Integration host factor is required for the activation of developmentally regulated genes in *Caulobacter*

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Several temporally controlled flagellar genes in *Caulobacter crescentus* require a σ^{54} promoter and upstream sites for transcription activation. We demonstrate here that in some of these genes, an AT-rich region containing an integration host factor (IHF) consensus binding site lies between the activator and the promoter, and that this region binds IHF in vitro. Analysis of mutations in the IHF-binding region of the hook operon demonstrated that an intact IHF-binding site is necessary for transcription in vivo. An adjacent and divergent promoter also has an IHF consensus sequence that binds IHF. The IHF and enhancer sites are 3' to the transcription start site in this promoter. We postulate that IHF mediates the formation of a higher order structure between the divergent promoter regions in a manner analogous to the nucleosome-like structure generated for λ -*Escherichia coli* DNA recombination and that this higher order structure modulates transcription.

[Key Words: *Caulobacter*; IHF; *fla* genes]

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Caulobacter crescentus forms morphologically distinct daughter cells at each cell division. Asymmetry is generated in the predivisional cell, resulting in a stalked cell and a motile, swarmer cell that contains a single polar flagellum. The best studied aspect of this simple developmental cycle is the temporal biogenesis of the flagellum. Genetic analysis has identified over 48 genes responsible for the formation of this structure (Ely et al. 1984; Ely and Ely 1989). A large number of these genes are transcribed during a discrete period in the cell cycle (Ohta et al. 1985; Loewy et al. 1987; Minnich and Newton 1987; Kaplan et al. 1989) and their expression is controlled by a *trans*-acting regulatory hierarchy (Champer et al. 1987; Newton et al. 1989; Xu et al. 1989). The positioning of the genes in the hierarchy reflects their order of transcription, which in turn reflects the order of assembly of their gene products into the flagellar structure. In addition to the modulation of the temporal control, the products of these genes are localized to one pole of the predivisional cell. In an effort to understand both the temporal and spatial control of gene expression we analyzed the factors that control the transcription of the divergent hook operons (*flbG* and *flaN*) and two flagellin genes (*flgK* and *flgL*).

The *Caulobacter* genes *flbG*, *flgK*, and *flgL* (Mullin et al. 1978) require σ^{54} -containing RNA polymerase for in vitro transcription (Ninfa et al. 1989). Site-directed mutagenesis (Mullin and Newton 1989) has revealed an ac-

tivator site in the 5' region of *flbG*, analogous to NtrC sites found upstream of the glutamine synthetase promoters of enteric bacteria (Hirschman et al. 1985; Reitzer and Magasanik 1986). The *Caulobacter* enhancer-like *flr* sequences are located ~100 bp from the start of transcription (Chen et al. 1986; Minnich and Newton 1987; Mullin and Newton 1989) and are required for transcription of *flbG* in vivo (Mullin and Newton 1989). Genetic evidence suggests that the regulation of transcription of *flbG* is under both positive and negative control and is likely to be a multicomponent process because mutations in at least six genes abolish transcription (Xu et al. 1989; Newton et al. 1989). The product of one of these genes is likely to encode the protein that binds to the *flr* element. We have recently purified a 95-kD protein, RF-1, that binds to this upstream region (J.W. Gober and L. Shapiro, unpubl.). This binding activity is under cell cycle control and thus contributes to the temporal transcription of *flbG* (J.W. Gober, H. Xu, A. Dingwall, and L. Shapiro, unpubl.).

We report here that an AT-rich region positioned between the σ^{54} promoter and the upstream *flr* site of these flagellar genes functions as a binding site for the basic, sequence-specific DNA-binding protein from *Escherichia coli* called integration host factor (IHF) (Nash and Robertson 1981). Western blot analysis of *C. crescentus* protein extracts with *E. coli* anti-IHF antibody revealed the presence of two cross-reacting polypeptides of a molecular weight similar to *E. coli* IHF, suggesting that *Caulobacter* possesses a homolog to this protein. Site-di-

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rected mutagenesis of the *flbG* IHF-binding region suggests that IHF is essential for in vivo transcription of the *flbG* operon. IHF is a heterodimer with subunits of 10.5 and 9.5 kD that are encoded by the *himA* and *himD* genes of *E. coli* (Nash and Robertson 1981; Nash et al. 1987). In *E. coli*, IHF forms a nucleosome-like structure that is required for integrase (Int)-mediated recombination of bacteriophage λ DNA (for review, see Friedman 1988). We postulate that the *flbG* and the divergent *flaN* promoter regions, both of which bind IHF, form a similar higher order structure that contributes to the transient temporal activation of these genes. The known diversity of IHF functions suggests that it might be a component of a global regulatory network for the temporal and spatial control of gene expression in *Caulobacter*.

Results

IHF binds to the region upstream of three flagellar promoters

The promoter and upstream regulatory region of the hook operon (*flbG*) and two flagellin genes (*flgK* and *flgL*) have a similar architecture (Fig. 1). The GG and GC conserved dinucleotides found in all σ^{54} -requiring promoters (Kustu et al. 1989) are underlined. A *ptr* consensus sequence reported by Mullin and Newton (1989) $\sim$ 100 bp upstream of the transcription start site, is indicated. These promoters also possess an AT-rich region that lies between the *ptr* and the σ^{54} promoter. In each promoter, the region extends $\sim$ 40–50 bp between -90 and -35 bp from the start of transcription. The AT content of this region is 58% in the *flbG* promoter and 62% and 70% in the *flgK* and *flgL* promoters, respectively. Stretches of AT sequence in a GC-rich (67%) organism such as *Caulobacter* are rare. Examination of these sequences re-

vealed that they all contain the consensus binding site for IHF (Table 1). Figure 1 shows that these potential IHF-binding sites (open boxes) can occur on both strands and in either orientation. *flbG* has two IHF consensus sequences located on the coding (a) and noncoding (b) strand. The *flgK* consensus sequence occurs on the coding strand and is oriented in the same direction as *flbG* sequence (a). The consensus sequence in the *flgL* promoter region is in the opposite orientation and is located on the noncoding strand.

To determine if IHF could interact with these consensus sequences, we assayed binding in vitro using purified IHF from *E. coli*. A uniquely end-labeled DNA fragment that contains the *flbG* promoter region (-120 to -8 bp from the transcription start site) was used as a probe for gel mobility-shift assays. Relatively low concentrations of IHF (5–25 nM) retarded the mobility of the *flbG* promoter fragment in a nondenaturing polyacrylamide gel (Fig. 2A). The *flgK* upstream region was also tested with a uniquely end-labeled 280-bp fragment as a probe in the binding assay (Fig. 2B). The migration of this probe was retarded by the same concentrations of IHF used for the *flbG* promoter region.

The sequences bound by IHF were determined by DNase I protection assays using the same -120 to -8 fragment of the *flbG* promoter (Fig. 3A). Purified IHF protected 38 bp, corresponding to sequences -38 to -76 bp from the *flbG* transcription start site. This is approximately the size of the region protected by a single heterodimer of IHF in the *attP* region of bacteriophage λ DNA (Craig and Nash 1984). Similarly, IHF protected 37 bp in the *flgK* regulatory region (Fig. 3B), and a 41-bp sequence in the AT-rich region between the *ptr* and the promoter of *flgL* (Fig. 3C). In each case, the protected regions extended beyond the IHF consensus se-

Figure 1. IHF sites in the upstream regulatory region of the flagellar genes *flbG*, *flgK*, and *flgL*. The 5' sequence of *flbG* and partial sequences of *flgK* (-134 to -90 and -40 to $+7$) and *flgL* (-140 to -100 and -40 to $+7$) are from Chen et al. (1986) and Minnich and Newton (1987). The 5' sequences of *flgK* and *flgL* were identified by the dideoxy sequencing method of Sanger et al. 1977. The σ^{54} promoter and the upstream *ptr* activator sequence were identified by Mullin and Newton (1989). The -12 , -24 conserved dinucleotide promoter sequences are underlined. Both strands of an AT-rich sequence between positions -38 and -90 bp from the start of transcription ($+1$) are shown and the consensus sequence for an IHF binding site is indicated with an open box. Two IHF consensus sequences, shown as *a* and *b*, are present in the *flbG* promoter region.

Figure 3. Identification of the IHF-protected region by DNase I footprinting assays. IHF binding protects the *flbG*, *flgK*, and *flgL* promoter regions from attack by DNase I. (A) *flbG* promoter. An end-labeled 130-bp fragment *Hind*III–*Eco*RI containing *flbG* sequences from –120 to –8 was incubated in either the absence (lanes 1 and 6) or presence of IHF (50 nM, lane 2; 100 nM, lane 3; 150 nM, lane 4; 200 nM, lane 5). The DNA–protein complexes were subjected to DNase I treatment, phenol/chloroform extracted, ethanol precipitated, and electrophoresed on an 8% acrylamide urea sequencing gel. The region protected from DNase I by IHF binding is shown as the open bracket to the right of the DNase I ladder. Numbers indicate the distance in bases from the start of transcription. Sequencing reactions are designated G and G + A (Maxam and Gilbert 1980). (B) *flgK* promoter. An end-labeled 280-bp *Nco*I–*Eco*RI fragment was used as a source of *flgK* promoter region DNA. The DNA fragment, containing *flgK* sequences from –145 to +135, was incubated in either the absence (lanes 1 and 6) or presence of IHF (50 nM, lane 2; 100 nM, lane 3; 150 nM, lane 4; 200 nM, lane 5) and then treated as described in (A). (C) *flgL* promoter. A 760-bp *Hind*III–*Eco*RI fragment, containing the *flgL* sequences from –650 to +110, was incubated in either the absence (lanes 1 and 6) or presence (50 nM, lane 2; 100 nM, lane 3; 150 nM, lane 4; 200 nM, lane 5). The DNA–protein complexes were then treated as described in A.

wild-type construction. These results support the conclusion that the ability to bind IHF is required for transcription of the *flbG* promoter in vivo.

flaN contains an IHF-binding site downstream of its promoter

The divergent *flaNQ* operon is located directly 5' to the *flbG* hook operon (Chen et al. 1986; Fig. 6). Newton and

his co-workers have shown that *flaN* is transcribed from a σ^{54} promoter (Mullin and Newton 1989; Ninfa et al. 1989). The *frt* element that lies between the divergent *flbG* and *flaN* promoters is not required for *flaN* transcription (Mullin and Newton 1989; J.W. Gober and L. Shapiro, unpubl.). Deletion of the two *frt* elements located 3' to the transcriptional start of *flaN*, however, eliminates transcriptional activity (Mullin and Newton 1989). It is therefore likely that *flaN* transcription is ac-

Figure 4. Summary of mutations introduced into the IHF-binding site of the *flbG* promoter. A schematic diagram of the *flbG* promoter region is shown. The IHF binding site, sequences -81 to -38, is the region that IHF protects from attack by DNase I. The σ^{54} promoter sequences are represented by filled boxes and the transcriptional start is indicated by an arrow. The wild-type promoter region and the mutagenized promoter regions were fused to a promoterless β -galactosidase gene (*lacZ*) as described in Experimental procedures. The IHF-binding site was mutagenized using oligonucleotide-directed site-specific mutagenesis (Kunkel and Roberts 1987). The wild-type sequence is shown on the first line, and the sequence that matches the *attP* IHF consensus site is boxed. Conserved bases are underlined. An inverted repeat within the IHF-protected region is designated with arrows. Mutagenized bases are shown in bold and are marked by arrowheads. IHF binding to each fusion was tested by DNase I protection assays shown in Fig. 5. A plus sign (+) in the IHF-binding column indicates that the promoter region was protected by IHF, at concentrations of 150 to 250 nM. A minus sign (-) indicates that IHF was unable to bind at these concentrations. β -Galactosidase activity was measured by assaying O-nitrophenyl galactoside (ONPG) hydrolysis in permeabilized cells obtained from log-phase cultures, as described in Experimental procedures. The values shown represent the mean value of 6 to 10 separate determinations for each mutant promoter.

tivated by proteins that bind to these downstream sites. *flaN* also possesses an AT-rich region that contains an IHF consensus sequence similar to those found upstream of *flbG*, *flgK*, and *flgL* (Table 1). The IHF consensus sequence for *flaN* lies between the promoter and the downstream *ftr* sites, and thus is located 3' to the transcription start site. Evidence that this sequence in fact functions as an IHF-binding site was demonstrated by the presence of a footprint in a DNase I protection assay (Fig. 7). When DNA from the *flaN* promoter region was incubated with IHF, a protected region of about 33 bp, +24 to +57 from the start of transcription, is observed (Fig. 7). Thus, two *cis*-acting elements normally found upstream of the σ^{54} promoters, as in the *flbG*, *flgK*, and *flgL* promoter regions, are found downstream of the *flaN* promoter, supporting the contention that these *cis*-acting sites function as enhancers.

Western blot analysis with anti-IHF antibody

To date, IHF has not been characterized in any organism other than *E. coli*. We used Western blot analysis with antibody to *E. coli* IHF to determine whether *Caulobacter* possesses an IHF homolog. Two polypeptides in *Caulobacter* cell extracts were found to react with anti-IHF antibody (Fig. 8). One band was the same molecular weight (10.5 kD) as the α -subunit of purified *E. coli* IHF, and the second band was slightly smaller than the β -subunit, suggesting that an IHF-like heterodimer is present in *Caulobacter*. An unidentified cross-reacting band was also present in the *Caulobacter* cell extracts.

Discussion

The timed expression of the flagellar components in *Caulobacter* is linked to other cell cycle events, including DNA replication, membrane biogenesis, and cell division. Localized flagellum formation is thus integrated into a number of global regulatory circuits that generate two distinct daughter cells at each cell division. In addition to responding to cell cycle cues, flagellar gene expression is also influenced by the assembly of the flagellum (Xu et al. 1989). Such complex control is thought to be accomplished at the level of transcription. An extensively studied flagellar promoter is that of the hook operon (*flbG*) (Chen et al. 1986). This promoter requires σ^{54} -containing RNA polymerase holoenzyme (Ninfa et al. 1989), in addition to sequences located at -100 bp from the transcription start for transcriptional activity. With analogy to the *ntr* system, these upstream sequences have been termed *ftr* elements (Mullin and Newton 1989). Several *trans*-acting factors are apparently necessary for transcription of *flbG*. The *flbD* gene, which is required for the transcription of the *flbG* operon, has been shown to encode a 52-kD protein that is homologous to the transcriptional activators *NtrC* and *NifA* (Ramakrishnan and Newton 1990). However, extracts of strains carrying mutations in *flbD* retain the ability to bind to the *ftr* element (J.W. Gober, H. Xu, A. Dingwall, and L. Shapiro, unpubl.). In addition, a 95-kD protein, RF-1, that binds to the *ftr* element, has been identified and purified (J.W. Gober and L. Shapiro, unpubl.). The binding activity is temporally regulated and

Figure 5. DNase I protection assays using IHF and mutant *flbG* promoter regions. Each panel represents *flbG* wild-type [A] or mutant promoter regions (HXr8, BK; HXΔ8, C; HX6M, D; I3216, E; I302, F) incubated with (150 nM, lane 2; 250 nM, lane 3) or without (lanes 1 and 4) IHF. Probes were prepared as described in Experimental procedures. The open bracket on the left shows the extent of the region protected by IHF from DNase I attack in the wild-type *flbG* promoter. Numbers indicate the distance from the start of transcription. G lane is a sequencing reaction (Maxam and Gilbert 1980).

appears to be an important component in the timed transcriptional activation of the *flbG* promoter (J.W. Goyer, H. Xu, A. Dingwall, and L. Shapiro, unpubl.). We have now identified an additional *cis*-acting element that is required for transcriptional activation of *flbG*.

An AT-rich region that lies between the σ^{54} promoter and the upstream *ptr* region binds IHF. This IHF-binding site is important for transcription *in vivo*, since site-specific mutations that do not permit IHF binding fail to activate transcription. IHF has been implicated in both transposon and Mu transposition, site-specific recombination of bacteriophages ϕ 80 and P22 (Leong et al. 1985), plasmid DNA replication (Stenzel et al. 1987), phage replication (Greenstein et al. 1988), and phase variation (Eisenstein et al. 1987). Elegant *in vitro* experiments by Nash and co-workers have demonstrated that IHF is required for the Int-mediated recombination between the *attB* and *attP* sites on the bacterial and lambda chromo-

somes (Nash and Robertson 1981). Structural studies have shown that IHF binding introduces a bend in the DNA, estimated to be $\sim 140^\circ$ (Robertson and Nash 1987; Stenzel et al. 1987; Thompson and Landy 1988; Higgins et al. 1989; Kosturko et al. 1989). Exchanging an IHF-binding site with DNA sequences that possess natural curvature can relieve the IHF requirement for Int-mediated recombination *in vitro*, thus demonstrating that the primary role of IHF is to organize higher order DNA structure (Goodman and Nash 1989).

The role of IHF in transcription is less well defined. IHF-binding sites have been identified in several bacterial σ^{70} promoter regions. These include the *E. coli* *ilvG*-MEDA operon, some bacteriophage Mu promoters, and the λ *P_L* and *pcin* promoters (Pereira et al. 1988; van Rijn et al. 1988; Peacock et al. 1984; Griffo et al. 1989). In the case of the *ilvG*-MEDA operon, IHF binding exerts a repressive effect on the transcription of the *ilvGp1* pro-

motor. Because IHF binds within the promoter sequence, it may prevent polymerase from binding. Transcription of this promoter only occurs in mutants lacking IHF or in vitro in the absence of IHF (Periera et al. 1988). The λ *P_{cin}* promoter may be repressed by IHF binding in a similar fashion (Griffo et al. 1989). In contrast, IHF binding to the μ and λ *P_L* promoters stimulates transcription. IHF-induced curvature is probably responsible for the stimulation of transcription of these σ^{70} promoters. This effect may be similar to that observed for transcription stimulated by catabolite activator protein (CRP) or factor for inversion stimulation (FIS), two proteins that induce bending of DNA (Bracco et al. 1989; Nilsson et al. 1990).

IHF-binding sites have been identified recently upstream of σ^{54} promoters in several *nif* operons (Santero et al. 1989; Hoover et al. 1990). Kustu and co-workers have analyzed the *Klebsiella nifH* promoter region and presented evidence that, in vitro, IHF does not influence binding of σ^{54} holoenzyme to the promoter, but that an IHF induced bend brings NifA in contact with RNA polymerase (Hoover et al. 1990). Presumably, the interaction between protein bound to an upstream enhancer and RNA polymerase at the promoter site is augmented by DNA looping. Indeed, such a model has been invoked for the *flbG* promoter in which looping is predicted to mediate interaction between a protein bound to *ftr* and RNA polymerase bound to both the *flbG* promoter and the divergent *flaN* promoter (Mullin and Newton 1989). The *flaN* promoter represents a variation on the structural motif found in the other flagellar promoters (Fig. 6). Here the IHF- and *ftr*-binding sites are downstream from the transcriptional start site. As diagrammed in Figure 9, bends caused by IHF binding to each of these DNA sequences would increase the likelihood of interactions

among the proteins bound at each promoter. A static bend in the *flbG* promoter sequences, such as one introduced by IHF binding, would thus increase the frequency of interactions between the RF-1 factor and RNA polymerase. It is likely that the interaction between these promoters is the consequence of DNA looping, since the *flaN* promoter, *ftr* elements, and IHF-binding site are all ~ 200 bp away from the analogous *cis* elements in the *flbG* promoter. In the case of λ recombination, looping permits Int protein to simultaneously interact with a high affinity arm-type site and a low-affinity core-type site (Moitoso de Vargas et al. 1989), facilitating recombination (Richet et al. 1986). Topological studies have demonstrated that the IHF-Int recombination complex forms a higher order structure similar to the nucleosome of eukaryotes, which has been termed an intasome (Better et al. 1983; Pollock and Nash 1983). The attainment of a higher order structure in the λ system is dependent on multiple protein molecules bound to DNA. By analogy, we propose that binding by RF-1, RNA polymerase and IHF influences the topological state of the *flaN*-*flbG* transcription complex. The proposed higher order structure facilitated by IHF binding to these sequences (Fig. 9) is reminiscent of the predicted intasome formed in the λ -*E. coli* recombination system (Better et al. 1983; Pollock and Nash 1983). For the *flaN*-*flbG* complex, however, protein-protein interaction through DNA binding would modulate the transcription of these promoters.

IHF binding is not a general requirement of all temporally controlled σ^{54} flagellar promoters in *Caulobacter*, because the σ^{54} promoter of *flbN*, a gene encoding one of the rings of the basal body gene, lacks an IHF-binding site (Dingwall et al. 1990). Some unrecognized feature of the upstream region or the promoter it-

Figure 6. Genetic map of the *flbG* hook operon and the divergent *flaNQ* operon (Ohta et al. 1985; Chen et al. 1986). A schematic of the region between *flaN* and *flbG* is shown below the genetic map (Mullin and Newton 1989). The *flaN* σ^{54} promoter is shown. The *ftr* at -120 bp from the *flbG* transcription start is about 70 bp upstream of the *flaN* promoter but is not required for transcription of *flaN* (Mullin and Newton 1989). Instead, sequences 3' to the promoter, presumably the two *ftr* sequences at +130 bp, are required for transcription (Mullin and Newton 1989). An IHF-binding site lies between these *ftr* sequences and the σ^{54} promoter. Shown below is the sequence of the *flaN* promoter region from -20 to +70 (Mullin and Newton 1989). The IHF-binding site is boxed and lies between +24 and +57 bp from the start of transcription.

Figure 7. DNase I footprinting of the *flaN* promoter with IHF. A 429-bp *Pst*I–*Eco*RI fragment (–70 to +350) with 9 bp of the polylinker was end-labeled and incubated with 50 nM (lane 2); 100 nM (lane 3); 150 nM (lane 4); 200 nM (lane 5); or without (lanes 1 and 6) IHF and treated as described in Experimental procedures. The protected region, +24 to +57, is indicated. G and G + A represent the products of chemical sequencing reactions (Maxam and Gilbert 1980).

self dictates the IHF requirement of a σ^{54} promoter. The *flbN* basal body gene is transcribed earlier than the *flbG* and *flaN* genes. Recent experiments with the *Klebsiella*

pneumoniae nifH promoter indicate that the promoter sequences themselves are an important influence on whether IHF is required for transcriptional activation (Hoover et al. 1990). In this case, if the promoter strength is increased by mutations in the σ^{54} recognition sequence, then IHF is no longer needed for open complex formation. The *flbG* hook promoter may be relatively weak, as evidenced by the low levels of β -galactosidase activity obtained with the transcription fusions (Fig. 4) and as reported by Newton et al. (1989). The temporal transcription of *flbG* might thus be controlled by the availability of IHF at unique times in the cell cycle. We have found recently that IHF is present in maximal amounts in predivisional cells at the time of *flbG* transcription (J.W. Gober, unpubl.). IHF is not present in the swarmer and stalked cell progeny. Because we know that a second *trans*-acting factor, RF-1, is also available only at the time when *flbG* transcription occurs normally (J.W. Gober, H. Xu, A. Dingwall, and L. Shapiro, unpubl.), the combination of these factors might fine tune the precise timing of gene expression.

Although the presence of IHF has not been demonstrated previously in nonenteric bacteria, anti-IHF antibody raised against purified *E. coli* IHF reacts with two proteins in *Caulobacter* that are similar in molecular weight to the subunits of the *E. coli* IHF heterodimer. We speculate that these two polypeptides represent the *Caulobacter* homolog of IHF. Extracts prepared from several strains that carry either Tn5 or deletion muta-

Figure 8. Western blot analysis with *E. coli* anti-IHF antibody. Crude cell extract (100 μ g of protein) from *Caulobacter* (lane 2) was electrophoresed in a SDS-polyacrylamide gel as described in Experimental procedures. The separated proteins were electrophoretically transferred to nitrocellulose and the filter was incubated with a 1 : 5000 dilution of anti-*E. coli* IHF serum. Pure IHF (100 ng) was electrophoresed next to the *Caulobacter* cell extracts (lane 1). The indicated molecular weights (10.5 kD and 9.5 kD) are those reported for the α and β subunits of the *E. coli* IHF, respectively.

Figure 9. Diagram of a possible higher order structure adopted by the *flaN*–*flbG* transcription units. A schematic of the *flaN* and *flbG* promoter regions is shown. The small open boxes represent the site of the -24 , -12 promoter sequences. The direction of transcription is indicated. RF-1 ovals represent the transcription factor, RF-1 occupying an *ptr* site. There are two sites downstream of the *flaN* transcription start and one site upstream of *flbG*. Each RF-1, as diagrammed, may represent a dimer. The distances between protein binding sites are proportionally correct. In addition, the face of the helix of each RF-1 binding site and σ^{54} promoter is accurately depicted. IHF-induced bends in this region would enable bound RF-1 molecules to interact with RNA polymerase bound at the σ^{54} promoter site. Furthermore, we propose that this structure would also permit interactions among the RF-1 proteins. This interaction, in turn, may influence the transcription initiation from either one or both of these promoters.

tions in flagellar regulatory genes (*flaS*, *flaO*, *flbD*, *flaW*, *flbF*, and *flbO*) all possess these two polypeptides, indicating that IHF is not encoded by the currently known genes at the top of the flagellar regulatory hierarchy. Given the diversity of functions ascribed to IHF, it is reasonable to conclude that this protein has many roles other than flagellar biogenesis in *Caulobacter*.

Experimental procedures

Bacterial strains, plasmids, and growth conditions

Wild-type *Caulobacter crescentus* (CB15N) was grown at 32°C in either PYE (Poindexter 1964) or in modified minimal M2 medium supplemented with 0.2% glucose (Contreras et al. 1978). *C. crescentus* cells harboring transcriptional fusions in Inc. P1 plasmids were grown in media supplemented with 1.5 μ g/ml tetracycline.

Preparation of flagellar gene promoter fragments for DNA binding assays

A probe that contains a 116-bp *SacI*–*DdeI* fragment of the *flbG* upstream region (see Fig. 1) cloned into the *SacI* and *EcoRV* sites of pKic7 was prepared from pHX120 (H. Xu and J.W. Gober, unpubl.). An end-labeled probe for the gel mobility-shift assay (Fig. 2) and the DNase I footprinting assay shown in Figure 3A, was prepared by digesting pHX120 with *HindIII* and labeling the end with [α - 32 P] deoxynucleotide triphosphates (Amersham, 3000–6000 Ci/mmol) using DNA polymerase I, Klenow fragment (Boehringer–Mannheim). Labeled DNA was then digested with *EcoRI* and a 130-bp fragment was isolated from a polyacrylamide gel and subsequently electroeluted, phenol/chloroform extracted, and precipitated with ethanol. This probe contains bases -120 to -8 from the transcription start of *flbG* plus 9 bases of pC19H polylinker (Marsh et al. 1984) on either side. A larger *flbG* promoter fragment was used in the DNase I protection assays shown in Figure 5. The fragment contained positions -120 to $+680$ of the *flbG* gene plus 9 bp of polylinker on the 5' end and 18 bp of polylinker on the 3' side subcloned into pKic7 (pJG61). For probe preparation, pJG61 was digested with *HindIII*, labeled, digested with *EcoRI*, and purified as described above.

A probe prepared from pJG81 was used as the source of *flgK* promoter DNA for both the gel mobility-shift assay (Fig. 2) and

the DNase I footprinting assay shown in Figure 3B. pJG81 contains a 580-bp *PstI*–*EcoRI* fragment of the 5' end of the *flgK* gene (Minnich and Newton 1987) inserted in the *PstI*–*EcoRI* site of pUC19. This plasmid was cut at an *NcoI* site located at -145 from the *flgK* transcription start site, labeled, cut with *EcoRI*, and purified as described above. This probe contains bases -145 to $+135$ of the *flgK* gene.

pJG72 was used as the source of *flgL* promoter DNA for the DNase I protection assay shown in Figure 3C. This plasmid contains a 760-bp *HindIII*–*EcoRI* fragment of *flgL* DNA (-650 to $+110$ from the transcription start site) cloned into pUC19. pJG72 was digested with *EcoRI*, labeled, digested with *HindIII*, and purified as described above.

pJG1901 was used as the source of *flaN* promoter DNA for the DNase I protection assay shown in Figure 7. pJG1901 contains a 420-bp *PstI*–*SacI* fragment of *flaN* DNA cloned into pUC19. To end-label probe, pJG1901 was digested with *EcoRI*, labeled, digested with *PstI*, and the fragment was gel purified. This probe contained *flaN* DNA from positions -70 to $+350$ plus 9 bases of pUC19 polylinker DNA at the 5' end.

DNA binding assays

Purified IHF from *E. coli* (which was the kind gift of Howard Nash) was used in all binding assays. Gel mobility-shift analysis was performed essentially as described by Fried and Crothers (1981). IHF (5 – 25 nM) was incubated at 25°C with 0.05 – 0.1 pmole of end-labeled DNA fragment in a total volume of 15 μ l for 20 min. The reaction buffer contained 20 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 0.1 mM dithiothreitol (DTT), 100 mM KCl, and 100 μ g/ml bovine serum albumin (BSA) (Fisher et al. 1988). Complexes were electrophoresed in a nondenaturing polyacrylamide gel in $1 \times$ TBE at 4°C (Maniatis et al. 1982).

DNase I footprinting assays (Galas and Schmitz 1978) were performed by incubating purified IHF (50 – 200 nM) with labeled DNA fragment (0.01 – 0.05 pmole; 0.5 – 1.0×10^5 cpm) for 20 min at room temperature. The reaction mixture (100 μ l) contains 10 mM Tris (pH 7.5), 100 mM KCl, 0.1 mM DTT, 10% glycerol, and 50 μ g/ml BSA. After incubation, the samples were placed on ice and mixed with an equal volume of a reaction mixture containing 20 mM Tris-HCl (pH 7.5), 200 mM KCl, 0.2 mM DTT, 20% glycerol, 100 μ g/ml BSA, 10 mM CaCl_2 , 10 mM MgCl_2 , and 15 units of DNase I (Boehringer–Mannheim). Reactions were terminated after 90 sec by the addition of 200 μ l of 0.6 M ammonium acetate, 0.1 M EDTA, and 20 μ g/ml of

sheared calf thymus DNA (Craig and Nash 1984). Samples were extracted with phenol/chloroform and ethanol precipitated. The pellets were washed twice with 70% ethanol, dried, and resuspended in 80% (vol/vol) formamide, 1 × TBE, 0.05% bromophenol blue, and 0.05% xylene cyanol FF. The samples were heated at 85°C for 5 min and electrophoresed in 8% acrylamide-urea sequencing gels in 1 × TBE (Maniatis et al. 1982). Sequencing reactions (G, G + A) were loaded simultaneously and performed as described by Maxam and Gilbert (1980).

flbG promoter mutagenesis and construction of transcription reporter gene fusions

The IHF-binding sequences within the *flbG* promoter were mutagenized using the oligonucleotide site-directed mutagenesis methods of Kunkel and Roberts (1987). Mutagenesis was performed on M13mp19 single strand DNA containing the 1.3-kb *Pst*I–*Xho*I of the *flbG* 5' region (Ohta et al. 1985). Mutations HXR8, HXΔ8, and XH6M were constructed by Hong Xu of this laboratory. All mutagenesis was confirmed by dideoxy DNA sequencing using a synthetic primer complementary to bases +40 to +59 of *flbG* (Sanger et al. 1977).

For construction of *lacZ* transcription fusions, the 805-bp *Sac*I–*Xba*I fragment from the mutagenized M13mp19 clones was subcloned into the *Sac*I–*Xba*I sites of pUC18. The fusions were constructed by ligation of an *Eco*RI–*Pst*I fragment from the pUC18 subclones into plasmid pRSZ3 (M.R.K. Alley, unpubl.), which contains a promoterless *lacZ* gene from *E. coli* in a wide host-range plasmid which is derived from pRK290 (Ditta et al. 1980). The transcription fusions were introduced into *C. crescentus* by mating with an *E. coli* donor. β-galactosidase activity was measured in mid-log phase cells (OD₆₆₀ = 0.5–0.9) grown in PYE. *C. crescentus* CB15N with plasmid-borne *flbG*–*LacZ* fusions were permeabilized by addition of 100 μl of chloroform to 1 ml of reaction mixture. Cell suspensions were incubated at 30°C for 5 min before the addition of substrate. The assay and calculation of unit activity was performed as described by Miller (1972).

Western blot analysis

Western blot analysis was performed as described by Towbin et al. (1979). Anti-IHF sera, provided by H. Nash, was incubated with blotted proteins at a 1 : 5000 dilution. Antibody protein complexes were detected using a secondary antibody conjugated to alkaline phosphatase (Bio-Rad). Alkaline phosphatase activity was visualized using a commercially available detection system (Boehringer–Mannheim).

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