ*, however, we found that *rpoN* expression is not negatively regulated by other genes at level II of the hierarchy (Table 2).

RpoN could directly regulate its own expression or, alternatively, the negative regulator could be the product of a gene dependent on RpoN for expression. We examined this latter possibility by testing the effect of mutations in *fla* genes that are below *rpoN* in the hierarchy. None of the mutations in the level III transcription units, such as *flgK*, *flbG*, *flgF*, or *flgI* (Fig. 1), altered the level of *rpoN* transcription (Table 2). We also examined *rpoN* expression in response to mutations in a group of *fla* genes known to be required for expression of level IV flagellin genes (Johnson and Ely 1979), but which have not been otherwise placed in the hierarchy (Fig. 1). None of these mutations had any effect on *rpoN* transcription (Table 2), and consequently, this set of 11 *fla* genes does not appear to participate in either negative or positive regulation of the *rpoN* gene. Thus, *rpoN* behaves as a level II gene in all of the epistasis experiments described here.

## Mapping the *rpoN* transcription start site

The orientation and start site of the *rpoN* transcript were mapped by nuclease S1 assays. The 378 bp *Bam*HI$_a$-*Pst*I$_a$ fragment a and the 390 bp *Eco*RI-*Pst*I$_a$ fragment b (Fig. 2D) from plasmid pDKA019 were used as 5′ end-labeled, double-stranded probes. Because the *Eco*RI site of fragment b lies in the vector polylinker and not in the genomic sequences, this probe should produce a partially protected, labeled fragment only if *rpoN* RNA is transcribed from left to right (see Fig. 2B), as predicted by the ORF (Fig. 3). Fragment a should produce a partially protected, labeled fragment from RNA transcribed in either direction. Protected DNA fragments of approximately 100 nucleotides were produced by both probes (data not shown), confirming the assigned direction of transcription and placing the transcription start site approximately 100 nucleotides to the left of the *Pst*I$_a$ site (Fig. 2B).

To map the 5′ end of the *rpoN* transcript more precisely, a 20 nucleotide strand was synthesized whose 5′ end corresponds to the 5′ end of the *Pst*I$_a$ restriction site of DNA fragment b (see also sequence in Fig. 3; Materials and methods). This oligonucleotide was used to generate a sequencing ladder from single-strand DNA which was electrophoresed in an 8% sequencing gel in parallel with the S1 nuclease reaction product from the *Eco*RI-*Pst*I$_a$

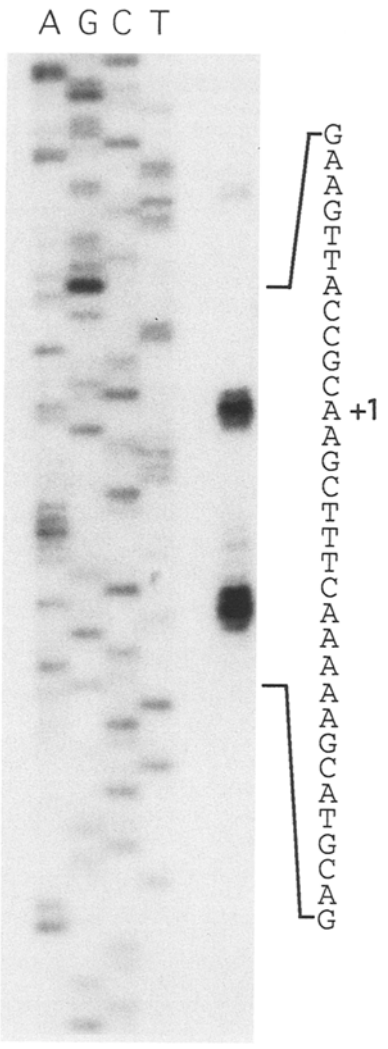


**Fig. 4** Nuclease S1 mapping of the *rpoN* transcription start site. Total RNA was prepared from *C. crescentus* and used in S1 nuclease assays. The 378 nucleotide *Eco*RI-*Pst*I$_a$ probe (fragment b, Fig. 2D) was 5′ end labeled and yielded the protected band shown, when annealed to 100 μg total RNA. The sequencing ladder was generated using an oligonucleotide whose 5′ end corresponds to the 5′ end of the probe. +1 indicates assigned transcription start site

```
                -30        -20        -10        +1
                 .          .          .
fliF  AAACGCCTCGTTTACCTTGTACTGGGTAAATCCTGCCT
fliL  AAACACATCGTTAACCATGCTTCGCGCATGAGTACGGGTA
fliQ  TAACGCCCTGTTAACCATATTTCGTCCATCTTCGAGCC
       AAC C    GTT ACC T      G    A
rpoN  TCGCGACCCGTTAACCAGAACCGCTCTTCAATGGCGT
        ↑*        ** *    ↑          ↑
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**Fig. 5** Alignment of *rpoN* 5′ nucleotide sequences with level IIB promoters. Level IIB promoter consensus. The 5′ sequences of *fliF* (VanWay et al. 1993), *fliL* (Stephens and Shapiro 1993), and *fliQ* (Dingwall et al. 1992b) were aligned to maximize similarities. The start of transcription (+1) is *underlined* at the right. Nucleotides conserved in all three promoters are in *bold* and shown as the level IIB consensus. Nucleotides in the *rpoN* 5′ region which match the consensus are also shown in *bold*. Consensus nucleotides in *rpoN* shown to be important for *fliL* transcription (Stephens and Shapiro 1993) are indicated with an *asterisk*; nucleotides not conserved in *rpoN* at positions shown to be important for *fliL* are indicated by a ↑

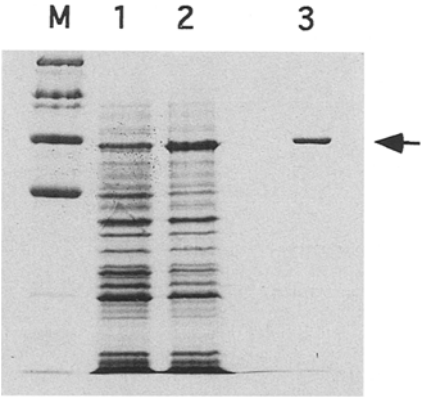


**Fig. 6** Purification of the *C. crescentus rpoN* gene product. Full-length *rpoN* was cloned into the T7 polymerase overexpression vector pRSETC and the resultant plasmid transformed into *Escherichia coli* strain E509. Equal amounts of uninduced cells (lane 1) and cells induced for expression of T7 RNA polymerase with IPTG for 60 min (lane 2) were electrophoresed through the gel. Lane 3 contains 7 μg of the purified protein. Molecular weight markers are shown at the left (lane M): 116, 97, 66, and 45 kDa, respectively

probe. A strong partially protected fragment that maps at nucleotide 326 was observed (Fig. 4). The faint bands at 325 and 327 could result from either multiple start sites or the action of nucleases. We consider the smaller transcript mapping to nucleotide 342 to result from rapid processing of the primary transcript. Rapid and specific processing of other *C. crescentus fla* gene transcripts, including the *flbG* transcript, have been reported (Chen et al. 1986). Thus we consider the strong band at 326 the most probable start site, and its position on the *rpoN* sequence is indicated in Fig. 3. The assigned +1 position is located in the left arm of an inverted repeat (Fig. 3), which could be involved in the observed negative autoregulation of *rpoN* (Table 2).

## Sequence requirements for *rpoN* expression

Level IIB *fliF*, *fliQ*, and *fliL* promoters contain a core consensus sequence with a degenerate *Hpa*I site at ca –25 (see Fig. 5). Nucleotides within this core are known to be essential for transcription of *fliF* (VanWay et al. 1993) and *fliL* (Stephens and Shapiro 1993). The 5′ sequence of *rpoN*, which our results suggest may be a level II gene, also contains a *Hpa*I site at ca –25 (Fig. 3). To determine if this sequence is important for *rpoN* transcription, we inserted a 6 nucleotide linker containing the *Xho*I restriction site at the *Hpa*I site at nucleotide 301 in the 2.0 kb *Eco*RV-*Sma*I fragment (Fig. 3). The ability of the *rpoN* gene to complement the *rpoN610*::Tn*5* mutation was totally abolished in the resulting plasmid pD-

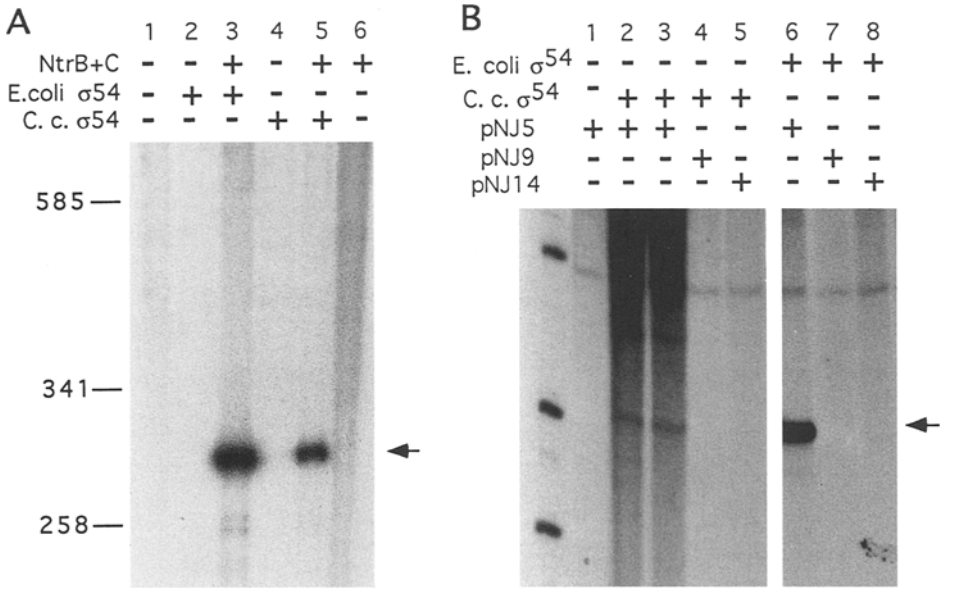


**Fig. 7A,B** In vitro transcription using purified transcription components. **A** Recognition of the *glnAp2* promoter. Single cycle transcription assays were performed on the supercoiled template pTH8. *E. coli* core RNA polymerase was used at 100 nM. Where present, NtrB and NtrC were used at 160 nM and 100 nM, respectively. *E. coli* and *C. crescentus* $\sigma^{54}$ was used at 400 nM. **B** Recognition of the *flbG* promoter in vitro by *C. crescentus* and *E. coli* $\sigma^{54}$. Single cycle transcription assays were performed using three different plasmids (Ninfa et al. 1989) in which the wild-type and mutant promoters of *flbG* gene are fused 365 nucleotides upstream of the Rho-independent terminator of the parental plasmid pTE103. Plasmid pNJ5 (lanes 1, 2, 3, 6) contains the wild-type *flbG* promoter. Plasmid pNJ9 (lanes 4, 7) has the mutant *flbG* promoter in which a GC dinucleotide at –12 and –11 is deleted. Plasmid pNJ14 (lanes 5, 8) contains a mutant *flbG* promoter in which a G residue at –26 is changed into a T residue. Templates were present at 5 nM in each reaction. *E. coli* core RNA polymerase was present in all reactions at 100 nM. *C. crescentus* transcriptional activator FlbD was added to each reaction at 1 μM. In lanes 2–5, the *C. crescentus* $\sigma^{54}$ factor was added to a final concentration of 800 nM and 1.6 μM in lane 3. In lanes 6–8, the *E. coli* $\sigma^{54}$ subunit was present at a final concentration of 400 nM. Neither *C. crescentus* nor *E. coli* $\sigma^{54}$ factor was added in lane 1. Size standards at the right are the same as those indicated in **A**

KA040 (Fig. 2B). We also constructed an *rpoN*p-*lacZ* fusion containing the *Xho*I linker inserted in the same *Hpa*I site (Fig. 2C). The derivative plasmid pANZ444 produced only 12 units of β-galactosidase activity, while its wild-type cognate, pANZ442, makes 150 units of enzyme activity in strain CB15 (Table 2). Thus the linker insertion at nucleotide 301 apparently disrupts sequence elements essential for *rpoN* transcription. In the Discussion we consider the similarity of the 5′ *rpoN* sequence to the level IIB promoters.

## Overexpression and purification of the *rpoN* gene product

The *C. crescentus rpoN* gene was cloned downstream of the inducible T7 promoter in pRSETC (Kroll et al. 1993) to create plasmid pDKA053 (Materials and methods). Induction of *rpoN* expression by the addition of IPTG to *E. coli* strain E509/pDKA053 produced a new protein which migrated with an apparent molecular weight of 67 kDa by SDS-PAGE (Fig. 6, compare lanes 1 and 2). Although this contrasts with the calculated molecular weight of 55 kDa, many sigma factors display mobilities expected of proteins larger than their predicted molecular weights (Burton et al. 1981; Gitt et al. 1985). The protein was subsequently purified to near homogeneity (Materials and methods; Fig. 6, lane 3). The N-terminal amino acid sequence was determined to be (Met) Ala Leu Ser His Arg Leu Glu Leu, which corresponds to residues predicted from the nucleotide sequence (Fig. 3) and confirmed that the protein purified is encoded by *rpoN*.

## Recognition of the $\sigma^{54}$-dependent *glnAp2* promoter by the *C. crescentus rpoN* gene product

The transcriptional activity of the purified *C. crescentus rpoN* gene product was examined using the *E. coli* core RNA polymerase in an in vitro transcription assay. Initially we used the well-characterized $\sigma^{54}$-dependent *glnAp2* promoter in the supercoiled plasmid pTH8 (Hunt and Magasanik 1985) as the template. With the *E. coli* $\sigma^{54}$ and transcription factors NtrB and NtrC, a transcript of 300 nucleotides was produced (Fig. 7A, lane 3), as expected if transcription were initiated at the start site previously characterized for this promoter (Ninfa et al. 1987). When the *C. crescentus* $\sigma^{54}$ was substituted for *E. coli* $\sigma^{54}$ under the same conditions, a transcript of the same size was produced (Fig. 7A, lane 5) and its production was absolutely dependent on the presence of NtrB and NtrC (Fig. 7A, lane 4). These biochemical results confirm that the *C. crescentus rpoN* gene product purified here catalyzes the initiation of transcription from a $\sigma^{54}$-dependent promoter.

Several explanations can account for the lower activity of the *C. crescentus* $\sigma^{54}$ compared to that of the *E. coli* $\sigma^{54}$. Recognition of either the *E. coli* promoter sequences or the *E. coli* core RNA polymerase by *C. crescentus* $\sigma^{54}$ may be relatively inefficient. Additionally, recognition between the NtrC activator protein and the holoenzyme containing *E. coli* core enzyme and *C. crescentus* $\sigma^{54}$ could be inefficient. As shown below, however, the *C. crescentus* $\sigma^{54}$ also gives lower activity even on a *C. crescentus* promoter with the *C. crescentus* FlbD as the activator (Fig. 7), which suggests that the *C. crescentus* $\sigma^{54}$ is less active with the *E. coli* core than is the *E. coli* $\sigma^{54}$.

### *C. crescentus* RpoN activates transcription from the *flbG* gene promoter

We examined the ability of the purified *C. crescentus* $\sigma^{54}$ protein to recognize *C. crescentus* $\sigma^{54}$-dependent promoters in vitro by carrying out single cycle transcription assays using the supercoiled plasmid template pNJ5. This plasmid contains the $\sigma^{54}$-dependent promoter from the level III *flbG* operon (Ninfa et al. 1989; Benson et al. 1994a). When purified RNA polymerase components consisting of *E. coli* core enzyme and the *C. crescentus* $\sigma^{54}$ and FlbD activator protein were used, we obtained a transcript of the size expected for initiation from the *flbG* promoter (Fig. 7B, lanes 2 and 3). No *flbG* transcript was detected when $\sigma^{54}$ was omitted from the reaction mixture (Fig. 7B, lane 1).

To verify that the transcript produced from the pNJ5 template was specific for the *flbG* promoter, we examined whether this highly purified transcription system recognized promoters with mutations known from previous studies to eliminate transcription in vitro and in vivo (Mullin and Newton 1989; Ninfa et al. 1989). A *flbG* template containing a T to G transversion at position –26 (pNJ9) was not recognized by RNA polymerase containing either *C. crescentus* $\sigma^{54}$ (Fig. 7B, lane 4) or *E. coli* $\sigma^{54}$ (Fig. 7B, lane 7). We also observed that deletion of the invariant GC dinucleotide at –12 (pNJ14) abolished initiation of transcription from the *flbG* promoter (Fig. 7B, lanes 5 and 8). Thus, the purified *rpoN* gene product is specific in its recognition of the *C. crescentus* $\sigma^{54}$-dependent *flbG* promoter in the pNJ5 template and is required for transcription from this promoter in vitro, as well as in vivo (Table 1).

## Discussion

RNA polymerases containing alternate $\sigma$ factors play a central role in the differentiation of prokaryotic cells, most notably in the $\sigma$ cascade regulating transcription of the sporulation-specific *Bacillus subtilis* genes (Haldenwang 1995). Analysis of *C. crescentus fla* genes and their promoters has suggested that the sequential activation and repression of these genes during the cell cycle are controlled by the activities of specialized RNA polymerases and their cognate regulatory proteins (see Introduction and Fig. 1). Genetic evidence, DNA sequence analysis, site-directed mutagenesis, and in vitro transcription results (Minnich and Newton 1987; Mullin et al. 1987; Mullin and Newton 1989; Ninfa et al. 1989) have shown that the *fla* genes at levels III and IV of the hierarchy are transcribed by RNA polymerase containing $\sigma^{54}$. We report here the cloning of *C. crescentus rpoN*, overexpression of the RpoN protein in *E. coli*, and in vitro transcription assays demonstrating that the purified *rpoN* gene product specifically recognizes $\sigma^{54}$-dependent promoters from both *E. coli* and *C. crescentus*. Experiments discussed in the next section suggest that *rpoN* itself should be placed at level II near the top of the *fla* gene hierarchy.

### Is *rpoN* a level II gene?

Results of extensive epistasis experiments have shown that *rpoN* behaves like a level II gene of the *fla* gene hierarchy (Tables 1, 2):

1. *rpoN* is required for expression of level III genes transcribed from the $\sigma^{54}$-dependent *flgK* and *flbG* promoters (Table 1); in addition *rpoN* is known to be required for expression of the level III *flgF* gene (Brun and Shapiro 1992) and the level IV *fljK* and *fljL* flagellin genes (Wingrove et al. 1993).
2. Genes at level II do not require *rpoN* for their expression: normal transcription from the level IIA *flhA* promoter and the level IIB *fliF* promoter occurs in a $\sigma^{54}$ mutant strain (Table 1). These genes are not negatively regulated by *rpoN*, unlike the level IIB promoters *fliL* and *fliQ*.
3. None of the level II transcription units identified, including *fliF*, *fliL*, *fliQ*, or the level IIA *flhA* gene, is required for *rpoN* transcription (Table 2).

Thus, *rpoN* behaves as a level II gene in these experiments (Fig. 1), but the fact that it is required for developmental functions such as stalk formation (Brun and Shapiro 1992) sets *rpoN* apart from other *fla* genes and suggests that it should be classified only nominally as a member of the *fla* gene family.

We observed that in an *rpoN* mutant strain, the level of *rpoN*p-*lacZ* fusion expression is about 3 times higher than in the wild-type strain CB15. We were, however, unable to identify any *fla* genes that are involved in the negative regulation of *rpoN* (Table 2). If the negative regulator is not the *rpoN* gene product itself, we can imagine that it could be the product of a *fla* gene not tested in these experiments, not yet identified or, alternatively, a gene required for some other cell cycle or developmental function.

### Promoter structure of level II genes

Level II genes are transcribed from what appear to be two promoter types. The level IIA gene *flhA* is expressed earlier in the cell cycle than any other *fla* gene, encodes a gene product implicated in export of the flagellar proteins, and contains a promoter similar in sequence to the $\sigma^{28}$-dependent promoters described in *E. coli* and *S. typhimurium* (Ramakrishnan et al. 1991; Sanders et al. 1992).

Level IIB genes *fliF*, *fliL*, and *fliQ* are expressed at about the same time in the cell cycle, after *flhA* and before the level III genes (Ramakrishnan et al. 1991; VanWay et al. 1993). These promoters share a 13 nucleotide consensus that extends from –10 to –36 on the *fliF* sequence (Fig. 5). Hallmarks of this consensus are a degenerate *Hpa*I recognition site GTTAAC centered at –24 and two shorter flanking motifs (Fig. 5). Extensive site-directed mutagenesis of *fliF* (VanWay et al. 1993) and *fliL* (Stephens and Shapiro 1993) has shown many of the conserved nucleotides, including those within the core consensus, are important for transcription. Comparison of the sequence 5′ to the *rpoN* transcription start site identified a *Hpa*I site that aligns with the IIB core consensus (Fig. 5). The effect of disrupting this *Hpa*I site suggests that the GTT-ACC motif is also part of a 5′ sequence essential for *rpoN* transcription (Fig. 2B; Table 2).

Mutagenesis studies similar to those carried out on *fliF* (VanWay et al. 1993) and *fliL* (Stephens and Shapiro 1993) will be required to define the *rpoN* promoter, but pairwise comparison of the 5′ nucleotide sequence of these genes reveals interesting similarities and differences. Although *rpoN* does not contain most of the conserved nucleotides flanking the *Hpa*I motif present in the level IIB consensus (Fig. 5), examination of the nucleotides between –1 and –37 shows it shares 18 nucleotides in common with *fliQ*, 14 with *fliL*, and 13 with *fliF*. A number of the non-consensus nucleotides shared between *rpoN* and the other promoters are either not essential for transcription or their role in transcription has not been examined. Thus, if *rpoN* is transcribed by the same RNA polymerase holoenzyme that recognizes level IIB operons, it may be subject to different regulation.

### In vitro transcription of $\sigma^{54}$-dependent promoters by *C. crescentus* RpoN

Temporal control of *fla* gene transcription is complex, as illustrated by the regulation of the *flbG* promoter, which is under positive regulation of genes at level II, and is negatively regulated by genes in the *flbG* operon and the divergent *flgK* operon (Newton et al. 1989; Mullin and Newton 1993). Phosphorylation has also been implicated in FlbD function (Wingrove et al. 1993; Benson et al. 1994b) and additional factors may play a role in the observed negative autoregulation of *flbG* (Mullin and Newton 1993). To understand these multiple layers of regulation fully, it will be necessary to reconstitute transcription in vitro using *C. crescentus* components. Ninfa et al. (1989) originally demonstrated that *E. coli* holoenzyme $E\sigma^{54}$ can recognize and transcribe the *flbG* promoter in the presence of *E. coli* proteins NtrB and NtrC. Our laboratory has recently purified the natural activator of *flbG* transcription, FlbD, and shown that it can replace NtrB and NtrC for in vitro transcription of *flbG* using the *E. coli* $E\sigma^{54}$ (Benson et al. 1994a). In the present work, we have taken a further step by purifying the product of *C. crescentus rpoN* and showing it can functionally replace *E. coli* $\sigma^{54}$ on an *E. coli* promoter, *glnAp2*, and direct transcription of *flbG* by *E. coli* core RNA polymerase in the presence of FlbD. This purified protein is an essential component needed to establish an in vitro transcription system constituted exclusively of *C. crescentus* transcription factors.

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