PspF and IHF bind co-operatively in the *psp* promoter-regulatory region of *Escherichia coli*

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Summary

PspF bound to the *psp* enhancer activates Eσ^{54} holo-enzyme-dependent transcription of the *Escherichia coli* phage-shock protein (*psp*) operon and autogen-ously represses its own σ^{70}-dependent transcription, thereby keeping its concentration at a low level. It has been demonstrated previously that integration host factor (IHF) bound to a DNA site located between the *psp* core promoter and the PspF binding sites stimu-lates *psp* expression. We show here that wild-type IHF strongly retards DNA containing the *psp* promoter region. *In vitro*, PspF binding to the *psp* enhancer facilitates IHF binding, while IHF binding to the *pspF–pspA-E* promoter-regulatory region increases the efficacy of PspF binding to the upstream activating sequences (UASs). This is the first demonstration of co-operative binding of an activator and IHF in a σ^{54}-dependent system. In the absence of IHF, *in vivo* autoregulation of *pspF* transcription is lifted and, con-sequently, PspF production is increased, indicating that IHF enhances PspF binding to the *psp* enhancer *in vivo*.

Introduction

Transcription from σ^{54}-dependent promoters requires the participation of an activator that binds to upstream ele-ments (Ninfa *et al.*, 1987; Sasse-Dwight and Gralla, 1988; Kustu *et al.*, 1989; Popham *et al.*, 1989; Weiss *et al.*, 1991; Austin and Dixon, 1992; Wedel and Kustu, 1995), which in several cases has been shown to depend upon contact between the activator and Eσ^{54} holoenzyme (Ninfa *et al.*, 1987; Kustu *et al.*, 1989; Popham *et al.*, 1989; Su *et al.*, 1990); this contact has been demonstrated to result from DNA looping, since the activator and the promoter are usually 100–200 bases away from each other (Buck *et al.*, 1986; Reitzer and Magasanik, 1986;

Sasse-Dwight and Gralla, 1988; Kustu *et al.*, 1989; Pop-ham *et al.*, 1989; Su *et al.*, 1990; Weiss *et al.*, 1991; Austin and Dixon, 1992; Charlton *et al.*, 1993). Formation of the loop can be facilitated either by a native DNA bend or, in many cases, by integration host factor (IHF) bound to a site between the activator and the promoter (reviewed in Freundlich *et al.*, Goosen and Putte, 1995). IHF is a sequence-specific DNA-binding and DNA-bending protein composed of two related but non-identical subunits encoded by the *himA* (*ihfA*) and *himD* (*ihfB*) genes (reviewed in Friedman, 1988). IHF binding induces a sharp DNA bend ($> 160°$) (Rice *et al.*, 1996) and can thus facilitate an interac-tion between proteins bound at distantly located elements. IHF does not stimulate transcription by itself (Goosen and Putte, 1995) and, in other promoters, does not affect the binding of either activator or Eσ^{54} holoenzyme to their respective DNA binding sites (Claverie-Martin and Magasanik, 1991; Santero *et al.*, 1992). These earlier results are specific to the promoters studied; there is no reason in principle why the specific organization of regula-tory elements in a σ^{54}-dependent promoter region should not facilitate co-operative binding of the activator, IHF, and polymerase. Results from *in vivo* experiments suggest that IHF might influence the binding of PspF (Jovanovic *et al.*, 1997), the activator of the *Escherichia coli* phage-shock protein (*psp*) operon (*pspA-E*) in which transcrip-tion is also σ^{54} dependent (Weiner *et al.*, 1991).

The *psp* operon encodes a number of proteins, one of which (PspA) has been postulated to protect the cell from loss of proton motive force (PMF) upon stress (Kleere-bezem *et al.*, 1996). This operon is strongly induced by filamentous phage pIV and by homologous bacterial secre-tins, all outer membrane proteins (Brissette *et al.*, 1990; Possot *et al.*, 1992; Russel and Kazmierczak, 1993). The *psp* operon also responds to a wide variety of other stimuli, most of which can be rationalized as having effects on the cell membrane and/or the cell's energy charge (Model *et al.*, 1997). PspF, an enhancer-binding protein, is required for activation of *pspA-E* transcription *in vivo* and *in vitro* (Jovanovic *et al.*, 1996; Dworkin *et al.*, 1997). The PspF protein itself lacks an N-terminal regulatory region and is constitutively active (Jovanovic *et al.*, 1996). PspF binding to the *psp* enhancer (upstream acti-vating sequences UAS I and UAS II), located -80 to -126 bp upstream of the *pspA-E* transcription start site, is essential for transcriptional activation of its target genes and for autogenous control of its own expression (Weiner

et al., 1995; G. Jovanovic and P. Model, unpublished; Jovanovic *et al.*, 1997). The consequence of *pspF* auto-regulation is that the intracellular concentration of PspF is maintained at a constant low level, and this could be essential for controlling the specificity of activation of target genes. $pspF_{877}$-encoded PspFΔHTH, a DNA-binding mutant protein, which lacks the helix–turn–helix (HTH) motif responsible for binding to the UAS, is 100-fold less active than wild-type activator *in vivo* and *in vitro* and is not capable of controlling its own transcription (Jovanovic *et al.*, 1996; 1997; Dworkin *et al.*, 1997). UAS site I is a strong binding site with C2 symmetry to which PspF can bind at low concentration. UAS II, a weaker site, has no C2 symmetry and PspF binds to it with higher affinity on supercoiled rather than on a linear DNA template (G. Jovanovic and P. Model, unpublished). PspF can activate the *psp* operon *in vitro* when UAS II alone is present on a template (Dworkin *et al.*, 1997).

pspA-E transcription is stimulated by IHF bound to DNA at a site located between the *psp* σ^{54} promoter and the *psp* enhancer (Weiner *et al.*, 1995). We have shown that binding of wild-type PspF to the UASs in the presence of IHF increases the effectiveness of *pspA-E* activation of transcription 100-fold (Dworkin *et al.*, 1997; Jovanovic *et al.*, 1997). UAS sites I and II, the IHF-binding site and the σ^{54} promoter sequences are on the same face of DNA. Hence, IHF plays a role in the activation of *pspA-E* by PspF, where it presumably bends the DNA to optimize the interaction between PspF and the polymerase. Moreover, removing the IHF and $E\sigma^{54}$ binding sites from the *psp* promoter-regulatory region makes autogenous control of PspF less effective *in vivo* (Jovanovic *et al.*, 1997).

The strength of IHF–DNA interactions depends on the recognition sequence and the availability of IHF hetero-dimers. Although the *in vivo* concentration of IHF is estimated to be 6–30 μM per cell (15–35 nM per site) (Ditto *et al.*, 1994), the number of specific and non-specific IHF-binding sites in the *E. coli* chromosome is unknown, and the effective concentration of free IHF could be very low. The IHF-binding site in the *psp* promoter region contains a Dam methylation site (GATC) that remains unmethylated during *E. coli* log-phase growth (Wang and Church, 1992), suggesting effective and continual occupancy by IHF.

Here, we study IHF and PspF binding in the *psp* promoter-regulatory region and the consequence that this binding has on the regulation of *pspF* transcription. The implications for *pspA-E* activation and *psp* promoter specificity are discussed.

Results

Protein binding in the pspF–pspA-E promoter-regulatory region

The interactions of proteins in cell extracts with the *psp* promoter-regulatory region were studied in gel mobility shift assays, using a polymerase chain reaction (PCR)-generated linear template (260) with a centrally located IHF-binding site (Fig. 1A). Extracts from a set of mutant strains were used to determine the contributions of IHF and/or PspF to these interactions (Fig. 1B). A crude cell extract from wild-type cells binds and strongly retards the template (complex 2 in lane 4; Fig. 1C), while an extract from a *himA himD* mutant lacking IHF retards the probe much less (Fig. 1B, complex 5 in lane 7). Use of a crude cell extract from a strain carrying the *himD* mutation alone shows that the IHF HimA (IHF α) subunit alone contributes to the retardation of the probe (complex 4 in Fig. 1B, lane 6). Incubation of the DNA mixture with purified His-tagged PspF (6×HisPspF) (3 nM) shows two highly retarded DNA–protein complexes, complex 2 and 1 (Fig. 1B, lane 2) that were previously predicted to represent complexes of the activator with UAS sites I and II respectively (G. Jovanovic and P. Model, unpublished). However, experiments with cell extract from a strain encoding the PspF DNA-binding mutant, PspFΔHTH (Fig. 1B, complex 3 in lane 5), shows that PspF binding contributes little to the retardation of the 260 fragment by the wild-type extract (Fig. 1B, lane 4). Since the most noticeable mobility difference was obtained between the reactions containing wild-type vs. IHF null mutant extracts, and since a crude cell extract from cells that are IHF⁺ but PspF⁻ retards the template to almost the same extent as does purified IHF by itself (Fig. 1B, complex 2 in lane 3), the retardation imposed by the IHF-directed bend dominates the shifts. These shifts are suppressed by specific competitor (Fig. 1C, lane 5) but not by non-specific DNA.

Co-operative DNA binding of PspF and IHF *in vitro*

We further explored the binding of PspF and IHF to the *psp* promoter-regulatory region using the proteins at concentrations estimated to be similar to their effective *in vivo* concentrations. IHF at 5 nM shifts little DNA by itself (Fig. 2A, lane 1), but the addition of PspF at 2.5 nM greatly increased the amount of retarded DNA (Fig. 2A, lane 3). Quantification of the complexes in lanes 1–4 showed that IHF in the presence of PspF (lane 3) shifts 48-fold times as much of the DNA probe than IHF by itself (lane 1). PspF alone shifts seven times as much DNA as does IHF alone (lane 2). The sum of the two individual shifts is much less than when the two proteins are added together (lane 3). We measured the relative amounts of PspF present in the shifted complexes either when PspF was used by itself or when IHF was also present. A scan of Western blots (Fig. 2B) of the gel used for the mobility shift assay showed that 2.5 times as much PspF was bound to the 260 probe in the presence of IHF (lane 3) than when it was added by itself (lane 2). Given that nothing is known

about the stability of the binding during the workup and the uncertainties of quantification of Western blots using enhanced chemiluminescence (ECL) detection, this number can only be taken as a crude estimate. It is, however, obvious that both proteins contributed to the large increase in the amount of probe shifted when they were used together. The contribution of IHF to the shift must be substantial.

Binding of PspF to UASs facilitates IHF binding

The co-operativity of PspF–IHF binding to DNA could be caused by direct protein–protein interactions or a consequence of changes in DNA topology upon binding of either of these proteins to the 260 template. To distinguish between these possibilities, mutant PspF that can activate *pspA-E* transcription *in vivo* (Jovanovic *et al.*, 1996) and *in vitro* (Dworkin *et al.*, 1997) but cannot bind the DNA (G. Jovanovic and P. Model, unpublished) was used. A gel mobility shift assay with His-tagged PspFΔHTH at 150 nM and IHF at 5 nM (Fig. 3, lane 5) shows that there is no increase in the quantity of DNA retarded (complex 1) by IHF and PspFΔHTH used together relative to a reaction in which IHF alone was added (Fig. 3, lane 2). The probe remaining at the top of the gel in samples containing PspF (lanes 3 and 4) results from the formation of a multimeric PspF–DNA complex (G. Jovanovic and P. Model, unpublished). A shorter template, '160', which contains the IHF-binding site at the end of the fragment (Fig. 1A), binds IHF at 5 nM alone (complex 1′ in Fig. 3, lane 8) as

efficiently as template 260 with a centrally located IHF-binding site in the presence of PspF (2.5 nM) (complex 1 in Fig. 3, lane 3). The addition of 2.5 nM PspF does not further increase IHF binding to probe 160 (Fig. 3, lane 9). (Purified His-tagged PspF does not bind to the 160 DNA fragment; G. Jovanovic and P. Model, unpublished.)

These results demonstrate that a PspF–IHF protein–protein interaction does not facilitate DNA binding of IHF in the presence of PspF. The experiment with the 160 fragment shows that IHF has a much higher affinity for its target site when it is placed at the end of the template, indicating that PspF binding to 260 can form an energetically preferred configuration of the DNA chain necessary for efficient IHF binding.

Gel retardation assays with templates in which UAS site I or sites I and II had been deleted were used to discover whether PspF must be bound to its UASs in order to stimulate IHF binding to the 260 DNA fragment (Fig. 4). PspF (2.5 nM) binds UAS II in the absence of UAS I and facilitates binding of IHF at low concentration (5 nM) (lane 6), increasing the amount of retarded DNA to a level similar

Fig. 1. Wild-type IHF strongly retards the *pspF–pspA-E* promoter-regulatory region DNA.
A. Schematic representation of the *pspF′–pspA-E* promoter-regulatory region and construction of the linear DNA templates, 260 (260 bp) and 160 (159 bp). P1–3, *pspF* σ[70] promoters; σ[54], sigma-54 recognition sequence and *pspA-E* promoter; rightward arrow (+1), *pspA-E* start of transcription; leftward arrows, *pspF* starts of transcription; hatched box, IHF (−25 to −60), IHF-binding site; GATC, methylation site inside the IHF-binding site; box with horizontal lines, UAS I and II (−80 to −126) *psp* enhancer; *pspA-E* upstream activating sequences (UASs) and PspF-binding sites; PspF-specific UASs were determined using gel mobility shift and DNase I footprinting assays with wild-type PspF, DNA-binding mutant, specific (260 DNA fragment) and non-specific (160 DNA fragment) DNA (G. Jovanovic and P. Model, unpublished).
B. Gel mobility shift assays with 2 ng of radioactive, γ-ATP-labelled DNA template 260, purified His-tagged PspF, purified IHF and crude cell extracts (cce) (4 μl). All reactions contained 1000× weight excess of poly(dI-dC). Lanes: 1, free DNA (260); 2, 6×HisPspF (3 nM); 3, IHF (10 nM); 4, K561 (WT) cce; 5, K1527 (*pspF*[877]; encodes PspFΔHTH) cce; 6, L108 (*himD*Δ3::*cam*; encodes HimA subunit) cce; 7, K1173 (*himA*Δ82::Tn*10 himD*Δ3::*cam*; IHF null mutant) cce.
C. Gel mobility shift assays with the wild-type (K561) cell extracts (the same preparation as in B) and DNA template 260 (2 ng). Lanes: 1, free DNA (260); 2, WT cce (1 μl); 3, WT cce (1 μl) + poly(dI-dC); 4, WT cce (2 μl) + poly(dI-dC); 5, WT cce (2 μl) + poly(dI-dC) + 150× specific competitor (cold DNA template 260). *WT cce–DNA complex.
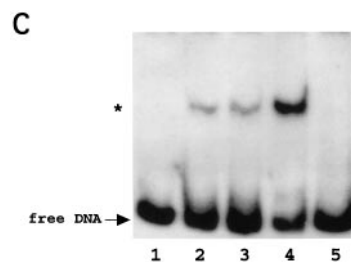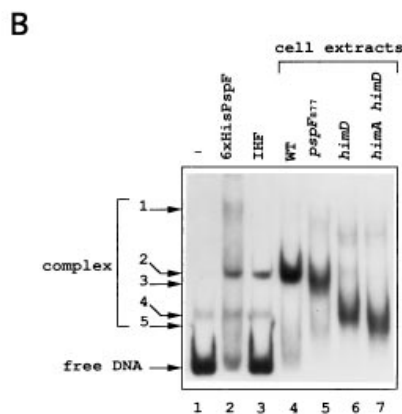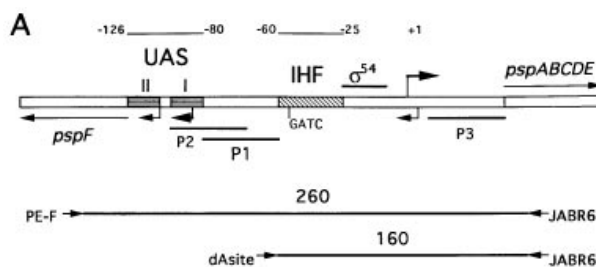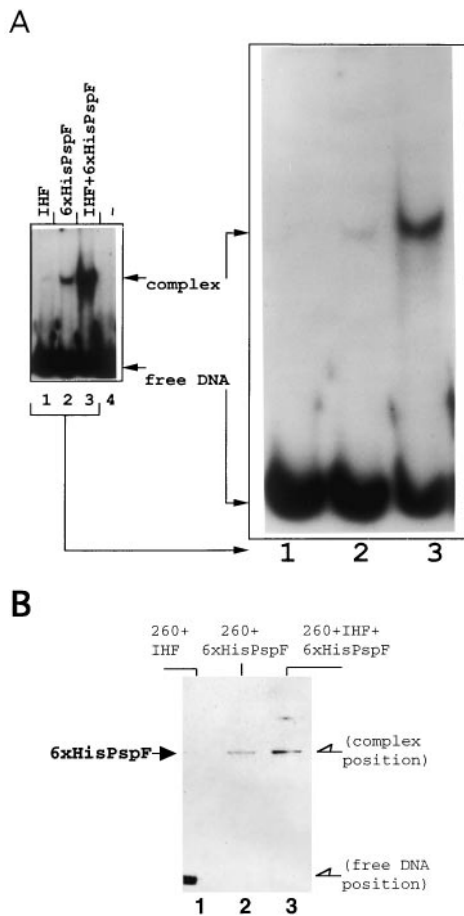
Fig. 2. PspF facilitates DNA binding of IHF *in vitro*.
A. Gel mobility shift assay performed using γ-ATP-radiolabelled template 260 (2 ng), 6×HisPspF (2.5 nM) and IHF (5 nM). Lanes: 1, IHF; 2, 6×HisPspF; 3, IHF and 6×HisPspF together; 4, free 260 DNA probe. (Gel was overexposed.) Phosphorimager quantification of the retarded band marked as 'complex' showed the relative values for lanes 1–4 as 1:7:48:0. Enlarged: shorter exposure of the same gel, lanes 1–3.
B. Western blot (with anti-PspF serum) of the non-denaturing acrylamide gel containing the probe retarded by 5 nM IHF (lane 1), 2.5 nM 6×HisPspF (lane 2) or by 2.5 nM 6×HisPspF and 5 nM IHF together (lane 3) in the gel mobility shift assay prepared as in A. PspF bands were quantified by microdensitometry and normalized to the background in a control lane containing the free 260 probe. The relative amounts of PspF (in arbitrary units) for lanes 1–3 are 0:1:2.5.

to that reached with a high concentration of IHF (50 nM) alone (lane 5). Although PspF at 2.5 nM does not bind the ΔUAS I/II template (data not shown) when introduced at 25 nM, it does bind DNA (lane 8). However, this PspF binding does not significantly facilitate binding of 5 nM IHF (lane 11), since the amount of retarded complex is similar to that retarded by IHF in the absence of PspF (lane 9). IHF at 50 nM effectively binds the ΔUAS I/II probe (lane 10). Hence, PspF must be bound specifically to UAS site I and/or site II to stimulate the co-operative binding of IHF (5 nM) *in vitro*.

IHF enhances the autogenous control of PspF production *in vivo*

To determine whether this PspF–IHF co-operativity occurs *in vivo*, a different approach was used. PspF autogenously represses its own *pspF* transcription and protein production *in vivo* and *in vitro* (Jovanovic *et al.*, 1997). We have postulated that this regulation is direct and is a consequence of PspF binding to the UASs, which overlap regulatory elements (Fig. 1A). On multicopy plasmids, less PspF is produced when DNA-binding sites for IHF and $E\sigma^{54}$ are present, suggesting that local binding of these proteins in the *psp* promoter-regulatory region influences *pspF* transcription (Jovanovic *et al.*, 1997). Hence, if PspF binding to the UASs is impaired, it should be possible to monitor this through increased PspF protein production. Western blot analysis using crude cell extracts shows that in a *himA himD* mutant strain (IHF null mutant) (Fig. 5, lane 3), PspF production is more than fivefold higher than in an IHF^+ strain (Fig. 5, lane 1). This indicates that IHF binding (and presumably DNA bending) and PspF DNA-binding are co-operative *in vivo*. Surprisingly, the production of PspF is increased only slightly in a *himD* mutant strain (Fig. 5, lane 4), presumably because the HimA subunit(s) bind and bend DNA to some extent (as shown in Fig. 1B). A strain that lacks σ^{54} (*rpoN* mutant) showed only slightly decreased PspF production (Fig. 5, lane 5), suggesting that $E\sigma^{54}$ holoenzyme contributes little, if at all, to PspF binding *in vivo*.

Discussion

Phage-shock protein σ^{54}-dependent transcription involves activation at a distance. The role of the *psp* enhancer is to increase the local concentration of the PspF activator in the vicinity of the promoter and to tether it in the right position to facilitate productive interaction with $E\sigma^{54}$ holoenzyme. IHF assists this interaction by bending the DNA at a specific site located between the UASs and the *psp* promoter. Thus, IHF plays an architectural role.

IHF facilitates binding of PspF to the *psp* enhancer on a linear template *in vitro* and it enhances PspF autogenous control, postulated to be a consequence of PspF binding to the enhancer, *in vivo* (Jovanovic *et al.*, 1997). Perhaps IHF, upon binding (and bending) DNA, favourably presents the UAS(s) (*psp* enhancer) to the DNA-binding HTH motif of the activator. IHF does not influence the binding of the activator and $E\sigma^{54}$ holoenzyme in the *glnHp2* and *nifH* promoters (Claverie-Martin and Magasanik, 1991; Santero *et al.*, 1992).

The IHF subunit HimA alone can bind DNA, but it retards DNA containing the *psp* promoter-regulatory region less than wild-type IHF, suggesting that it is less effective in bending DNA. *In vivo*, the *himD* mutant is ineffective in supporting activation of *pspA-E* transcription (Jovanovic
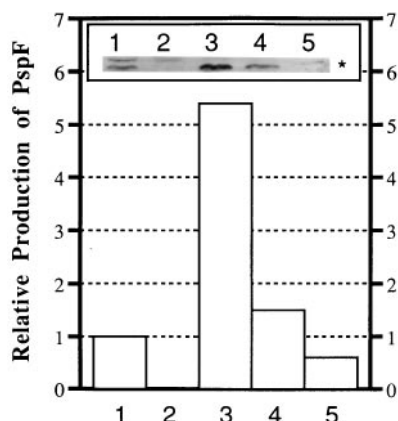
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<-pspF        RBS                    A
.CATGATGAAATTCGCCACTTGTTAGTGTAATTCGCTAACTCATCCTGGCATGTTGCTGTTGATTCTTCAATCA
    *-126                                              *-80              *-60

        _____        _____
        ___UAS II___        ___UAS I_____
        region protected by PspF - psp Enhancer


            IHF
GATCTTTATAAATCAAAAAGATAN5TTGGCACGCAAATTGTAN10AGCN6ATCCTGN16GGACAACATTATG..
                     *-25              *+1                      pspA->
```

IHF   − + + − + + − + +
6xHisPspF   − − + + − + − − +
6xHisPspFΔHTH − − − − + − − − −
cold 260   − − − − − + − − −

complex 1

free DNA:260
complex 1'

free DNA:160

1 2 3 4 5 6 7 8 9

Fig. 3. PspF binding to DNA enhances IHF binding.
Top. DNA sequence of the *pspF−pspA-E* promoter-regulatory region. *pspA* start of transcription (+1), major *pspF* start of transcription (A), *pspF* ribosome-binding site (RBS; underlined), IHF-binding site (−25 to −60) and *psp* enhancer (−80 to −126) are indicated.
Bottom. Gel mobility shift assay prepared using γ-ATP-radiolabelled 260 and 160 templates (2 ng), and 6×HisPspF (2.5 nM), 6×HisPspFΔHTH (150 nM) and IHF (5 nM) proteins in different combinations. Lanes: 1, free DNA (260); 2, IHF (complex 1); 3, 6×HisPspF and IHF (complex 1); 4, 6×HisPspF (complex 1); 5, 6×HisPspFΔHTH and IHF together; 6, 6×HisPspF and IHF together, preincubated with specific competitor DNA (cold 260) in excess of 100 times; 7, free DNA (160); 8, IHF (complex 1'); 9, 6×HisPspF and IHF together (complex 1').

et al., 1996) but is almost as efficient as wild-type IHF in the autoregulation of *pspF* expression. These results indicate that strong DNA bending is required for proper initiation of *psp* transcription but not for facilitation of PspF binding to the UAS(s). Zulianello *et al.* (1994) reported that the separate subunits of IHF, HimA (IHF α) and HimD (IHF β), specifically bind to *ihf* sites as homodimers and bend DNA to the same extent as wild-type IHF. In the *psp* system, this appears not to be the case. Although the gel shifts suggest that the degree of bending obtained with HimA alone is less than with the IHF heterodimer, it is also possible that the specific geometry necessary for interaction between PspF and the Eσ54 holoenzyme is not provided by DNA binding of the HimA subunit.

PspF bound to its UASs facilitates binding of IHF *in vitro*. *In vivo* and *in vitro* IHF binding to DNA deforms the helical axis, and IHF binds preferentially to a prebent DNA target site (Yang and Nash, 1989; Kahn and Crothers, 1992; Engelhorn *et al.*, 1995; Rice *et al.*, 1996). This may be the basis for the preponderance of A-T basepairs within typical IHF-binding sites (Goodrich *et al.*, 1990; Nunes-Duby *et al.*, 1995). Moreover, it has been shown that the DNA sequence surrounding an IHF-binding site influences its affinity for IHF. For example, the presence of an adjacent dAdT-rich element increases the flexibility of the DNA and enhances IHF binding (Hales *et al.*, 1994). Transcriptional activators bound to DNA induce bending (e.g. 69–90°, catabolite gene activator protein (CAP); Lutter *et al.*, 1996). In the case described here, it is likely that PspF binding to the UAS(s) (and presumably bending

the DNA) overcomes the restriction imposed by the DNA conformation of the sequence surrounding the IHF-binding site in the *psp* promoter-regulatory region (see Fig. 3, top) and consequently facilitates binding of IHF. Deletion of the UASs by themselves does not appreciably improve binding of IHF. However, removal of the entire DNA sequence upstream of the IHF-binding site (including the UAS sites),

ΔUAS I   ΔUAS I/II

complex →

free DNA →

1 2 3 4 5 6 7 8 9 10 11

Fig. 4. PspF binding to the UAS(s) is required for the facilitation of IHF binding in the *pspF−pspA-E* promoter-regulatory region *in vitro*. Gel mobility shift assay prepared using γ-ATP-radiolabelled ΔUAS I (lanes 1–6) and ΔUAS I/II (lanes 7–11) templates (2 ng), and 6×HisPspF and IHF proteins at varying concentrations. [Linear DNA fragments ΔUAS I (260 fragment from which the UAS I was deleted) and ΔUAS I/II (260 fragment from which the UAS I and II were deleted) were generated by PCR amplification as described in *Experimental procedures*.] Lanes: 1, free DNA (ΔUAS I); 2, 6×HisPspF (2.5 nM); 3, 6×HisPspF (2.5 nM) in the presence of 100-fold excess of ΔUASI/II competitor DNA; 4, IHF (5 nM); 5, IHF (50 nM); 6, 6×HisPspF (2.5 nM) and IHF (5 nM) together; 7, free DNA (ΔUAS I/II); 8, 6×HisPspF (25 nM); 9, IHF (5 nM); 10, IHF (50 nM); 11, 6×HisPspF (25 nM) and IHF (5 nM). (Gel was overexposed to show bands in lanes 9 and 11.)

Fig. 5. IHF downregulates PspF production *in vivo*. Graphical representation of Western blot analysis (window) of the PspF protein production (asterisk) using anti-PspF polyclonal antibodies and crude cell extracts. Lanes: 1, K561 (*pspF*$^+$); 2, K1247 (Δ*pspF*); 3, K1173 (*pspF*$^+$ *himA*Δ82::Tn*10 himD*Δ3::*cam*; IHF null mutant); 4, L108 (*pspF*$^+$ *himD*Δ3::*cam;* encodes HimA subunit); 5, L57 (*pspF*$^+$ *rpoN*::Tn*10*; σ^{54} null mutant). Lanes in window correspond to lanes on graph. PspF bands were quantified by microdensitometry and normalized to a background in control lane 2, containing the strain K1247 with *pspF* gene deleted. The relative amounts of PspF protein (in arbitrary units) were normalized to a background protein band (because of the cross-reactivity of the PspF antibodies) and the relative values for lanes 1–5 are 1, 0, 5.4, 1.5 and 0.6. (The production of PspF was measured in three independent experiments, and the average relative values for lanes 1–5 are 1, 0, 6 ± 1.2, 1.2 ± 0.5 and 0.4 ± 0.3.)

which leaves it at the end of the template (160 DNA fragment), significantly increases the effectiveness of IHF binding. The literature does not suggest that an IHF-binding site placed at the end of a DNA fragment has a higher affinity for IHF (the 'end effect'). Therefore, it is likely that the presence of the surrounding sequences restricts IHF binding and that removing them increases the affinity of the binding site for IHF. This is consistent with the proposition that PspF binding to the UASs and consequent DNA bending of the region adjacent to the IHF-binding site might form a DNA structure favourable for IHF binding. The regulatory elements in the *psp* promoter region are tightly clustered, and the binding sites for IHF, PspF and Eσ^{54} are on the same face of DNA. Hence, if IHF and PspF DNA bending is congruent, each could use the bending energy resulting from the other's binding. Although this mutual interaction might be unique to the *psp* promoter region, it is likely to occur in other σ^{54} systems with promoter-regulatory elements organized in a similar fashion.

In the absence of IHF, PspF and NR$_I$ cross-activate the *glnA* and *psp* σ^{54} transcription from supercoiled templates *in vitro*, and *psp* activation by NR$_I$ is repressed by IHF (J. Dworkin *et al.*, unpublished). Hence, specific and effective binding of IHF to its target site in the *psp* promoter-regulatory region, facilitated by PspF bound to the UAS(s), may play a direct role in preventing inappropriate activation of

transcription, as has been shown for the xylene-activated Pu promoter of *Pseudomonas putida* (Perez-Martin and Lorenzo, 1995) and the *glnHp2* promoter lacking binding sites for NR$_I$ (Claverie-Martin and Magasanik, 1991). Supercoiling alone can permit the accidental encounter of Eσ^{54} holoenzyme and heterologous activator(s) bound to cryptic site(s) on DNA. Presumably, IHF fixes local DNA topology by restricting DNA flexibility and thus stabilizes the assembly of the *psp*-specific transcriptional machinery. This would increase target gene activation specificity by preventing the access of heterologous activator(s). This is in agreement with the proposal (Perez-Martin and Lorenzo, 1995) that IHF acts as an active 'restrictor' of inappropriate activation by cross-reactive heterologous σ^{54}-transcriptional activator(s).

PspF–IHF co-operativity may improve the specificity of *psp* σ^{54}-dependent transcription both directly, by increasing the *pspA-E* expression, and indirectly, by making autogenous repression of *pspF* expression more effective.

Experimental procedures

Bacterial strains and plasmids

E. coli strains K561 [HfrCλ$^+$ *relA1 spoT1* T$_2$^{r} (*ompF627 fadL701*) *lacI*^{q}] (Davis *et al.*, 1985), K1527 (K561 *pspF*::mTn*10-tet* (*pspF*$_{877}$) (Tc^r)) (Jovanovic *et al.*, 1996), K1247 (PK2212) (Δ*kdpABC-5 thi-1* Δ*trkE* tet^s *rha4 lacZ48 gal-33 malA35 zci-233*::Tn*10* Δ*aldH-ordL-goaG-pspF-pspA-E* λ^r λ$^-$) (Jovanovic and Model, 1997), K1173 (W3110 [K-12λ$^-$ IN (*rrnD–rrnE*)]str^R *himA*Δ82::Tn*10 himD*Δ3::*cam* (Cm^r)) (Greenstein *et al.*, 1988), L57 (K561 *rpoN*::Tn*10* (Tc^r)) (Weiner *et al.*, 1991) and L108 (K561 *himD*Δ3::*cam* (Cm^r)) (L. Weiner, unpublished results) have been described previously. *E. coli* strains were grown at 37°C in rich Luria–Bertani (LB) medium. When appropriate, antibiotics were used at the following concentrations: ampicillin (100 µg ml^{-1}), chloramphenicol (25 µg ml^{-1}), tetracycline (15 µg ml^{-1}).

Plasmid pBRPS-1 (Weiner and Model, 1994) carries the complete *pspF* and *pspA-E* coding sequences and the entire *psp* promoter-regulatory region. pJD11 and pJD12 were described previously (Dworkin *et al.*, 1997) and carry the *pspF–pspA-E* promoter-regulatory region with UAS I (sequence −107 to −89) or UAS I and II (sequence −126 to −89) deleted respectively. Transformation of *E. coli* cells was performed as described previously (Sambrook *et al.*, 1989).

DNA manipulations

For PCR amplification (Pfu polymerase; Stratagene), preparation of plasmid DNA, digestion with restriction enzymes (New England Biolabs), electroelution and 5' end-labelling of DNA fragments, ligation (T4 DNA ligase; BRL) and other recombinant DNA techniques, previously described procedures were used (Ausubel, 1989; Sambrook *et al.*, 1989). The synthetic oligonucleotides, PE-F, JABR6 and dAsite (Operon Technologies), used for PCR amplification have been described previously (Jovanovic *et al.*, 1996).

DNA fragments used in gel mobility shift assays were constructed by using PCR amplification and the following templates and pairs of primers: fragments 260 (260 bp; carries the entire *pspF–pspA-E* promoter-regulatory region containing the *psp cis*-acting sequence) and 160 (159 bp; carries the *pspF–pspA-E* promoter-regulatory region with the *psp cis*-acting sequence deleted) were generated using pBRPS-1 as a template and PE-F and JABR6, and dAsite and JABR6 oligonucleotides respectively; fragments ΔUAS I and ΔUAS I/II were amplified using PE-F and JABR6 primers and templates pJD11 and pJD12 respectively.

Gel mobility shift assays

The 260, 160, ΔUAS I and ΔUAS I/II PCR-generated fragments were end-labelled with [γ-^{32}P]-ATP (3000 Ci mmol^{-1}; New England Nuclear) using T4 polynucleotide kinase (New England Biolabs). Crude cell extracts from 5-ml night cultures were resuspended in 800 μl of buffer containing 10 mM Tris-HCl, pH 8, and 20% glycerol. The binding reaction was done in 10 mM Tris-HCl, pH 7.5, 70 mM KCl, 5 mM MgCl$_2$, 1 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% (v/v) Triton X-100 and 12.5% (v/v) glycerol. Cellular proteins and a 1000× weight excess of poly(dI-dC) (Pharmacia) were mixed at room temperature and incubated for 30 min. Labelled DNA fragment (260) was then added and the incubation was continued for 20 min.

The gel mobility shift assay using purified proteins was carried out as described previously (Prentki *et al.*, 1987). His-tagged PspF and PspFΔHTH were purified by affinity chromatography on a Ni-NTA resin column (G. Jovanovic and P. Model, unpublished). Briefly, 2 ng of each fragment (260, 160, ΔUAS I and ΔUAS I/II) were incubated at 25°C for 20 min with different combinations and at the desired concentrations of 6×HisPspF, 6×HisPspFΔHTH and IHF (kindly provided by H. A. Nash, NIMH, Bethesda, MD, USA) in 50 mM Tris-HCl (pH 7.4), 70 mM KCl, 10% glycerol, 1 mM EDTA, 1 mM β-mercaptoethanol, 7 mM MgCl$_2$ and 200 μg ml^{-1} bovine serum albumin (BSA) (20 μl final volume). All reaction mixtures contained 3 mM ATP.

Two microlitres of 50% glycerol and 0.1% xylene cyanol solution was added to the binding reactions at 20 min, and the mixture was loaded on a 5% non-denaturing acrylamide gel. Electrophoresis was carried out at 4°C in 0.5× TBE buffer (Sambrook *et al.*, 1989) at 14 V cm^{-1} for 2 h. The gels had been prerun for several hours to stabilize the conductivity. Following electrophoresis, the gels were dried, autoradiographed on radiographic film or exposed to a phosphorimager screen (PhosphorImager, Molecular Dynamics).

Western blotting

Western blot analysis was performed either on the gel used for the gel retardation experiment using a 260 DNA fragment and purified His-tagged PspF and native IHF proteins, or on a sodium dodecyl sulphate (SDS) protein gel containing the crude cell extracts. Following electrophoresis, the non-denaturing acrylamide gel containing the DNA (260) and purified 6×HisPspF and IHF were washed in transfer buffer three times for 10 min.

Cell extracts were diluted in SDS sample buffer (60 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.01% bromophenol blue) and subjected to electrophoresis through a 12.5% (w/v) polyacrylamide gel containing SDS. Proteins from either the non-denaturing or the SDS protein gel were transferred to polyvinylidene difluoride (PVDF) membranes (Schleicher and Schuell Westran) using a Bio-Rad Trans-Blot apparatus at 200 V for 50 min in 1× blotting buffer (15 mM Tris-HCl, pH 10.5, 120 mM glycine, 20% methanol). Membranes were blocked for 2 h with 5% (w/v) dried milk in TBS-Tween 20 buffer (10 mM Tris-HCl, pH 8.0, 300 mM NaCl, 0.05% Tween 20), washed three times for 10 min with 20 ml of TBS-Tween 20, and then incubated with a 1:2000 dilution of anti-PspF polyclonal serum in 20 ml of TBS-Tween 20 for 1 h. The membranes were washed three times for 10 min with 20 ml of TBS-Tween 20, incubated with goat anti-rabbit secondary antibody–horseradish peroxidase conjugate (IgG–HRP reagent from Amersham) and washed as above. Western blot signals were visualized by enhanced chemiluminescence (ECL) detection reagent (Amersham), used as specified by the manufacturer and quantified on a double-beam recording Microdensitometer MK III C (Joyce, Loebl).

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