A Developmentally Regulated *Caulobacter* Flagellar Promoter is Activated by 3' Enhancer and IHF Binding Elements

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The transcription of a group of flagellar genes is temporally and spatially regulated during the *Caulobacter crescentus* cell cycle. These genes all share the same 5' cis-regulatory elements: a σ^{54} promoter, a binding site for integration host factor (IHF), and an enhancer sequence, known as the *ftr* element. We have partially purified the *ftr*-binding proteins, and we show that they require the same enhancer sequences for binding as are required for transcriptional activation. We have also partially purified the *Caulobacter* homolog of IHF and demonstrate that it can facilitate in vitro integrase-mediated lambda recombination. Using site-directed mutagenesis, we provide the first demonstration that natural enhancer sequences and IHF binding elements that reside 3' to the σ^{54} promoter of a bacterial gene, *fla*NQ, are required for transcription of the operon, in vivo. The IHF protein and the *ftr*-binding protein is primarily restricted to the predivisional cell, the cell type in which these promoters are transcribed. *fla*NQ promoter expression is localized to the swarmer pole of the predivisional cell, as are other flagellar promoters that possess these regulatory sequences 5' to the start site. The requirement for an IHF binding site and an *ftr*-enhancer element in spatially transcribed flagellar promoters indicates that a common mechanism may be responsible for both temporal and polar transcription.

INTRODUCTION

The bacterium *Caulobacter crescentus* undergoes a simple developmental program within each cell cycle. Cell division results in the formation of distinct daughter cells, a sessile stalked cell and a motile swarmer cell that possesses a single polar flagellum. The biogenesis of the polar flagellum occurs in the predivisional cell and requires ~50 genes (Ely and Ely, 1989). These genes are expressed in an ordered sequence during each cell division cycle. The temporal control of expression of many of these genes is regulated at the transcriptional level and is subject to a trans-acting regulatory hierarchy (reviewed in Bryan *et al.*, 1990; Newton and Ohta, 1990; Gober *et al.*, 1991a; Gober and Shapiro, 1991). Within this hierarchy, the temporal order of flagellar gene transcription during the cell cycle approximates the order

of assembly of their gene products into the flagellar structure (Champer *et al.*, 1985, 1987; Chen *et al.*, 1986; Newton *et al.*, 1989; Xu *et al.*, 1989). For example, a gene that encodes a basal body ring protein is transcribed earlier than genes that encode proteins that are assembled after the basal body is formed, such as those in the hook and the flagellar filament (Dingwall *et al.*, 1990).

In addition to temporal control of gene expression, a subset of flagellar genes are subject to spatial regulation. The asymmetric positioning of proteins within the predivisional cell is a critical aspect of flagellar biogenesis and the *Caulobacter* developmental program in general. At least two different mechanisms are used to generate polarity in the predivisional cell. Several flagellar structural proteins (Loewy *et al.*, 1987) and chemoreceptor proteins (Nathan *et al.*, 1986; Alley *et al.*, 1992) are specifically targeted the swarmer pole of the predivisional cell. A second mechanism that serves to generate polarity is the localization of the mRNA of at least two flagellar genes to the incipient swarmer pole of the pre-

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divisional cell (Milhausen and Agabian, 1983; Gober *et al.*, 1991b). The two genetic loci that exhibit this asymmetric positioning of their mRNA are *flgK*, which encodes a 25-kDa flagellin (Milhausen and Agabian, 1983), and the hook operon (Gober *et al.*, 1991b). The hook promoter sequences alone are sufficient to accomplish polar mRNA localization (Gober *et al.*, 1991b). These results suggest that transcription of genes that exhibit mRNA localization is specifically limited to the chromosome in the swarmer portion of the predivisional cell and that cell division traps the transcript in the resulting progeny swarmer cell. The hook operon is not transcribed in the progeny swarmer cell.

The promoters that exhibit spatially restricted expression possess a similar sequence architecture (Gober *et al.*, 1991b). They all have the bacterial σ^{54} -promoter consensus sequence (Mullin *et al.*, 1987; Minnich and Newton, 1987) and require σ^{54} , the product of the *rpoN* gene, for transcription activity in vivo (Gober and Brun, unpublished data) and in vitro (Ninfa *et al.*, 1989). To initiate transcription, σ^{54} -containing RNA polymerase must interact with a transcriptional activator protein (reviewed in Kustu *et al.*, 1989; Popham *et al.*, 1989). The binding site for the transcriptional activator, or enhancer sequence, for σ^{54} promoters is located 5' relative to the promoter sequence at a distance of ~ 100 base pairs (bp) (Collado-vidos *et al.*, 1991). The *C. crescentus*, *flgK*, and hook operon promoters both possess the same putative enhancer sequence located at ~ 100 bp from the transcription start site (Minnich and Newton, 1987; Mullin and Newton, 1989). This enhancer sequence, or *ftt* element, is required for transcription of the hook operon promoter in vivo (Mullin and Newton, 1989; Gober *et al.*, 1991c). Two proteins, a 95- and 55-kDa protein, were found to bind to this region of the hook operon promoter in a southwestern assay (Gober *et al.*, 1991c). Gel-shift experiments have demonstrated that at least one of these binding activities is under cell-cycle control (Gober *et al.*, 1991c). We report here the partial purification of these binding proteins and show that the proteins that bind to the enhancer element require the same sequences for binding activity as are necessary for transcriptional activation.

A binding site for integration host factor (IHF), located between the enhancer sequence (*ftt*) and the σ^{54} promoter, is essential for wild-type levels of hook operon promoter transcription in vivo (Gober and Shapiro, 1990). IHF is a small heterodimeric DNA binding protein that was initially identified as a host requirement for bacteriophage lambda integration (reviewed in Landy, 1989). We demonstrate here that the partially purified IHF homolog from *C. crescentus* can substitute for *Escherichia coli* IHF in an in vitro bacteriophage lambda recombination assay. Upon binding to DNA, IHF induces a large bend (Stenzel *et al.*, 1987; Robertson and Nash, 1988; Thompson and Landy, 1988; Kosturko *et al.*, 1989) that serves to facilitate the recombination reaction (Ri-

chet *et al.*, 1986) by permitting integrase to simultaneously interact with distantly spaced DNA sequences (Moitoso de Vargas *et al.*, 1989). Hoover *et al.* (1990) have proposed that IHF plays an analogous role in σ^{54} promoters, in that the bend induced by IHF delivers transcriptional activators, bound at enhancer sequences, to RNA polymerase, bound at the promoter. In support of this, IHF was shown to be required for transcription of the *nifH* promoter of *Klebsiella* in an in vitro transcription-translation system (Hoover *et al.*, 1990).

The IHF- and enhancer-binding proteins were found predominantly in the predivisional cell, the cell type in which the genes that they regulate are transcribed. In addition, we report that the flagellar *flaNQ* operon is transcribed in the incipient swarmer cell portion of the predivisional cell. The promoter region of this operon contains the same cis-regulatory elements as the two previously described spatially expressed promoters, but these conserved sequences were observed 3' to the transcription start site (Mullin and Newton, 1989; Gober and Shapiro, 1990). Using in vitro mutagenesis, we demonstrate for the first time that native bacterial 3'-enhancer sequences and IHF binding sites are required for maximal levels of transcription of a σ^{54} promoter.

METHODS

Bacterial Strains, Plasmids, and Growth Conditions

A synchronizable strain (NA1000) derived from wild-type CB15 (Evinger and Agabian, 1977) was grown at 32°C in either peptone yeast extract (PYE) (Poindexter, 1964) or modified minimal M2 medium (Contreras *et al.*, 1978). For *lacZ* transcriptional fusions, promoter fragments were cloned into plasmid *placZ/290* (Alley and Gober, unpublished data). This plasmid consists of the promoterless *E. coli lacZ* gene from pRSZ3 (Alley, unpublished data; Gober and Shapiro, 1990) subcloned into pRK290 (Ditta *et al.*, 1980). *C. crescentus* cells harboring *placZ/290* and its derivatives were grown in medium supplemented with 2 μ g/ml tetracycline.

Fractionation of the *ftt* Binding Proteins

C. crescentus NA1000 cells were grown to mid-log phase in PYE in a 200-l fermenter. The cells were harvested by centrifugation and suspended in an equal volume of 40 mM tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 7.4), 2 mM EDTA, 20% glycerol, and were frozen in liquid nitrogen. Cells (100 g) were disrupted in a french pressure cell. The protein was precipitated by the addition of $(\text{NH}_4)_2\text{SO}_4$ to 50% saturation, followed by centrifugation. Precipitated extract was dialyzed against buffer A50 (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 50 mM $(\text{NH}_4)_2\text{SO}_4$, 10% glycerol, 1 mM dithiothreitol (DTT), 0.1 mM PMSF). This was applied to a heparin agarose column (80 ml) equilibrated with A50 (Shore *et al.*, 1987). The column was washed with one volume of A50. Proteins that remained bound to the column were eluted with a linear gradient (80 ml) of A50–A600 [A50 with 600 mM $(\text{NH}_4)_2\text{SO}_4$] and 1-ml fractions were collected (Shore *et al.*, 1987). Activity in the column fractions was monitored by gel mobility shift assays (Fried and Crothers, 1981) with labeled synthetic double-stranded *ftt* oligonucleotide as a binding substrate, as described previously (Gober *et al.*, 1991c). *ftt*-binding activity eluted in a broad peak at ~ 400 –450 mM $(\text{NH}_4)_2\text{SO}_4$. The active fractions were pooled, concentrated by precipitation in $(\text{NH}_4)_2\text{SO}_4$ (50% saturation), and dialyzed against A50 containing 0.05% octylglucoside. This fraction was then subjected to DNA sequence-specific chromatography (Kadonaga and Tjian, 1986). The column (2 ml) consisted

of an oligonucleotide that contained the core *flr* sequences, in addition to some adjacent sequence (–120 to –80 bp from the hook promoter transcription start site) coupled to sepharose. Protein was applied to the oligonucleotide column in A50 buffer plus 0.05% octylglucoside. The column was washed with five volumes of A50 plus 0.05% octylglucoside. Protein bound to the column was then eluted with a linear KCl gradient (0–500 mM), and the active fractions (300–400 mM KCl) were pooled and used for subsequent analysis.

Partial Purification of IHF

The *C. crescentus* homolog of IHF was partially purified by using the methods of Nash and Robertson (1981). Cells were grown, and extracts were prepared as described above. The crude extract was subjected to precipitation with Polymyxin P followed by chromatography on a phosphocellulose column. Extracts were assayed for IHF by Western blots with anti-*E. coli* IHF antibody (gift of H. Nash) and by an in vitro lambda intermolecular recombination assay (Nash and Robertson, 1981). *attP* and *attB*-containing plasmids, pure integrase, and pure *E. coli* IHF were the generous gift of H. Nash. The reaction consisted of 0.25 μ g of linearized pBB105 (*attB*) and 0.25 μ g supercoiled pHN823 (*attP*), incubated in the presence of integrase (35 ng) and either *E. coli* IHF (15 ng) or *C. crescentus* extract (50 μ g protein) from the phosphocellulose column (Nash and Robertson, 1981). Reactions were incubated 1 h at room temperature and electrophoresed in a 1% agarose gel in Tris-boric acid-EDTA buffer at 60 V overnight. To detect recombination products with *C. crescentus* extracts, the DNA was transferred to nitrocellulose and hybridized to ³²P-labeled pBB105.

Mutagenesis of the *flbG* and *flaNQ* Promoter Regions and Construction of Transcription Fusions

Site directed mutagenesis was performed on a 1.3-kb *Pst* I-*Xho* I M13mp19 clone of *flaNQ-flbG* promoter DNA (Gober and Shapiro, 1990) grown in *E. coli* dut ung strain CJ236 (Kunkel and Roberts, 1987). In vitro mutagenesis was performed on single-stranded DNA isolated from this strain, as described by Kunkel and Roberts (1987). All mutagenesis was confirmed by dideoxy DNA sequencing (Sanger *et al.*, 1977). To test mutant promoters for relative transcription activity, they were cloned upstream of a promoterless *E. coli lacZ* reporter gene (*placZ/290*). An 805-bp *Sac* I-*Xho* I fragment containing *flbG* hook operon DNA from –120 to +685 bp was used for testing hook promoter transcription in the absence of upstream *flaNQ* sequences (see Fig. 3A). To test for *flaNQ* transcription in the absence of hook promoter sequences, a 420-bp *Sac* I-*Pst* I DNA fragment that contained from –81 to +339 bp of *flaNQ* DNA was cloned upstream of the promoterless *lacZ* reporter gene in *placZ/290* (see Figs. 6–8). The transcription fusions were introduced into *C. crescentus* NA1000 by conjugation with an *E. coli* donor. β -galactosidase activity was determined in mid-log phase cultures (OD₆₆₀ = 0.5–0.9) grown in PYE, as described previously (Gober and Shapiro, 1990). Calculation of unit activity was performed as described by Miller (1972).

Synchronization of Cell Cultures

Cells were synchronized by isolating pure populations of swarmer cells by the use of density centrifugation in colloidal silica (Ludox LS, Du Pont, Wilmington, DE) as described previously (Evinger and Agabian; 1977; Shapiro *et al.*, 1982). For assaying localized expression of *flaNQ*, cells harboring a *flaNQ-lacZ* transcription fusion on plasmid *placZ/290* were grown in Medium M2 (1 l), and swarmer cells were isolated. The swarmer cell suspension was placed in fresh M2 and permitted to progress through the cell cycle at 32°C. At the predivisional stage (120–150 min), proteins were labeled with [³⁵S]-trans-label (methionine) (ICN Biomedical, Irvine, CA), and the label was chased as described previously (Gober *et al.*, 1991b). The predivisional cells were allowed to divide. The progeny swarmer and stalked cells were separated by centrifugation through Ludox LS, and labeled β -galactosidase was immunoprecipitated as described (Gomes and

Shapiro, 1984) with monoclonal anti- β -galactosidase antibody (Promega, Madison, WI).

For preparation of protein extracts from synchronized populations, 10 l of *C. crescentus* NA1000 were grown to late log phase (OD₆₆₀ = 1.0) in PYE. Swarmer cells were isolated by centrifugation through Ludox LS. Isolated swarmers were resuspended in fresh PYE medium and allowed to progress through the cell cycle at 32°C. After another round of cell division, the newly divided progeny swarmer and stalked cells were separated by centrifugation through a Ludox gradient. The stalked cell fraction was further fractionated to remove predivisional cells. To accomplish this, the stalked cell fraction was diluted with medium M2 and was centrifuged at 4000 $\times$ g for 5 min. The supernatant, which is enriched for stalk cells, was removed and centrifuged again. This was repeated four more times. The resulting supernatant was judged, by light microscopy, to be >95% stalk cells. Cells were lysed by sonication and extracts were prepared as described by Gober *et al.* (1991c).

Other Assays

IHF binding to wild-type and mutant *flaNQ* promoters was assayed with *E. coli* IHF in a DNase I protection assay (Galas and Schmitz, 1978). The 420-bp *Sac* I-*Pst* I DNA fragment of wild-type *flaNQ* DNA, as well as the IHF binding site mutants, NIN-1 and NIN-2 (see Fig. 8), were cloned into pUC19. The resulting plasmid(s) were digested with *Eco*RI, labeled with [α -³²P]-deoxynucleotide triphosphates (3000–6000 Ci/mmol, Amersham, Arlington Heights, IL) with DNA polymerase I, Klenow fragment (Boehringer-Mannheim, Indianapolis, IN). The labeled DNA was then digested with *Pst* I and purified from an acrylamide gel after electrophoresis. The DNase I protection assay was performed essentially as described previously (Gober and Shapiro, 1990), with 0.1 pmol of labeled promoter DNA and pure *E. coli* IHF (200 nM) (gift of H. Nash).

Southwestern assay for DNA binding proteins (Miskimins *et al.*, 1985) was performed on crude cell extracts prepared from synchronized populations. A synthetic double stranded oligonucleotide representing the hook promoter upstream region (from –120 to –80 bp) was used as a binding substrate. The assay was performed as described previously (Gober *et al.*, 1991c).

Western blot analysis (Towbin *et al.*, 1979) was performed on extracts prepared from synchronized populations. The assay employed anti-IHF antibody raised against *E. coli* IHF (gift of H. Nash) and was performed as described previously (Gober and Shapiro, 1990).

RESULTS

flr Sequences are Required for Enhancer Binding Activity

The organization of the divergent *flbG* hook and *flaNQ* operons is shown in Fig. 1A. Two binding proteins of 95 and 55 kd that specifically interact with the hook operon promoter *flr* region were identified by gel mobility shift assays and southwestern assays (Gober *et al.*, 1991a). These binding activities are not detectable in cell extracts prepared from strains carrying mutations in class I flagellar genes, *flaO* (SC290) and *flaW* (SC297) (Gober *et al.*, 1991c). To determine if the ability of the 95- and the 55-kDa proteins to bind to the *flr* site is periodic, Southwestern analysis was performed on cell extracts obtained from synchronized populations. Both the 95- and the 55-kDa binding activities are regulated similarly (Fig. 2). DNA binding activity is maximal in the predivisional stage and decreases to lower levels after cell division (Fig. 2).

Figure 1. Genetic organization of the divergent *flbG* hook and *flaNQ* flagellar operons. (A) Genetic map of the divergent *flbG* hook and *flaNQ* operons (Ohta *et al.*, 1985; Chen *et al.*, 1986). A schematic of the promoter regions between the transcription start sites is shown below the genetic map (Mullin and Newton, 1989; Gober and Shapiro, 1990). Both the operons use σ^{54} promoters, (Mullin and Newton, 1989; Ninfa *et al.*, 1989) which are denoted by two black boxes upstream of the transcription start site (designated +1). The cis-regulatory elements of the *flbG* promoter, an IHF binding site and an *ftr*-enhancer sequence, exist 5' to the transcription start (Mullin and Newton, 1989; Gober and Shapiro, 1990; Gober *et al.*, 1991c). In the divergent *flaNQ* promoter, these sequences are located 3' to the transcription start (Mullin and Newton, 1989; Gober and Shapiro, 1990). The *flaNQ* regulatory region contains two copies of the *ftr* enhancer with a dyad symmetry element between them. (B) Sequence of the *flaNQ* promoter region. The DNA sequence shown is from Mullin and Newton (1989). The region shown is from -117 to +181 from the transcription start site. The *ftr*-sequence elements are boxed. An *ftr* element located upstream at -96 bp is critical for transcription of the divergent hook promoter (Mullin and Newton, 1989; Gober *et al.*, 1991c; Fig. 3A). There are two additional elements located 3' to the *flaNQ* transcription start site at +104 and +137 bp (Mullin and Newton, 1989). There exist several regions of dyad symmetry denoted by half arrows. An IHF binding site is located from +24 to +57 from the transcription start (Gober and Shapiro, 1990). The region underlined represents the sequences that IHF binding protects from digestion by DNase I (Gober and Shapiro, 1990). All of these 3' cis-elements occur upstream of the ribosome binding site (denoted S.D.) and the translation initiation codon.

To determine if the sites within the region between the *flaNQ* and the hook operon promoters that interact with specific binding activities are the same sites that are required for transcriptional activation, competition gel mobility shift assays were performed with wild-type and mutant enhancer sequences. It was necessary to use the hook operon promoter for these assays, instead of the *flaNQ* *ftr* sequences, because the hook promoter contains only one *ftr* element. Utilizing DNA sequence affinity chromatography, we fractionated the *ftr*-binding activities, as described in MATERIALS AND METHODS, and used the pooled fraction in the gel mobility shift competition assays (Figure 3B). A 112-bp fragment of hook promoter DNA was used as a radiolabeled sub-

strate for DNA binding. This DNA fragment contained hook promoter sequences from -120 to -8 bp from the transcription start. These include a single *ftr* enhancer element, an IHF binding site, and a σ^{54} promoter. The unlabeled competitor DNAs were the double-stranded *ftr* oligonucleotide (representing -120 to -80 bp) and two plasmids containing either wild-type or mutant hook operon DNA. Plasmid pG0.7 contained wild-type hook operon DNA from -120 to +685 bp from the transcription start site. Plasmid pGRF-2 contained the identical DNA fragment, except the GG sequence in the *ftr* element (bases -115 and -114) was changed to AA by site-directed mutagenesis. In the gel mobility shift competition assay shown in Figure 3B, *ftr* oligonucleotide

Figure 2. Cell-type expression of *ftr*-binding activities in a South-western assay. Extracts were prepared from predivisional cells and progeny swarmer and stalked cells. Proteins in the extract (25 μ g/lane) were separated by SDS-polyacrylamide gel (10%) electrophoresis, transferred to a nitrocellulose filter, and assayed for DNA binding activity, as described previously (Gober *et al.*, 1991c). A [32 P]-labeled double-stranded oligonucleotide representing the hook operon promoter upstream region (–120 to –80 bp) was used as a DNA binding substrate. The apparent molecular weights of the separated proteins are indicated in kilodaltons. The predominant cell types used for extract preparation are shown above each autoradiogram; at left, the predivisional stage followed by progeny stalked and swarmer cells. These progeny cells were separated after cell division, as described in MATERIALS AND METHODS.

and plasmid DNA containing the wild-type hook promoter, competed for the binding activity. In contrast, the plasmid that contained the altered sequences in the *ftr* element was unable to compete for DNA binding activity. This mutant promoter, when fused to a promoterless *E. coli lacZ* reporter gene (pGRF-2), generated 71 units of β -galactosidase activity, compared with 902 units generated by a *lacZ* transcriptional fusion using a wild-type hook promoter pG0.7 (Figure 3A). It was previously shown that mutagenesis of one of these bases, results in a decrease in hook transcription (Mullin and Newton, 1989). These results show that the same *ftr* sequence that is required for hook promoter transcription is also specifically recognized by DNA binding protein(s). By analogy to other σ^{54} -dependent promoters, these DNA binding protein(s) may act as transcriptional activator(s) of the hook promoter.

Caulobacter crescentus Possesses a Functional IHF Homolog

Although the IHF binding sites in flagellar σ^{54} promoters and those in σ^{54} promoter regions from other bacteria are important for transcription activity (Santero *et al.*, 1989; Gober and Shapiro, 1990; Hoover *et al.*, 1990), IHF protein has yet to be identified outside of the enteric bacteria. Western blot analysis, employing antibody directed against *E. coli* IHF, shows that *C. crescentus* cell extracts possess the homologs of two polypeptides of similar molecular weight to the α and β subunits of *E.*

coli IHF (Gober and Shapiro, 1990). To obtain evidence that *C. crescentus* possesses a functional homolog of IHF, we have partially purified IHF from *C. crescentus* and tested its activity in an in vitro lambda recombination assay.

Figure 3. Gel mobility shift assays with *ftr*-binding proteins and a wild-type and mutant hook operon *ftr* element. (A) A schematic of the hook operon promoter fused to a reporter *lacZ* gene (pG0.7) is shown. The conserved sequence of dyad symmetry that is present in all *ftr* elements, –CGGC–, was mutagenized to –GAAG–. This mutant promoter was also fused to the promoterless *lacZ* reporter gene in *placZ*/290 to yield plasmid pGRF-2. The activity of β -galactosidase (Miller, 1972) in cells containing each of these plasmids is shown. (B) Subclones of these mutant promoters were used in gel mobility shift competition experiments. Partially fractionated *ftr*-binding protein (12.5 ng protein) from the pooled active fraction eluted from the oligonucleotide column described in MATERIALS AND METHODS, was used in conjunction with a labeled DNA fragment that contained sequences from –120 to –8 bp from the hook promoter transcription start site. F indicates unbound DNA. B indicates a labeled DNA-protein complex. In lane 1, no protein was added. Lane 2, protein extract plus labeled hook promoter DNA (0.09 pmol). In lanes 3 and 4, the reaction consisted of labeled hook promoter DNA (0.09 pmol), protein extract, and unlabeled double-stranded *ftr* oligonucleotide (–120 to –80) as a competitor (lane 3; 26 pmol, lane 4; 390 pmol). In lanes 5 and 6, a plasmid that contained wild-type hook DNA in pG0.7 from –120 to +685 bp was used as an unlabeled competitor at 0.17 pmol and 0.5 pmol, respectively. In Lanes 7 and 8, a plasmid that contained mutant hook promoter DNA from GRF-2 from –120 to +685 bp was used as an unlabeled competitor at 0.17 pmol and 0.5 pmol, respectively.

After polymin P precipitation, fractionated cell extracts were subjected to phosphocellulose column chromatography. Column fractions were tested for the presence of the IHF protein by use of Western blot analysis with anti-IHF antibody (Figure 4B) and for IHF function by use of the in vitro lambda recombination assay (Figure 4A). Using antibody raised against *E. coli* IHF, *C. crescentus* column fractions showed only two cross-reacting species. Fraction 30 showed the highest amount of both subunits. The relative amounts of the two subunits detected by Western blot differed in the column fractions. We do not know if this reflects differences in

Figure 4. Partially purified IHF assayed by Western blots and an in vitro lambda intramolecular recombination reaction. *C. crescentus* proteins fractionated by phosphocellulose chromatography were tested in A an in vitro lambda recombination assay and B a Western blot with anti-*E. coli* IHF antibody. (A) Recombination assay conditions were those of Robertson and Nash (1981). Reaction products were separated by electrophoresis in an agarose gel and visualized by hybridization to a radioactive probe as described in MATERIALS AND METHODS. The reaction consists of 0.25 μ g of a linear plasmid (pBB105) that contains *attB* sequences and 0.25 μ g of a supercoiled (Sc.) plasmid (pHN823) that contains *attP* sequences. Incubation of these substrates with either integrase alone (first lane) or IHF alone (not shown) results in no recombination product formed. However, incubation together with DNA results in the formation of a recombination product denoted with an arrow (second lane). In the remaining lanes *C. crescentus* fractions (50 μ g protein) from a phosphocellulose column were substituted in the recombination reaction for pure *E. coli* IHF. Numbers above these lanes indicate fraction numbers from the phosphocellulose column. (B) Western blots with anti-IHF antibody raised against *E. coli* protein. The first lane contained 0.1 μ g of pure *E. coli* IHF. Lanes from phosphocellulose column of *Caulobacter* extracts contained 0.4 μ g protein.

the absolute amount of each subunit or if there are differences in the activity of the antibody for each subunit. It is also possible that each subunit elutes differently from the phosphocellulose column.

We attempted to measure the activity of column fractions in gel mobility shift assays, but because of the presence of many proteins in these fractions, we could not establish that the observed binding activity was due to IHF. To assay IHF function we instead used the in vitro lambda intermolecular recombination assay described in MATERIALS AND METHODS and shown in Figure 4A. When a supercoiled plasmid that contains *attP* sequences and a linear plasmid that contains *attB* sequences are incubated with lambda integrase and IHF, a recombination product is formed that can be detected by agarose gel electrophoresis. No product is formed when the DNA is incubated with either integrase alone (Figure 4A) or IHF alone. Detectable recombination activity is dependent on IHF binding to specific sequences on substrate DNA and inducing a sharp bend. Figure 4A shows that fractionated *C. crescentus* protein from the phosphocellulose column can substitute for *E. coli* IHF in facilitating the intermolecular recombination activity. A peak of activity is evident in fraction 30 from the phosphocellulose column that coincides with the presence of both the 9.5- and 10.5-kDa proteins, as detected by Western blots with anti-IHF antibody (Figure 4B). The recombination product formed with *C. crescentus* protein is apparently the same size as the product formed with *E. coli* IHF (Figure 4A). To facilitate integrase-mediated recombination in this assay, IHF bends the DNA at specific locations (Moitoso de Vargas *et al.*, 1989). We presume that the *C. crescentus* IHF is binding to the same sequences recognized by the *E. coli* protein. Based on the ability of *C. crescentus* extracts to facilitate the same reaction, we conclude that *C. crescentus* possesses a functional homolog of the IHF protein.

IHF Level Changes upon Asymmetric Cell Division

The amount of IHF heterodimer, as determined by Western blot assays with anti-IHF antibody was found to change during the cell cycle (Figure 5). Assays of synchronized populations revealed that IHF levels are highest in the predivisive cell stage and decrease to low levels after cell division. IHF is almost undetectable in the swarmer cells. It then reappears after swarmer make their transition into predivisive cells later in the cell cycle. This pattern of expression is similar to that of the 95- and the 55-kDa binding proteins shown in Figure 2. The genes requiring the *flr*- and IHF-binding activities are not transcribed in either the progeny swarmer cell or stalked cell but are only transcribed in the predivisive cell when the binding proteins are most abundant.

Spatial Expression of the *fla*NQ Operon

The hook operon and *flgK* (flagellin) promoters appear to be expressed only from the chromosome in the

Figure 5. Cell-type expression of IHF. Western blots were done on extracts of predivisional cells and progeny swarmer and stalked cells with the use of anti-IHF antibody. Extracts were prepared as described above and in MATERIALS AND METHODS. Protein (100 μ g per lane) was separated by electrophoresis in a 15% SDS-polyacrylamide gel, transferred to nitrocellulose, and the presence of IHF was assayed with anti-*E. coli* IHF antibody, in conjunction with an alkaline phosphatase enzymatic detection system (Gober and Shapiro, 1990). The cell types present at the time of extract preparation are shown in schematic form above each lane. The molecular weight of cross-reacting bands was determined from their relative mobilities to pre-stained molecular weight standards and pure *E. coli* IHF.

swarmer pole of the predivisional cell (Gober *et al.*, 1991b). The predivisional cell contains two chromosomes one in each portion of the cell: the "swarmer" portion and the "stalked" portion. It appears that late in the cell cycle the swarmer- and stalked-cell portions of the predivisional cell exist as separate compartments (Maddock and Shapiro, unpublished data). To test for the localized expression of the *fla*NQ operon, a transcriptional fusion was constructed that consisted of a 550-bp *Sac* I-*Pst* I fragment of *fla*NQ DNA (–81 to +470) ligated 5' to a promoterless *E. coli lacZ* reporter gene in plasmid *placZ*/290 (Figure 6). This fusion does not contain any known 5' cis-regulatory elements (see Figure 1) yet actively expresses β -galactosidase in vivo (see Figure 7). The only apparent enhancer sequences present are located 3' of the promoter. Cells harboring this fusion were synchronized and allowed to progress to the predivisional stage where expression of the *fla*NQ operon is maximal (Ohta *et al.*, 1991). The *fla*NQ operon is not transcribed in the progeny swarmer or stalked cells. Proteins synthesized in the predivisional cell were pulse labeled, the label was chased, the cells were permitted to divide, and the progeny swarmer and stalked cells were separated. The labeled β -galactosidase in extracts of the swarmer cell and stalked cell progeny was immunoprecipitated with antibody to β -galactosidase and visualized by autoradiography after sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Figure 6). The presence of labeled β -galactosidase in one or the other or both of the progeny cells provides a record of the location of *fla*NQ-*lacZ* transcription and translation in the predivisional cell. Previous experiments

have demonstrated that reporter genes driven by their own promoters, including those encoding neomycin phosphotransferase II, luciferase, or β -galactosidase are found in both progeny cell types (Gober *et al.*, 1991b). In this experiment, the distribution of reporter gene product in the progeny cells reflects the position of its expression in the predivisional cell, and it is dictated solely by the expression of the flagellar gene promoter in the transcriptional fusion. Transcriptional fusion of *lacZ* to the *gyrB* (gyrase B), promoter in *C. crescentus* results in the appearance of labeled β -galactosidase in the progeny stalked cell (Rizzo, Shapiro, and Gober, in preparation). In contrast, assay of the *fla*NQ fusion to *lacZ* showed that the majority of labeled β -galactosidase is present in the progeny swarmer cell (Figure 6). Therefore, this fusion is apparently transcribed and translated preferentially in the swarmer pole of the predivisional cell, and subsequent cell division traps the labeled β -galactosidase in the progeny swarmer cell. This result is the same as that obtained with the spatially expressed *flgK* and hook operon promoters (Gober *et al.*, 1991b), indicating that all three of these promoters are subject to similar regulatory controls.

*fla*NQ Expression Requires σ^{54} Promoter Sequences

An important consideration in the use of transcription fusions to assay *fla*NQ gene expression is that the assay

Figure 6. Localized expression of a *fla*NQ-*lacZ* transcription fusion. Predivisional cells were isolated from a culture of wild type *C. crescentus* CB15N harboring a plasmid that contains a *fla*NQ-*lacZ* transcriptional fusion. The predivisional cells were pulse-labeled with [35 S]-trans-label (methionine) for 10 min and then chased with 10 μ M unlabeled methionine and 0.2% peptone. The cells were permitted to divide, the progeny stalked and swarmer cells were isolated, and the segregation of β -galactosidase (synthesized in the predivisional cell) to the two progeny cells was assayed by immunoprecipitation, as described previously (Gomes and Shapiro, 1984).

Figure 7. Mutagenesis of the *flaNQ* σ^{54} promoter sequences and 3' cis-regulatory sequences. (A) A schematic diagram of the *flaNQ* promoter region (Mullin and Newton, 1989) is shown. The sequence of the σ^{54} promoter region is shown below. The conserved GC and GG dinucleotides at -12 and -24 , respectively, are underlined. The sequences of two mutant promoters, generated by site-directed mutagenesis are also shown. In one mutant, NPRD-1, the conserved GC at -12 is changed to TT. The other mutant, NPRD-2, possesses a 1-bp deletion between the -12 and -24 regions. The wild-type and mutant promoters were cloned upstream of the promoterless *lacZ* reporter gene of *placZ/290*. The fusion contained a 420-bp *Sac*I/*Pst*I DNA fragment representing sequences from -81 to $+339$ bp from the transcription start of *flaNQ*. The in vivo β -galactosidase level generated by this wild-type fusion was compared with that generated by fusions with the two mutant promoters. Units of β -galactosidase are that of Miller (1972). The activity represents the mean β -galactosidase activity obtained from at least three different midlog phase cultures. Standard deviation, in all cases, was $<5\%$. (B) A schematic diagram of the *flaNQ* promoter region is shown with the detailed sequence of the putative downstream enhancer elements shown below. W.T. represents the wild-type sequence. Below this are three mutant sequences created by site-directed mutagenesis. In mutant NRF1, the conserved $-GCCG-$ of the most proximal *ftr* element is changed to $-GAAG-$. In mutant NRF2, the analogous bases in the distal *ftr* element are altered. In mutant NRF5, the sequence with dyad symmetry, located between the *ftr* elements has been mutated. The wild-type and mutant promoters were fused to the reporter *lacZ* gene, and the levels of β -galactosidase generated by each fusion were assayed in *C. crescentus*.

reflects the activity of the σ^{54} promoter and not other unknown promoter activities within the transcriptional fusion. To establish that the σ^{54} promoter is the only sequence contributing to β -galactosidase expression, site-directed mutagenesis was performed (Figure 7A). Previously, the T at -15 (e.g., the first T in the sequence $-TTGC-$) was shown to be important for expression of *flaNQ* (Mullin and Newton, 1989). However, this base, although conserved in many σ^{54} promoters, is not necessarily essential for transcription. Indeed, the *nifH* σ^{54} promoter of *Klebsiella* possesses a C not a T at this position (Sundaresan *et al.*, 1983). It is established, that

the GC and GG dinucleotides at -12 and -24 , respectively, are important for transcription activity (reviewed in Dixon, 1984, Johnston and Downie, 1984, Gussin *et al.*, 1986). Additionally, the 10-bp spacing between these elements is also critical. To confirm that the apparent σ^{54} promoter in *flaN* was in fact important for transcription, we mutagenized the -12 GC to TT and tested for transcription activity in vivo, by use of the *lacZ* transcriptional fusion. Additionally, the spacing between the conserved GG and GC dinucleotides was altered by deleting a single base pair between the -24 and -12 regions. Both of these mutations almost com-

pletely abolished the synthesis of β -galactosidase (Figure 7A). Finally, the wild-type *flaNQ-lacZ* fusion was assayed for expression in a newly identified σ^{54} mutant of *C. crescentus* (Brun and Shapiro, in preparation). There was a similarly low level of β -galactosidase expression in this mutant. These experiments indicate that the promoter activity within the 550-bp *Sac* I-*Pst* I fragment of *flaNQ* DNA derives from the identified σ^{54} promoter.

Transcription of the *flaNQ* Promoter is Activated by Downstream Enhancer Elements

The 550-bp *Sac* I-*Pst* I DNA fragment contains all the cis-regulatory sequences required for temporal and spatial expression of the *flaNQ* promoter. No apparent enhancer-like sequences exist upstream of the σ^{54} promoter in this DNA fragment. Previous experiments showed that a deletion of most of the sequence 3' to the *flaNQ* promoter results in a decrease in expression (Mullin and Newton, 1989). Here, we directly test whether the 3'-enhancer elements participate in transcriptional activation. Site-directed mutagenesis was performed on the enhancer-like sequences located from +90 to +137 bp from the transcription start site (Figure 7B). The bases mutagenized in the *flr* elements were the CC in the sequence GCCG. This is the conserved element of dyad symmetry in all *flr* enhancer elements (reviewed in Newton and Ohta, 1990). In addition, these two bases in the dyad symmetry were shown to be important for transcriptional activation of the hook operon promoter (Mullin and Newton, 1989; Figure 3A) and for *flr*-binding activity (Figure 3B). As shown in Figure 8A, the *flaNQ* promoter derivatives were fused to the promoterless *lacZ* reporter gene, and β -galactosidase activity was measured in wild-type *C. crescentus*. The native promoter generated 516 units of β -galactosidase activity. A 2-bp mutation in the *flr* element that lies closest to the promoter (+90 to +104 bp) (NRF1) abolishes transcription, generating only 15 units of β -galactosidase activity. These results demonstrate that this 3'-enhancer sequence is essential for transcriptional activation of the *flaNQ* promoter. A mutation in the distal *flr* element (NRF2) decreases the rate of *flaNQ* transcription to about one-third that of wild type. A sequence with dyad symmetry that could also be a protein binding site lies between the two *flr* elements. A mutation in this region (NRF5) yielded transcriptional activity that is similar to that obtained with the mutation in the more distal *flr* element (NRF2) (Figure 7B). These results demonstrate that transcription of the *flaNQ* promoter is activated by downstream enhancer sequences.

A 3' IHF Binding Site can Facilitate Transcriptional Activation

The IHF binding site, downstream of the *flaNQ* promoter, exists in the same basic configuration as is found

in other σ^{54} promoters (Santero *et al.*, 1989; Gober and Shapiro, 1990; Hoover *et al.*, 1990; Claverie-Martin and Magasanik, 1991; deLorenzo *et al.*, 1991). Here also, it is located between the enhancer sequences and the σ^{54} -promoter sequences. After establishing that the most proximal *flr* element, 3' to the *flaNQ* promoter, functions as its principle enhancer, we next tested whether the 3'-IHF binding site was required for maximal levels of transcription. Previous experiments with the hook operon promoter demonstrated that its 5'-IHF binding site is necessary for maximal levels of transcription in vivo (Gober and Shapiro, 1990). Two different two-base-pair changes were made in the A+T-rich IHF binding region between the σ^{54} promoter and the enhancer elements of the *flaNQ* promoter region (Figure 8A). These mutations are within the sequences that are protected in DNA footprinting experiments with IHF (Gober and Shapiro, 1990, see Figure 8B). The mutant promoters (NIN1 and NIN2) were assayed for relative transcription activity by utilizing the promoterless *lacZ* reporter gene construction described above. Both mutant promoters generated β -galactosidase activities that were approximately one-third that of wild type (Figure 8A). With a DNase I protection assay, we tested whether IHF could bind to these mutant promoters (Figure 8B). With the wild-type promoter *E. coli* IHF (200 nM) protects ~32 bp from attack by DNase I between +24 and +57 bp from the transcription start site (Figure 8B). Both mutant promoters (NIN1 and NIN2) do not bind IHF at this concentration (200 nM), as evidenced by the inability of IHF to protect from attack by DNase I (Figure 8B). We conclude that the IHF-binding site downstream of the *flaNQ* promoter is necessary for maximal levels of transcription in *Caulobacter*. These results are analogous to those obtained in experiments with the hook-operon promoter in which IHF resides 5' to the transcription start site (Gober and Shapiro, 1990). Therefore, these experiments show that IHF can apparently facilitate the interactions between enhancer elements and polymerase bound at the promoter sequences, independent of their location relative to the transcription start site.

DISCUSSION

Localized Transcription of Flagellar Genes

The *flaNQ* promoter, like the hook and *flgK* promoters, is transcribed only in the predivisional cell. These genes appear to be expressed exclusively in the swarmer pole of the predivisional cell, and their gene products are sequestered to the swarmer cell progeny on division. The localization of gene expression is due to the promoter sequence (Gober *et al.*, 1991b). The transcription of each of these genes occurs late in the cell cycle, just before cell division. We assume that local expression, as demonstrated by our assay, is occurring after either a membrane barrier or septum has formed between the

Figure 8. Mutagenesis of the 3' IHF binding site in the *flaNQ* promoter region. (A) A schematic diagram of the *flaNQ* promoter region is shown with the detailed sequence of the IHF binding region. Below the wild-type sequence is shown the sequence of two mutated binding sites, NIN1 and NIN2, generated by use of site-directed mutagenesis. The wild-type and mutant promoters were fused to the *lacZ* reporter gene of *placZ*/290 and assayed for β -galactosidase activity. The activity represents the mean β -galactosidase activity obtained from at least three different mid-log phase cultures. The standard deviation, in all cases, was <5%. (B) DNase I protection assay of IHF binding. The *Sac* I-*Pst* I DNA fragment from the wild-type *flaNQ* promoter, and the two mutants, NIN-1 and NIN-2, were uniquely end-labeled with [32 P]. This labeled DNA was gel purified and digested with DNase I in the absence (-) or presence (+) of IHF (200 nM). Note that the pattern of DNase I digestion is different in the mutant promoters. The protected region of wild-type DNA in the presence of IHF, represents sequences +57 to +24 bp from the *flaNQ* transcription start, and was determined by subjecting the labeled DNA fragment to chemical sequencing (G and G + A reactions) (Maxam and Gilbert, 1980).

swarmer and stalked cell poles. Recently, we have obtained evidence with the use of electron microscopy of immunogold-labeled cells that such a barrier forms at what will become the cell division plane, although at this time we do not know the nature of this barrier (Maddock and Shapiro, unpublished data). The compartmentalization of the predivisional cell would account for the fact that the reporter gene product apparently does not diffuse between the stalked and

swarmer poles of the predivisional cell (Gober and Shapiro, 1991b).

All of the flagellar promoters that appear to be transcribed from the chromosome in the "swarmer" portion of the predivisional cell contain the same cis-regulatory elements, suggesting that a common mechanism is responsible for differential transcription of the two chromosomes. Three models can account for the localized transcription of these promoters. First, a critical transcription factor, perhaps one that binds to an enhancer element, may be specifically localized to the swarmer pole of the predivisional cell. There is precedent for protein localization in *Caulobacter*: the chemosensory receptors are specifically localized to the swarmer pole of the predivisional cell (Nathan *et al.*, 1986; Alley *et al.*, 1992). Alternatively, a critical transcription factor may be active only in the swarmer pole. The NtrC protein of enteric bacteria activates transcription only when it is phosphorylated (reviewed in Kustu *et al.*, 1989, 1991). Phosphorylation of NtrC, in turn, is governed by the availability of ammonium in the cell. By analogy, the transcriptional activator of these flagellar promoters may itself be activated by a spatial cue that is unique to the swarmer pole. One candidate for this activation cue is flagellar assembly. All of the locally expressed flagellar promoters require flagellar assembly for transcription. Last, there may be a unique feature of the DNA in the swarmer cell that makes it accessible to the transcription factors. In fact, the physical properties of the swarmer cell chromosome appear to be different than those of the stalked cell. For example, the sedimentation coefficient of the isolated swarmer cell nucleoid is greater than that of the stalked cell nucleoid (Evinger and Agabian, 1977, 1979; Swoboda *et al.*, 1982; Gober and Shapiro, 1991). The differences in swarmer and stalked cell chromosome structure may influence the program of gene expression in each cell type. For this mechanism to be operating, the differences in the physical properties of the chromosomes would also have to apply to plasmids in the two portions of the predivisional cell, because localized expression can be demonstrated when the reporter gene fusions are carried on plasmids (Gober *et al.*, 1991b). We are currently testing each of these models to determine how each contributes to localized flagellar gene expression.

Transcription of the flaNQ Promoter is Regulated by 3' Enhancer and IHF Binding Sequences

The *flaNQ* promoter, which is both temporally and spatially controlled, contains its cis-regulatory elements in a novel configuration. This is the first natural bacterial σ^{54} promoter whose expression has been shown to depend on regulatory elements that are located 3' to the transcription start site. Previous experiments showed that a large deletion of DNA downstream of the *flaNQ* promoter abolished transcription (Mullin and Newton,

1989). We demonstrated here that the critical 3' sequences required for transcriptional activation are two *ptr* elements and another newly identified element with dyad symmetry that lies between the *ptr* sequences. The base pair changes in the *ptr* elements that effect *flaNQ* transcription are analogous to those that are important for transcription of the hook promoter (Figure 3A; Mullin and Newton, 1989). These sequences are also required for the binding proteins to interact with the *ptr* element (Figure 3B). It is therefore likely that the regulation of *flaNQ* transcription by 3' elements is similar to that of the hook operon and *flgK*, both of which require the same elements, but in a 5' configuration (Mullin and Newton, 1989; Gober and Shapiro, 1990).

The NtrC binding sites of the σ^{54} *glnA* promoter of enteric bacteria, that are similar to the *ptr* elements of *Caulobacter*, have been shown to possess the characteristics of eukaryotic enhancer elements (Ninfa *et al.*, 1987). In vitro experiments demonstrated that they are capable of participating in transcriptional activation when placed either upstream or downstream of the promoter (Ninfa *et al.*, 1987). Furthermore, NtrC binding sites can activate transcription of the *glnA* promoter when each are present on different circles of DNA (Wedel *et al.*, 1990). Activation of transcription occurs only when the circles are interlinked. This experiment provides evidence that proteins like NtrC activate transcription by contacting RNA polymerase via a DNA loop. The enhancer sequence serves to increase the concentration of activator proteins in the vicinity of the promoter (Wedel *et al.*, 1990). The finding that downstream sequences participate in the transcriptional activation of *flaNQ*, is consistent with the view that these sequences function as enhancer elements. The *ptr* elements can apparently activate transcription regardless of their relative location to promoter sequences.

IHF is another factor that facilitates transcriptional activation of σ^{54} promoters. The proposed role of IHF is to actuate loop formation, resulting in an increase in the interactions between the enhancer binding protein and RNA polymerase (Hoover *et al.*, 1990). One prediction of this looping model for IHF action is that IHF should function to facilitate transcription activation, whether it is located downstream or upstream of the promoter, the only requirement being that its binding site lie between the enhancer sequence and the promoter. The participation of the 3' IHF binding site in *flaNQ* transcription is consistent with the proposed mechanism of IHF function.

Trans-Acting Factors That Activate Spatially Restricted Transcription

The flagellar genes that appear to be differentially transcribed from the chromosome in the "swarmer" portion of the predivisional cell have the same set of regulatory elements in their promoter regions. Because the proteins

that bind to these regulatory elements might contribute to spatially controlled transcription, we explored their function and time of expression.

Two proteins, 95 and 55 kDa, specifically bind to *ptr* elements and are subject to temporal regulation (Gober *et al.*, 1991c). The presence of maximal binding activity in the predivisional cell may contribute to the temporal pattern of transcription of these flagellar σ^{54} promoters. DNA binding activities were fractionated on the basis of their ability to bind to an oligonucleotide affinity column composed of DNA sequences from the *ptr* region of the hook operon promoter. DNA binding competition experiments show that binding activity requires the same 2 bp in the *ptr* element that are required for hook promoter transcription. The sequence that is required for binding activity is part of a dyad symmetry present in all *ptr* sequences (reviewed in Newton and Ohta, 1990). These data implicate these DNA binding activities in the transcriptional activation of these promoters. However, without more direct evidence, such as demonstration of its function in an in vitro assay or a mutation in the gene that encodes these proteins, their relationship to transcriptional activation is, at present, circumstantial.

It is possible that the 55-kDa binding activity is encoded by the *flbD* gene. Based on DNA sequence homology, the product of the *flbD* gene encodes a 52-kDa protein that shares sequence similarity with the transcriptional activators of σ^{54} promoters of other bacteria (Ramakrishnan and Newton, 1990). The FlbD protein can function as a transcriptional activator of both *E. coli* and *Caulobacter* σ^{54} promoters in *E. coli* (Ramakrishnan and Newton, 1990). In addition, genetic evidence suggests that FlbD regulates flagellar gene expression and has been placed near the top of the regulatory hierarchy (Xu *et al.*, 1989; Newton *et al.*, 1989). However, its target for transcriptional activation in *C. crescentus* remains unknown. For example, strains containing a mutation in the *flbD* gene do not express several genes that are lower in the hierarchy (Xu *et al.*, 1989; Newton *et al.*, 1989), and some of these genes, such as the basal body gene *flgH* and the basal body operon, *flgE*, *flgG*, and *flaD*, are transcribed from σ^{54} promoters but have a different enhancer element (Dingwall *et al.*, 1990; Dingwall, Garman, and Shapiro, unpublished data).

To obtain evidence that *Caulobacter* possesses an IHF-like binding protein, we have fractionated an activity from *C. crescentus* extracts that can faithfully substitute for the *E. coli* IHF protein in an in vitro lambda intermolecular recombination assay. This assay is a rigorous test of the sequence-specific DNA binding and bending functions of IHF. Fractions that participated in the recombination assay had peak concentrations of the IHF subunits, based on immunoassay with anti-IHF antibody. The *Caulobacter* IHF proteins, like the *ptr*-binding activities, are subject to differential availability during the cell cycle. As detected by Western blots, IHF levels

are maximal in predivisional cells, coincident with the transcription of the relevant flagellar promoters and are barely detectable in the progeny cells after cell division. The disappearance of IHF, perhaps upon cell division, is probably due to a specific proteolytic event. Recent experiments with the methyl-accepting chemotaxis proteins has revealed a program of developmentally controlled proteolysis that occurs at specific stages of the *Caulobacter* cell cycle (Alley, Maddock, and Shapiro, in preparation). The loss of *flr*-binding activity in the swarmer and stalked progeny cells could be due to the actual loss of protein, or it could be due to the absence of a critical modification that is needed for binding activity. The loss of IHF and *flr*-binding activities after cell division is consistent with the observations that these factors are responsible for activating transcription of the *flaNQ* and *flbG* operons, an event which occurs exclusively in the predivisional cell.

The availability of IHF may mediate the temporal expression of IHF-requiring promoters. The regulation of IHF expression in *E. coli* is not well understood. The *himA* gene contains binding sites for the SOS-responsive repressor, LexA (Mechulam *et al.*, 1987), suggesting that the regulation of IHF synthesis may be a component of this global regulatory network (Miller *et al.*, 1981). The temporal expression of IHF in *C. crescentus* indicates that, here too, IHF may be subject to global regulatory controls that in turn function to integrate flagellar gene expression into the cascade of cell cycle events.

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