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

James W. Gober and Lucille Shapiro

Department of Developmental Biology, Stanford University School of Medicine, Stanford, California 94305-5427 USA

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]

Received May 23, 1990; revised version accepted July 6, 1990.

*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 activator 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 itr 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 itr 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 itr 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-
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 ε54-requiring promoters [Kustu et al. 1989] are underlined. A ftr consensus sequence reported by Mullin and Newton (1989) ~100 bp upstream of the transcription start site, is indicated. These promoters also possess an AT-rich region that lies between the ftr and the ε54 promoter. In each promoter, the region extends ~40–50 bp between ~90 and ~35 bp from the start of transcription. The AT content of this region extends ~40–50 bp between -90 and -35 bp.

**Results**

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 completed by the dyeoxy sequencing method of Sanger et al. 1977. The ε54 promoter and the upstream ftr 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 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 completed by the dyeoxy sequencing method of Sanger et al. 1977. The ε54 promoter and the upstream ftr 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.
Table 1. Comparison of IHF consensus sequences in Caulobacter flagellar promoters

| flbG1  | flbG2  | flbK | flbL | flaN |
|-------|-------|------|------|------|
| -68   | -65   | -87  | -49  | -49  |
| T A A A G T C A T T G A T | T T A A T G G C T T G T | G C A A T T A C C T T G G T | A A T A T C T A T T G T | G C A A T T A C C T T G G T |
| -55   | -78   | -64  | -62  | +52  |
|       |       |      |      |      |

quence as has been found for the attP sites in λ DNA [Craig and Nash 1984].

Effect of mutations in the IHF-protected region on binding affinity and in vivo transcription

To determine if IHF-binding sequences are required for transcription, site-directed mutagenesis was performed on the flbG promoter region [Fig. 4]. Mutagenized promoter regions were fused to a β-galactosidase transcription reporter gene and assayed for both the ability to bind IHF and β-galactosidase activity in vivo.

Because the precise contacts of IHF within the protected region are unknown, we first chose to introduce mutations that changed the consensus sequence. Using DNase I protection assays, we found that a mutation in the consensus sequence that changed AA to GG in I302 was unable to bind IHF [Figs. 4 and 5F]. IHF has been shown to interact primarily with adenine residues in the minor groove of the DNA helix [Yang and Nash 1989]. We also changed the AT content of the region normally bound by IHF, but outside of the consensus sequence. Specifically, the adenine residues that lie 9 to 11 bp from the right end of the λ attP core consensus are important for IHF binding [Yang and Nash 1989]. The flbG IHF-binding site possesses an analogous set of adenine residues located on the noncoding strand, −47 to −45 from the transcriptional start [see Fig. 1]. These nucleotides are within a short inverted repeat sequence at the boundaries of the IHF-protected region. Three of the five changes in mutant HXR8 alter the AT content from TTA to CGG. This results in a sequence [shown on the coding strand] that is no longer bound by IHF [Figs. 4 and 5B]. A deletion of 11 bases in this region in mutant HXÅ8, also abolished IHF binding [Figs. 4 and 5C]. This deletion restored two T residues mutagenized in HXR8, indicating that additional bases within this region are important for IHF binding. These mutations lie within a conserved 13-mer sequence that is found in the 5’ region of flbG and several other fla genes, including flbN and flaE [Kaplan et al. 1989] that do not contain an IHF core sequence. The binding of alternate proteins to this region may play a role in the differential control of fla gene expression. Although IHF was unable to bind the flbG mutant sequences at the concentrations (150–250 nM) tested in Figure 5, it could yield a partial, but specific DNase I footprint when tested at a concentration of 800 nM [data not shown]. Gardner and Nash (1986) also found differences in binding affinity when mutated attP-binding sites were assayed for IHF binding.

Two mutations within the IHF-protected region do not affect the affinity of IHF for the binding site [Figs. 4 and 5D,E]. One of these, a GG to TC change [HX6M] is in a region not expected to contact IHF, according to previous studies with the λ attP sites [Yang and Nash 1989]. The other mutation that failed to affect binding, I3216, is an alteration in the left arm of the inverted repeat sequence shown in Figure 4. Although an alteration in the right arm of the inverted repeat sequence in HXR8 had a strong effect on binding ability, an AA to CC change in I3216 had no effect. Because the mutated sequences in I3216 are located within consensus sequence b [shown in Fig. 1], these data indicate that IHF is utilizing the first consensus listed in Table 1 [sequence a] as its binding site in the flbG promoter.

Mutant flbG promoter regions fused to a promoterless β-galactosidase (lacZ) reporter gene were placed in a wild-type background and cultures were assayed for β-galactosidase activity [Fig. 4]. The extent of β-galactosidase activity in these fusions is a direct reflection of the extent of transcription. Mutant promoters, HXR8, HXÅ8, and I302, that had a significant reduction in binding affinity for IHF, also had five- to ninefold lower levels of β-galactosidase activity. The two mutant promoters that bound IHF with relatively high affinity retained about one-half the β-galactosidase activity observed with the

Figure 2. Gel mobility-shift assays with IHF and flagellar gene promoters. (A) flbG promoter. A 130-bp HindIII–EcoRI DNA fragment containing the flbG promoter region [−120 to −8] flanked by 9 bp of pIC19H polylinker, was end-labeled and purified as described in Experimental procedures. The labeled probe was incubated in either the absence or presence of purified E. coli IHF. (Lanes 1–5) Probe incubated with 0, 5, 10, 15, or 25 nM IHF, respectively. (B) flgK promoter. An end-labeled 280-bp NcoI–EcoRI DNA fragment that contains the flgK promoter region [−145 to +135] was used as probe to test IHF binding. Binding conditions were as described in Experimental procedures. (Lanes 1–4) Probe incubated with 0, 10, 15, 25 nM of IHF, respectively.
IHF is required for transcription of *fla* genes

**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 HindIII–EcoRI 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 NcoI–EcoRI 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 HindIII–EcoRI 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 ρ-independent promoter (Mullin and Newton 1989; Ninfa et al. 1989). The *ftr* 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 *ftr* 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-
Gober and Shapiro

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^34 promoter sequences are represented by filled boxes and the transcriptional start is indicated by an arrow. The wild-type promoter region is shown. The IHF binding site, sequences −81 to −38, is the region that IHF protects from attack by DNase I. 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.

**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.

**IHF Binding**

| Mutant | IHF Binding | β-galactosidase activity (units) |
|--------|-------------|---------------------------------|
| wild type | + | 204 |
| HXr8 | − | 37 |
| HXΔ8 | − | 42 |
| HXGf1 | + | 127 |
| I3216 | + | 104 |
| I302 | − | 22 |

**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^34-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
IHF is required for transcription of *fla* genes

Figure 5. DNase I protection assays using IHF and mutnat *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. Gober, 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 $\sigma^{34}$ promoter and the upstream *ftr* 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 $\phi$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 binding exerts a repressive effect on the transcription of the *ilvGMP* operon, some bacteriophage Mu promoters, and the $\lambda$ 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 *ilvGMP* operon, IHF binding exerts a repressive effect on the transcription of the *ilvGPl* pro-
moter. 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 Pcin promoter may be repressed by IHF binding in a similar fashion [Griffo et al. 1989]. In contrast, IHF binding to the Mu and λ P2 promoters stimulates transcription.

IHF-induced curvature is probably responsible for the stimulation of transcription of these σ70 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 σ54 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 σ54 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 σ54 flagellar promoters in Caulobacter, because the σ54 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 σ54 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 σ54 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.

1500 GENES & DEVELOPMENT
IHF is required for transcription of *fla* genes

*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 σ44 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 7.** DNase I footprinting of the *flaN* promoter with IHF. A 429-bp PstI–EcoRI 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 σ44 promoter. The *flbN* basal body gene is transcribed earlier than the *flbG* and *flaN* genes. Recent experiments with the *Klebsiella*...
promoter DNA for both the gel mobility-shift assay (Fig. 2) and phenol/chloroform extracted, and precipitated with ethanol. This probe contains bases -120 to +680 from the transcription start of the flaN promoter region cloned into pJG61. For probe preparation, a 420-bp SacI-DdeI fragment of the flaN 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 ftr 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^4 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^4 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.

**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 μg/ml tetracycline.

*Preparation of flagellar gene promoter fragments for DNA binding assays*

A probe that contains a 116-bp SacI-Ddel 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) was prepared from pJG81 was used as the source of flaN promoter DNA for the DNase I protection 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.

pG1901 was used as the source of flaN promoter DNA for the DNase I protection assay shown in Figure 7. pG1901 contains a 420-bp PstI–SacI fragment of flaN DNA cloned into pUC19. To end-label probe, pG1901 was digested with EcoRI, labeled, digested with PstI, and the fragment was gel purified. This probe contains 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 μl 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 μg/ml bovine serum albumin (BSA) (Fisher et al. 1988). Complexes were electrophoresed in a nondenaturing polyacrylamide gel in 1 × TBE at 4°C (Maniatis et al. 1982).
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 x 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 x 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 PstI–XhoI of the flbG 5′ region (Ohta et al. 1985). Mutations HX8, HX8A, and HX6M 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 SacI–XbaI fragment from the mutagenized M13mp19 clones was subcloned into the SacI–XbaI sites of pUC18. The fusions were constructed by ligation of an EcoRI–PstI 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 pKK290 (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).

Acknowledgments

We thank Howard Nash for kindly providing IHF protein and antibody to IHF and Sydney Kustu for helpful discussions. We also thank Hong Xu for subcloned regions of the mutagenized C. crescentus hook operon. This investigation was supported by U.S. Public Health grant GM32506 from the National Institutes of Health and grant MV408 from the American Cancer Society. J.W.G. is a Helen Hay Whitney Fellow.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

Better, M., S. Wickner, J. Auerbach, and H. Echols. 1983. Role of the Xis protein of bacteriophage λ in a specific reactive complex at the attR prophage attachment site. Cell 32: 161–168.

Bracco, L., D. Kotlarz, A. Kolb, S. Diekmann, and U. Buc. 1989. Synthetic curved DNA sequences can act as transcriptional activators in Escherichia coli. EMBO J. 8: 4259–4266.

Champer, R., A. Dingwall, and L. Shapiro. 1987. Cascade regulation of Caulobacter flagellar and chemotaxis genes. J. Mol. Biol. 194: 71–80.

Chen, L.-S., D. Mullin, and A. Newton. 1986. Identification, nucleotide sequence and control of developmentally regulated promoters in the hook operon region of Caulobacter crescentus. Proc. Natl. Acad. Sci. 83: 2860–2864.

Craig, N.L. and H.A. Nash. 1984. E. coli integration host factor binds to specific sites in DNA. Cell 39: 707–716.

Contreras, I., L. Shapiro, and S. Henry. 1978. Membrane phospholipid composition of Caulobacter crescentus. J. Bacteriol. 135: 1130–1136.

Dingwall, A., J.W. Gober, and L. Shapiro. 1990. Identification of a Caulobacter basal body structural gene and a cis-acting site required for activation of transcription. J. Bacteriol. [in press].

Ditta, G., S. Stanfield, D. Corbin, and D.R. Helinski. 1980. Broad host range cloning system for gram-negative bacteria: Construction of a gene bank for Rhizobium meliloti. Proc. Natl. Acad. Sci. 77: 7347–7351.

Eisenstein, B.I., D.S. Sweet, V. Vaughan, and D.I. Friedman. 1987. Integration host factor is required for the DNA inversion that controls phase variation in Escherichia coli. Proc. Natl. Acad. Sci. 84: 6506–6510.

Ely, B. and T.W. Ely. 1989. Use of pulsed field gel electrophoresis and transposon mutagenesis to estimate the minimal number of genes required for motility in Caulobacter crescentus. Genetics 114: 717–730.

Ely, B., R.H. Croft, and C.J. Gerardot. 1984. Genetic mapping of genes required for motility in Caulobacter crescentus. Genetics 100: 523–532.

Fisher, R.F., T.T. Egelhoff, J.T. Mulligan, and S.R. Long. 1988. Specific binding of proteins from Rhizobium meliloti cell-free extracts containing NodD to DNA sequences upstream of inducible nodulation genes. Genes Dev. 2: 282–293.

Fried, M. and D.M. Crothers. 1981. Equilibria and kinetics of lac repressor-operator interactions by polycracylamide gel electrophoresis. Nucleic Acids Res. 9: 6505–6525.

Friedman, D.I. 1988. Integration host factor: A protein for all reasons. Cell 55: 545–554.

Galas, D. and A. Schmitz. 1978. A simple method for the detection of protein—DNA binding specificity. Nucleic Acids Res. 5: 3157–3170.

Gardner, J.F. and H.A. Nash. 1986. Role of Escherichia coli IHF protein in lambda site specific recombination: A mutational analysis of binding sites. J. Mol. Biol. 191: 181–189.

Goodman, S.D. and H.A. Nash. 1989. Functional replacement of a protein-induced bend in a DNA recombination site. Nature 341: 251–254.

Greenstein, D., N.D. Zinder, and K. Horiiuchi. 1988. Integration host factor interacts with the DNA replication enhancer of filamentous phage F1. Proc. Natl. Acad. Sci. 85: 6262–6266.

Grifo, G., A.B. Oppenheim, and M.E. Gottesman. 1989. Repression of the λ pcin promoter by integrative host factor. J. Mol. Biol. 209: 55–64.

Higgins, N.P., D.A. Collier, M.W. Kilpatrick, and H.M. Krause. 1989. Supercoiling and integration host factor change the phospholipid composition of Caulobacter crescentus. Proc. Natl. Acad. Sci. 86: 7410–7414.

Hirschman, J., P.-K. Wong, K. Sei, J. Keener, and S. Kustu. 1985. Products of nitrogen regulatory genes ntrA and ntrC of en-
teric bacteria activate \( glnA \) transcription \textit{in vitro}: Evidence that the \( ntrA \) product is a \( \sigma \) factor. \textit{Proc. Natl. Acad. Sci.} \textbf{82}: 7525–7529.

Hoover, T.R., E. Santero, S. Porter, and S. Kustu. 1990. The integration host factor (IHF) stimulates interaction of RNA polymerase with \( NifA \), the transcriptional activator for nitrogen fixation operons. \textit{Cell} (in press).

Kaplan, J.B., A. Dingwall, R. Bryan, R. Champer, and L. Shapiro. 1989. Temporal regulation and overlap organization of two \textit{Caulobacter} flagellar genes. \textit{J. Mol. Biol.} \textbf{205}: 71–83.

Kosturko, L.D., E. Daub, and H. Murialdo. 1989. The interaction of \textit{E. coli} integration host factor and \( \lambda \) cos DNA: Multiple complex formation and protein-induced bending. \textit{Nucleic Acids Res.} \textbf{17}: 313–334.

Kunkel, T.A. and J.D. Roberts. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. \textit{Methods Enzymol.} \textbf{154}: 367–382.

Kustu, S., E. Santero, J. Keener, D. Popham, and D. Weiss. 1989. Expression of \( \sigma^{\text{ntr}} \) [\( ntrA \)]-dependent genes is probably united by a common mechanism. \textit{Microbiol. Rev.} \textbf{53}: 367–376.

Leong, J.M., S. Nunes-Duby, C.F. Lesser, P. Youderian, M.M. Susskind, and A. Landy. 1985. The phi 80 and \( P_{22} \) attachment sites. Primary structure and interaction with \textit{Escherichia} coli integration host factor. \textit{J. Biol. Chem.} \textbf{260}: 4468–4477.

Leowy, Z.G., R.A. Bryan, S.H. Reuter, and L. Shapiro. 1987. Control of synthesis and positioning of a \textit{Caulobacter crescentus} flagellar protein. \textit{Genes Dev.} \textbf{1}: 626–635.

Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Marsh, J.L., M. Evile, and J.G. Wykes. 1984. The \( P_{C} \) plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation. \textit{Gene} \textbf{32}: 481–485.

Maxam, A. and W. Gilbert. 1980. Sequencing end-labelled DNA with base-specific chemical cleavages. \textit{Methods Enzymol.} \textbf{65}: 499–560.

Miller, J.H. 1972. Assay of \( \beta \)-galactosidase. In \textit{Experiments in molecular genetics}, pp. 352–355. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Minnich, S.A. and A. Newton. 1987. Promoter mapping and cell regulation of flagellin gene transcription in \textit{Caulobacter crescentus}. \textit{Proc. Natl. Acad. Sci.} \textbf{84}: 1142–1146.

Moitoso de Vargas, L., S. Kim, and A. Landy. 1989. DNA looping generated by DNA binding protein IHF and the two domains of lambda integrase. \textit{Science} \textbf{244}: 1457–1461.

Mullin D.A. and A. Newton. 1989. \( ntr \)-like promoters and upstream regulatory sequence \( ftr \) are required for transcription of a developmentally regulated \textit{Caulobacter crescentus} flagellar gene. \textit{J. Bacteriol.} \textbf{171}: 3218–3227.

Mullin, D., S. Minnich, S. Chen, and A. Newton. 1987. A set of positively regulated flagellar gene promoters in \textit{Caulobacter crescentus} with sequence homology to the \( nif \) gene promoters of \textit{Klebsiella pneumoniae}. \textit{J. Mol. Biol.} \textbf{195}: 939–943.

Nash, H.A. and C.A. Robertson. 1981. Purification and properties of the \textit{Escherichia coli} protein factor required for \( \lambda \) integrative recombination. \textit{J. Biol. Chem.} \textbf{256}: 9246–9253.

Nash, H.A., C.A. Robertson, E. Flamm, R.A. Weisberg, and H.I. Miller. 1987. Overproduction of \textit{Escherichia coli} integration host factor, a protein with nonidentical subunits. \textit{J. Bacteriol.} \textbf{169}: 4124–4127.

Newton, A., N. Ohta, G. Ramakrishnan, D. Mullin, and G. Raymond. 1989. Genetic switching in the flagellar gene hierarchy of \textit{Caulobacter} requires negative as well as positive regulation of transcription. \textit{Proc. Natl. Acad. Sci.} \textbf{86}: 6651–6655.

Nilsson, L., A. Vanet, E. Viijenboom, and L. Bosch. 1990. The role of FIS in trans activation of stable RNA operons of \textit{E. coli}. \textit{EMBO J.} \textbf{9}: 727–734.

Ninfa, A.J., D.A. Mullin, G. Ramakrishnan, and A. Newton. 1989. \textit{Escherichia coli} \( \sigma^{\text{ntr}} \) RNA polymerase recognizes \textit{Caulobacter crescentus} \( ftrB \) and \( ftrN \) flagellar gene promoters \textit{in vitro}. \textit{J. Bacteriol.} \textbf{171}: 383–391.

Ohta, N., L.S. Chen, E. Swanson, and A. Newton. 1985. Transcriptional regulation of a periodically controlled flagellar gene operon in \textit{Caulobacter crescentus}. \textit{J. Mol. Biol.} \textbf{186}: 107–115.

Peacock, S., H. Weissbach, and H.A. Nash. 1984. \textit{In vitro} regulation of phase \( \lambda \) cII gene expression by \textit{Escherichia coli} integration host factor. \textit{Proc. Natl. Acad. Sci.} \textbf{81}: 6009–6013.

Pereira, R.F., M.J. Ortuno, and R.P. Lawther. 1988. Binding of integration host factor [IHF] to the \( ilvGp1 \) promoter of the \( ilv \) GMEDA operon of \textit{Escherichia coli} K12. \textit{Nucleic Acids Res.} \textbf{16}: 5973–5989.

Pfojndexter, J.S. 1964. Biological properties and classification of the \textit{Caulobacter} group. \textit{Bacteriol. Rev.} \textbf{28}: 231–295.

Pollock, T.J. and H.A. Nash. 1983. Knotting of DNA caused by genetic rearrangement. Evidence for a nucleosome-like structure in site-specific recombination of bacteriophage lambda. \textit{J. Mol. Biol.} \textbf{170}: 1–18.

Ramakrishnan, G. and A. Newton. 1990. \( FlBD \) of \textit{Caulobacter crescentus} is a homologue of the \textit{NtrC} [\( N_{R} \)] protein and activates \( \sigma^{\text{ntr}} \)-dependent flagellar gene promoters. \textit{Proc. Natl. Acad. Sci.} \textbf{87}: 2369–2373.

Reitzer, L.J. and B. Magasanik. 1986. Transcription of \( glnA \) in \textit{E. coli} is stimulated by activator bound to sites far from the promoter. \textit{Cell} \textbf{45}: 785–792.

Richet, E., P. Abercian, and H.A. Nash. 1986. The interaction of recombinant proteins with supercoiled DNA: defining the role of supercoiling in lambda integrative recombination. \textit{Cell} \textbf{46}: 1011–1021.

Robertson, C.A. and H.A. Nash. 1988. Bending of the bacteriophage lambda attachment site by \textit{Escherichia coli} integrase host factor. \textit{J. Biol. Chem.} \textbf{263}: 3554–3557.

Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. \textit{Proc. Natl. Acad. Sci.} \textbf{74}: 5463–5467.

Santero, J. and E. Santero. 1989. \textit{In vitro} activity of the nitrogen fixation regulatory protein \( NIFA \). \textit{Proc. Natl. Acad. Sci.} \textbf{86}: 7346–7350.

Stenzel, T.T., P. Patel, and D. Bastia. 1987. The integration host factor of \textit{E. coli} binds to bent DNA at the origin of replication of the plasmid pSC101. \textit{Cell} \textbf{49}: 709–717.

Thompson, J.F. and A. Landy. 1988. Empirical estimation of protein-induced DNA bending angles: Applications to lambda site-specific recombination complexes. \textit{Nucleic Acids Res.} \textbf{16}: 9687–9705.

Towbin, H., T. Staehlin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. \textit{Proc. Natl. Acad. Sci.} \textbf{76}: 4350–4354.
Integration host factor is required for the activation of developmentally regulated genes in Caulobacter.

J W Gober and L Shapiro

*Genes Dev.* 1990, 4:
Access the most recent version at doi:10.1101/gad.4.9.1494

References
This article cites 54 articles, 26 of which can be accessed free at:
http://genesdev.cshlp.org/content/4/9/1494.full.html#ref-list-1

License

Email Alerting Service
Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here.](#)