A protein-induced DNA bend increases the specificity of a prokaryotic enhancer-binding protein

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Control of transcription in prokaryotes often involves direct contact of regulatory proteins with RNA polymerase from binding sites located adjacent to the target promoter. Alternatively, in the case of genes transcribed by *Escherichia coli* RNA polymerase holoenzyme containing the alternate sigma factor σ^{54} , regulatory proteins bound at more distally located enhancer sites can activate transcription via DNA looping by taking advantage of the increasing flexibility of DNA over longer distances. While this second mechanism offers a greater possible flexibility in the location of these binding sites, it is not clear how the specificity offered by the proximity of the regulatory protein and the polymerase intrinsic to the first mechanism is maintained. Here we demonstrate that integration host factor (IHF), a protein that induces a sharp bend in DNA, acts both to inhibit DNA-looping-dependent transcriptional activation by an inappropriate enhancer-binding protein and to facilitate similar activation by an appropriate enhancer-binding protein. These opposite effects have the consequence of increasing the specificity of activation of a promoter that is susceptible to regulation by proteins bound to a distal site.

[**Key Words:** Integration host factor; σ^{54} -RNA polymerase; upstream activation sequence; transcriptional activator]

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The ability of transcriptional regulatory proteins to act at a distance via DNA looping is characteristic of both prokaryotic and eukaryotic promoters (Matthews 1992; Schleif 1992). Over distances less than the persistence length of DNA (~150 bp), the DNA is relatively stiff, both torsionally and laterally (Wang and Giaever 1988), and looping is typically aided by proteins that bind to specific sequences on the DNA and bend it with a characteristic stereospecificity (Perez-Martin et al. 1994). However, at larger distances, the intrinsic flexibility of the DNA allows the formation of loops in the absence of a DNA-bending protein (Bellomy and Record 1990). These loops, which lack the stereospecificity provided by a specific, protein-induced DNA bend, may result in inappropriate protein-protein contacts. Thus, specific bends may act not only to facilitate correct protein-protein interactions, but also to prevent the stable formation of loops that result in inappropriate protein-protein interactions.

Transcription by prokaryotic RNA polymerase (RNAP) holoenzyme containing the alternate sigma fac-

tor, σ^{54} , requires contact between an enhancer-binding protein (EBP) bound at upstream activation sites (UAS) and the holoenzyme (Buck et al. 1986; Reitzer and Magasanik 1986; Ninfa et al. 1987). Through an ATP-hydrolysis-dependent mechanism, activation by the EBP transforms the closed complex formed by σ^{54} -RNAP at the promoter to an open complex permissive for transcriptional initiation (Popham et al. 1989). A DNA loop is thought to facilitate this interaction by increasing the local concentration of the EBP in the vicinity of the RNAP holoenzyme (Buck et al. 1987; Su et al. 1990; Wedel et al. 1990; Rippe et al. 1997).

Many prokaryotic species, including *Escherichia coli*, have multiple EBPs with distinct regulatory roles (Morett and Segovia 1993; Kaufman and Nixon 1996). Their specificity is thought to be largely a consequence of binding to a particular associated UAS sequence upstream of their target promoter(s) through a carboxy-terminal DNA-binding domain (Morett et al. 1988). While DNA binding is not essential for activation, mutant EBPs lacking the DNA-binding domain require far higher concentrations and show a loss of specificity (Dworkin et al. 1997; North and Kustu 1997).

Integration host factor (IHF), a heterodimeric protein that bends DNA by >160°, (Rice et al. 1996) binds in the promoter region of many σ^{54} -dependent operons at a site

This paper is dedicated to the memory of Brian Laurence Selsky.

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typically located between the promoter and the UAS sites (Gralla and Collado-Vides 1996). The sharp bend generated by IHF is thought to facilitate the interaction between the UAS-bound EBP and the σ^{54} -RNAP holoenzyme, particularly at weaker promoters with a lower affinity for the σ^{54} -RNAP holoenzyme (Hoover et al. 1990; Santero et al. 1992). This facilitation depends on the specific geometry of the interaction: If the UAS sites are moved so that the EBP binds on the opposite face of the DNA, then IHF inhibits activation (Claverie-Martin and Magasanik 1992). EBPs can activate from templates missing specific UAS sites; however, this activation is weaker than activation from wild-type templates and it is also inhibited by IHF (Claverie-Martin and Magasanik 1992; Dworkin et al. 1997). By contrast, activation by an EBP lacking a DNA-binding domain is unaffected by IHF (Dworkin et al. 1997); thus, IHF-mediated inhibition is presumably the result of EBPs binding to nonspecific (or cryptic) sites that are not appropriately spaced relative to the IHF bend and the σ^{54} -RNAP bound at the promoter.

The *hycA* and *hypA* promoters of the formate hydrogenase system of *E. coli* are regulated by IHF and the EBP, FhlA. In the presence of nitrate, expression from these promoters is significantly reduced, and strains lacking IHF (carrying a *himA* mutation) show an increase (three- to fourfold) in transcription from these promoters over wild-type strains (Hopper et al. 1994). In addition, a *Pseudomonas putida* strain carrying a *himA* mutation shows increased basal activation of the σ^{54} -dependent *Pu* promoter in the absence of the cognate EBP, XylR, and this increase was attributed to activation by heterologous EBPs that might be normally inhibited by IHF (Perez-Martin and de Lorenzo 1995). Similarly, we observed that *pspA* transcription measured under noninducing conditions (using a *pspA-lacZ* promoter fusion) in a strain carrying a *himA* mutation, along with a deletion of the gene encoding the associated EBP, PspF, showed a twofold increase over a strain carrying only the *pspF* deletion (Dworkin 1997). We, as well as the previous investigators, were unable, however, to attribute this increased basal expression to activation by a particular EBP. We therefore decided to examine whether, in vitro, IHF could inhibit activation by a specific heterologous EBP and whether this inhibition would result in an increase in the specificity of transcriptional activation.

Results

The *pspA* gene of *E. coli* is transcribed by σ^{54} -RNAP (Weiner et al. 1991) and is under control of the constitutively active EBP PspF (Jovanovic et al. 1996; Model et al. 1997). The *pspA* promoter region contains two binding sites for PspF (Jovanovic 1997), as well as a binding site for IHF (Fig. 1A,B) (Weiner et al. 1995). In vitro transcriptional activation by PspF at the *pspA* promoter is facilitated threefold by IHF (Fig. 2A, lanes 3,4; see also Dworkin et al. 1997). We replaced PspF with the heterologous EBP NR₁ of *E. coli*. Under conditions of nitrogen limitation, phosphorylated NR₁ activates transcription at several σ^{54} -dependent promoters involved in the metabo-

lism of nitrogen-containing compounds (Ninfa and Magasanik 1986). While phosphorylated NR₁ can activate transcription at the *pspA* promoter (Fig. 2A, lane 1), IHF inhibits this activation fivefold (lane 2).

This inhibition suggests that NR₁ is bound to site(s) in the *pspA* promoter region that do not result in an appropriate EBP-IHF-RNAP geometry. The ability of NR₁ to activate at low concentrations (<100 nM) from templates lacking specific NR₁ binding sites has been attributed to its ability to bind to the DNA nonspecifically (Weiss et al. 1992). In addition, since EBPs can act at distances of up to 2 kb upstream (or downstream) of their target promoters (Buck et al. 1986; Ninfa et al. 1987), the possible sites are not restricted to those in near proximity to the RNAP bound at the promoter, as is the case for another prokaryotic transcriptional activator, CAP (Busby and Ebright 1994).

In an effort to extend the generality of this observation, we examined the *E. coli* σ^{54} -dependent *glnH* promoter. The *glnH* promoter region contains two strong and two weak binding sites for NR₁ as well as an IHF site (Fig. 1A,B) (Claverie-Martin and Magasanik 1991). As has been demonstrated (Claverie-Martin and Magasanik 1991), NR₁ activates transcription from the *glnH* promoter in vitro and this activation is facilitated fourfold by IHF (Fig. 2B, lanes 3,4). By contrast, PspF-dependent activation of the *glnH* promoter is inhibited 2.5-fold by IHF (Fig. 2B, lanes 1,2). This inhibition suggests that PspF is binding to site(s) that result in a geometry of the EBP-IHF-RNAP interaction that is unfavorable for activation. The NR₁ and PspF UAS sites are distinct (Fig. 1B) and neither the *pspA* nor the *glnH* promoter region contains sequences matching the heterologous UAS sites.

When the sequence comprising the IHF site in the *pspA* promoter is replaced with a sequence lacking an IHF site, both the stimulatory effect of IHF on PspF-dependent activation (Fig. 2C, lanes 5–8) and the inhibitory effect of IHF on NR₁-dependent activation (Fig. 2C, lanes 1–4) are eliminated. Thus, IHF inhibition of NR₁-dependent *pspA* transcription is not a consequence of binding site competition or protein-protein interaction between IHF and NR₁.

The opposite effects of IHF on activation by two different EBPs at a single promoter suggest that IHF could help determine the specificity of activation. That is, in addition to the specificity resulting from EBP binding to a cognate UAS, the bend generated in the promoter region by the binding of IHF would prevent activation by EBPs bound nonspecifically. We examined this question using in vitro transcription reactions containing multiple templates with different promoters. In the absence of IHF, the EBP NR₁ activates transcription from promoters containing either PspF-specific (*pspA*) or NR₁-specific (*glnH* and *glnA*) binding sites comparably (Fig. 3A, lane 1). We take as a measure of specificity the ratio of appropriate to inappropriate transcripts, which, in this case (no IHF), is the ratio *glnH* : *pspA* = 1.6 : 1. In the presence of IHF (lane 2), however, this ratio increases tenfold to 15 : 1. This increase is a result of IHF acting both to facilitate NR₁-dependent activation of *glnH* and to in-

Figure 1. Organization of σ^{54} -dependent promoters and upstream activation sequences. (A) The *pspA* promoter contains an IHF-binding site (–30 to –65) (Weiner et al. 1995) and two high-affinity binding sites (–89 to –126) for the PspF transcriptional activator (Jovanovic 1997). The *glnH* promoter contains an IHF-binding site (–33 to –59), two overlapping high-affinity binding sites for the NR_I transcriptional activator (–100 to –129), and two lower affinity NR_I sites (not shown) (Claverie-Martin and Magasanik 1991). The *glnAp2* promoter contains two high affinity NR_I sites (–100 to –147) as well as three weaker NR_I sites (not shown) (Reitzer and Magasanik 1986). The *pspA*– Δ UAS promoter is identical to the *pspA* promoter except that the sequences spanning the PspF binding sites were deleted (Dworkin et al. 1997). (B) The upstream activation sequences (in bold) of the promoters schematized in A. Note that the *glnH*UAS sequences are overlapping and that, whereas the *glnH* and *glnAp2* UAS sequences are similar, they both differ from the *pspA* UAS sequences.

hibit NR_I-dependent activation of *pspA*. The *glnA* promoter contains no IHF-binding site (Fig. 1C) and thus is unaffected by IHF (Fig. 3A).

When the EBP is PspF, a similar effect of IHF is seen. In this case, the appropriate transcript is from the *pspA* promoter and the inappropriate transcript is from the *glnH* promoter. Quantification of these transcripts yields the specificity ratio of *pspA* : *glnH* is 3 : 1 in the absence of IHF (Fig. 3B, lane 1), and 14 : 1 in its presence (Fig. 3B, lane 2). A comparison of the *pspA* and *glnH* transcripts in the presence and absence of IHF demonstrates that IHF acts to increase PspF-dependent activation of *pspA* while simultaneously inhibiting PspF-dependent activation of *glnH*.

When the PspF UAS sequences in the *pspA* promoter are removed, IHF inhibits PspF-dependent transcription (Dworkin et al. 1997). In a transcription reaction containing both this template (*pspA*ΔUAS) and a *glnH* promoter template (Fig. 3C), there is no change in the specificity ratio *pspA*ΔUAS : *glnH* in the presence of IHF. Thus, the effect of IHF to increase the specificity of activation is dependent on the presence of a specific UAS.

Discussion

IHF facilitates EBP-dependent activation of the weak, σ^{54} -dependent *nifH* promoter and at such weak promoters, EBPs that do not have an appropriate binding site properly positioned to take advantage of the IHF-generated bend are unable to activate efficiently (Santero et al. 1992). However, not all promoters regulated by IHF are weak; for example, the two promoters analyzed here, *pspA* and *glnH*, are stronger than *nifH*, and, as predicted, are less dependent on IHF (Fig. 2A,B; threefold) than

Figure 2. Effect of IHF on PspF- and NR_I-dependent in vitro transcription from *pspA* and *glnH* promoters. (A) NR_I- or PspF-dependent activation assayed on a supercoiled *pspA* promoter template (pJD10) in the presence or absence of IHF. (B) PspF- or NR_I-dependent activation assayed on a supercoiled *glnH* promoter template (pJD37) in the presence or absence of IHF. In the absence of any EBP, *pspA*- or *glnH*-specific transcription is abolished (Claverie-Martin and Magasanik 1991; Dworkin et al. 1997). (C) NR_I- or PspF-dependent activation assayed on a supercoiled *pspA* promoter template (wt; pJD10) or on a supercoiled *pspA* promoter template lacking the IHF binding site (*ihf*–; pJD28). Quantified RNA transcripts (in normalized arbitrary units): (A) (lane 1) 1.0; (lane 2) 0.19; (lane 3) 2.3; (lane 4) 6.4. (B) (lane 1) 1.0; (lane 2) 0.41; (lane 3) 1.4; (lane 4) 5.3.

Figure 3. IHF increases specificity of activation by PspF and NR₁. (A) NR₁-dependent activation assayed on supercoiled templates (3 nM) containing the *pspA* promoter (pJD10), the *glnH* promoter (pJD37), and the *glnA2* promoter (pTH8) in the presence or absence of IHF. The specific transcripts generated from each of these templates are identified. (B) PspF-dependent activation assayed on supercoiled templates (3 nM) containing the *pspA* promoter (pJD10) and the *glnH* promoter (pJD37) in the presence or absence of IHF. (C) PspF-dependent activation assayed on supercoiled templates (3 nM) containing the *pspA*ΔUAS promoter (pJD12) and the *glnH* promoter (pJD37) in the presence or absence of IHF. Quantified RNA transcripts (in decreasing order of size and in normalized arbitrary units): (A) (Lane 1) 0.61; 1.0, 0.75; (lane 2) 0.11, 1.6, 0.85; (B) (Lane 1) 1.0, 0.33; (lane 2) 1.8, 0.13; (C) (Lane 1) 1.0, 0.63; (lane 2) 0.29, 0.23.

weaker promoters (e.g., *nifH*, >20-fold; Hoover et al. 1990). The potential susceptibility of stronger promoters to activation by heterologous activators raises the question of how specificity of activation is maintained (Hoover et al. 1990; Santero et al. 1990). The experiments presented here are consistent with a model in which activation by inappropriate EBPs bound to nonspecific sites is inhibited by IHF.

While binding of proteins to upstream (or downstream) sequences can increase their effective local concentration at the promoter (Mossing and Record 1986; Rippe et al. 1995), we would argue that binding of an EBP to a nonspecific site(s) in the presence of IHF results in a DNA geometry that decreases the local concentration of the EBP near the promoter. Previous explanations of IHF inhibition of background activation have focused on the formation of a specific complex higher-order structure resulting from the relatively unique juxtaposition of several σ^{54} promoters (Hopper et al. 1994) or on the ability of IHF to occlude access to the RNAP holoenzyme by interposing a segment of DNA that blocks interactions between an EBP acting from solution and the RNAP (de Lorenzo and Perez-Martin 1996; Perez-Martin and de Lorenzo 1995). While the data presented here do not directly evaluate the second model, heterologous EBPs, as DNA-binding proteins, can be assumed from thermody-

namic principles to bind DNA nonspecifically (von Hippel et al. 1974; Lin and Riggs 1975). Further, IHF does not negatively regulate a mutant EBP lacking a DNA-binding domain (Dworkin et al. 1997).

A localized DNA bend can have implications for DNA structure beyond its direct effect; a DNA sequence with intrinsic curvature is sufficient to determine the plectonemic structure of a supercoiled plasmid (Laundon and Griffith 1988). In fact, DNA-bending proteins that act to inhibit specific loop formation include the Nac protein of *Klebsiella aerogenes*, which prevents the interaction of NR₁ bound at a specific enhancer in the *nac* promoter region with the σ^{54} -RNAP holoenzyme (Feng et al. 1995), and the CAP protein, which disrupts the repression loop formed by AraC dimers bound simultaneously to two sites in the *araBAD* promoter region (Lobell and Schleif 1991). Thus, the bend generated by IHF acts both to increase contacts between UAS-bound EBPs and σ^{54} -RNAP at weaker promoters and to reduce contacts between nonspecifically bound heterologous EBPs and the σ^{54} -RNAP at stronger promoters.

These observations may be particularly relevant for the regulation of eukaryotic genes, where it has been suggested that protein-protein interactions between the basal transcriptional machinery and proteins bound to distal enhancers can be modulated by the topology of the intervening DNA (Echols 1986; Ptashne 1986). In the specific case of the chicken β^A -globin promoter, enhancer-dependent in vitro transcription requires that the intervening DNA be supercoiled (Barton et al. 1997). Consistent with this observation, Monte Carlo-based simulations of DNA dynamics demonstrate that the effective local concentration of two sites on DNA is far greater on supercoiled DNA than on relaxed DNA (Vologodskii et al. 1992). This increase is largely unaffected by the contour separation between the sites (Vologodskii et al. 1992), suggesting that the basal transcription apparatus could be susceptible to inappropriate contacts from proteins bound at nonspecific sites located far upstream (or downstream). In vivo, where the chromosome is primarily supercoiled [and particularly upstream of promoters where transcription-induced negative supercoiling occurs (Wu et al. 1988)], mechanisms that restrict inappropriate protein-protein interactions must therefore exist.

The data presented here suggest that a general mechanism of negative regulation of loop formation may play an important role in determining the specificity of activation of promoters utilizing DNA looping. It is therefore of interest that the nucleosome appears to block access to promoters by transcription factors not by preventing binding, but instead through a distortion of the DNA structure (Luger et al. 1997).

Materials and methods

DNA manipulation

All DNA manipulations were conducted according to established protocols (Sambrook et al. 1989). All enzymes were ob-

tained from New England Biolabs (Beverly, MA) unless noted. Primers were synthesized by Operon Technologies (Alameda, CA). Template DNA for in vitro transcription reactions was purified by use of either the Wizard Megaprep or Midiprep DNA purification systems (Promega).

Plasmids

pJD10 (*pspA*) contains a 580-bp PCR-generated fragment encompassing the *pspA* promoter region (−458 to +122) cloned into the vector pGZ119EH (Lessl et al. 1992) upstream of the *rnnBT1* terminator (Dworkin et al. 1997). pJD12 (*pspAΔUAS*) is identical to pJD10 except that sequences −89 to −126 were replaced with an *NdeI* site (Dworkin et al. 1997). pTH8 (*glnAp2*) contains a 600-bp *HaeIII* fragment spanning the *glnA* promoter region cloned into the vector pTE103 upstream of the bacteriophage T7 early terminator (Hunt and Magasanik 1985). pJD37 (*glnH*) was constructed by cloning the *EcoRI*–*HindIII* fragment from pFC50 (Claverie-Martin and Magasanik 1991) spanning the *glnH* promoter into the *EcoRI* and *HindIII* sites of pGZ119EH. pJD28 (*pspA*–*ihf*) was constructed by PCR mutagenesis, which replaced the sequence −34 to −58 of the *pspA* promoter in pJD10 with a different sequence (5′-GGATCCTC-TAGAGTCGACCTGCAG-3′) of the same length not containing an IHF-binding site. Primers JD54 (5′-GGCTGGTACCTA-GCGAGTTCATCAAGAAATA-3′) and JD101 (5′-GGCGGAT-CCCTGATTGAAGAATCAACAGC-3′) were used in a PCR reaction with Taq polymerase and pBRPS-1 (Brissette et al. 1991) as template. The PCR product was cleaved with *Bam*HI and *Kpn*I and cloned into pGZ119EH cleaved with *Bam*HI and *Kpn*I. This plasmid was cleaved with *Pst*I and *Hind*III and ligated to a fragment generated by PCR with primers JD102 (5′-GGCG-GATCCCTGCAGGATAAAAAATTGGCAGCAAATTG-3′) and JD103 (5′-GGCAAGCTTCAGTTTCTGTGGATCTTCC-3′), Taq polymerase, and pJD10 as template, which was then cleaved with *Pst*I and *Hind*III. The transcript from pJD28 is 28 nucleotides shorter than that from pJD10 as a result of the cloning strategy employed. The lengths of the RNA transcripts from the respective plasmids are pJD10 (*pspA*) ~360 nucleotides; pJD37 (*glnH*) ~340 nucleotides; pTH8 (*glnAp2*) ~300 nucleotides; pJD12 (*pspAΔUAS*) ~360 nucleotides; pJD28 (*pspA*–*ihf*) ~330 nucleotides.

In vitro transcription

Reactions were carried out in a buffer containing 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM MgCl₂, and 0.1 mM EDTA. All reactions also contained 0.5 mM GTP, 0.5 mM UTP, 2 mM ATP, 2 mM DTT, and 0.3 units of recombinant RNAsin (Promega). Core RNAP (Epicentre Technology, Madison WI) and σ⁵⁴ [purified according to Hunt and Magasanik (1985)] were added at 10 nM and 14 nM, respectively. When used, PspF (gift of G. Jovanovic) was at 4 nM, NR_I and NR_{II} [both purified according to Ninfa et al. (1987)] were at 100 nM each, and IHF (gift of H. Nash) was at 15 nM. Typically, all components were incubated with supercoiled template (5 nM) at 37°C for 10 min. Then 0.5 μl of [α-³²P]CTP (10 μCi/μl; New England Nuclear) was added [except for reactions containing NR_I where heparin (Sigma) was added to 100 μM simultaneously], and the reaction allowed to incubate 10 min more at 37°C. Cold CTP was added to 1 mM and reaction continued at 37°C for 10 min. Reactions then were placed on ice, and an aliquot was treated with phenol-chloroform and precipitated in ethanol with tRNA (50 μg/ml). The pellet was resuspended in RNase-free glass-distilled H₂O, mixed with formamide loading buffer, loaded on 4% polyacryl-

amide/7 M urea gels, and run at 225 V in 0.5× TBE as described (Sambrook et al. 1989). Gels were subjected to autoradiography or exposed to a Molecular Dynamics Storage Phosphor Screen and analyzed on a Molecular Dynamics PhosphorImager using ImageQuant software. Quantification of PhosphorImager data was as follows: A box was drawn around the band representing the transcription terminating at *rnnBT1* and the pixel values were integrated. Background was calculated by integration of a same-sized box below the *rnnBT1* band in each lane and subtraction from the *rnnBT1* band.

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Dworkin et al.

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