

# Analysis of the proteins and *cis*-acting elements regulating the stress-induced phage shock protein operon

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## ABSTRACT

The phage shock protein operon (*pspABCE*) of *Escherichia coli* is strongly induced by adverse environmental conditions. Expression is controlled principally at the transcriptional level, and transcription is directed by the sigma factor  $\sigma^{54}$ . PspB and PspC are required for high-level *psp* expression during osmotic shock, ethanol treatment and f1 infection, but heat-induced expression is independent of these proteins. We report here that the promoter region contains an upstream activation sequence (UAS) that is required for *psp* induction and has the enhancer-like ability to activate at a distance. A DNA-binding activity is detected in crude protein extracts that is dependent on the UAS and induced by heat shock. We further show that integration host factor (IHF) binds *in vitro* to a site between the UAS and  $\sigma^{54}$  recognition sequence. In bacteria lacking IHF, *psp* expression is substantially reduced in response to high temperature and ethanol. During osmotic shock in contrast, *psp* expression is only weakly stimulated by IHF, and IHF mutants can strongly induce the operon. The dependence of *psp* expression on IHF varies with the inducing condition, but does not correlate with dependence on PspB and PspC, indicating distinct, agent-specific activation mechanisms.

## INTRODUCTION

In both prokaryotes and eukaryotes, exposure to a diverse set of toxic agents results in the vigorous induction of a set of proteins known collectively as heat shock proteins [Hsps; for reviews see (1–3)]. A conserved feature of this response is the increased transcription of heat shock genes, and in *Escherichia coli*, heat shock gene expression is regulated by at least three minor sigma factors (the subunits of RNA polymerase conferring promoter specificity)— $\sigma^{32}$ ,  $\sigma^E$  ( $\sigma^{24}$ ) and  $\sigma^{54}$ .  $\sigma^{32}$  controls the major heat shock regulon, which consists of ~17 genes (4–6), and RNA

polymerase (E) containing  $\sigma^E$  transcribes at least two heat shock genes, including the gene for  $\sigma^{32}$  (7–9).

$\sigma^{54}$ , which controls the transcription of a diverse array of bacterial genes (10), participates in the heat shock response by directing the expression of the phage shock protein (*psp*) operon (11). The *psp* operon is strongly induced in response to a variety of stressful conditions or agents, including high temperature, ethanol, osmotic shock, stationary phase incubation, the gene IV protein of filamentous phages, homologs of the gene IV protein from other gram-negative bacterial species and the inhibition of protein secretion or lipid synthesis (12–17). The role of the operon in *E. coli* physiology is not clear, but *psp* mutant bacteria exhibit a reduction in the efficiency of translocation (14) and decreased viability in stationary phase at alkaline pH (16). The operon consists of at least four genes, designated *pspABCE*, and is regulated primarily at the transcriptional level (12,18). The PspB and PspC proteins cooperatively activate *psp* expression, while PspA negatively regulates the operon (11). The  $\sigma^{32}$ -dependent heat shock proteins also play a role in repressing the *psp* genes following induction by heat shock (12,18). The level of Psp production is more directly determined by the level of PspC, and PspB enhances, but is not essential for, this PspC-dependent gene expression (11). Thus PspB and PspC, though not homologous to other bacterial proteins, may be functionally analogous to the two-component regulatory pathways utilized by other bacterial systems, in which a kinase modulates the activity of a regulatory protein (19,20). The inactivation of PspA results in constitutive *psp* expression that is not dependent on PspB and PspC, suggesting that the roles of PspB and PspC may be to counteract the negative feedback mechanism controlled by PspA (11).

$\sigma^{54}$  is distinct from other bacterial sigma factors in both its sequence (21,22) and mechanism of action. All known  $\sigma^{54}$ -dependent genes require an activator protein for transcription to proceed (10,23).  $\sigma^{54}$  can bind stably to its recognition sequence (forming the closed complex) in the absence of activator proteins, but requires these proteins to melt in and form the open complex (24,25). Similar to eukaryotic enhancers, the sequences that bind the activator proteins continue to stimulate

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transcription when their distance and orientation are changed with respect to the promoter (26–30). The factors bound to the activation sequences contact  $\sigma^{54}$  at the promoter through the formation of DNA loops (31–33). Binding sites for integration host factor (IHF), a histone-like protein that bends DNA (34), are frequent features of  $\sigma^{54}$ -dependent promoters (23) and are usually located between the  $\sigma^{54}$  and activator recognition sequences. In the cases studied to date, IHF stimulates transcription only when the activator is present, and does not enhance the binding efficiency of either the activator or  $\sigma^{54}$  to the promoter (30,35,36). It was thus proposed that by bending DNA, IHF promotes DNA looping and contact between the activator and polymerase (30,35).

In this report, we analyze the *psp* promoter region and the factors that regulate the operon. We identify an element upstream from the  $\sigma^{54}$  promoter that is necessary for activation and binds a factor induced by heat shock. This element, similar to the activation sequences of other  $\sigma^{54}$ -dependent promoters, possesses the enhancer-like ability to activate at a distance. We also show that IHF binds downstream from the activator binding site, and that IHF is required for high level *psp* expression in response to some, but not all, inducing agents. Hence *psp* expression may be activated by both IHF-dependent and IHF-independent mechanisms.

## MATERIALS AND METHODS

### Bacterial strains and phages

K561 [HfrC  $\lambda^+$  *phoA lacI<sup>q</sup> tonA22 garB10 ompF627 relA1 pit-10 spoT1 fadL701 phoM510 mcrB rrmB2*] (37), L106 [K561  $\Delta$ *pspABC*  $\Delta$ *himD3::cat*], L24 [HfrH  $\Delta$ *pspABC relA1 spoT1*] (11), K361 [W3110 *str<sup>R</sup>*] (39), K1173 [K361  $\Delta$ *himA82::Tn10*,  $\Delta$ *himD3::cat*] (39), K1108 [K561 *dam-13::Tn9 lacI<sup>+</sup>*], HB101 F<sup>+</sup> [F<sup>+</sup>:*kan*] (38), and f1 phage are from our laboratory collection. Transductions were performed according to Miller (40).

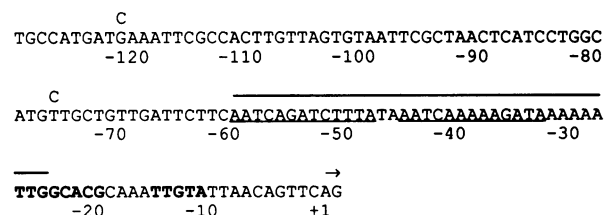
To assay the rate of Psp synthesis, bacteria were pulse-labelled with <sup>35</sup>S-methionine as previously described (11); immunoprecipitations were performed according to Davis *et al.* (37).

### Plasmid constructions

Restriction enzymes were purchased from New England Biolabs. Protruding 5' ends were filled in with the Klenow fragment of DNA polymerase I, and protruding 3' ends were removed with T4 DNA polymerase; both enzymes were from BRL. Bluescript (pBS) was from Stratagene. Plasmid DNA was purified according to Maniatis *et al.* (41).

pBRPS-1 (the complete *psp* operon on pBR322) was constructed by ligating a 4.5 kb *EcoRI* genomic fragment, isolated from a Charon 32 library and described in Brissette *et al.* (18), to *EcoRI*-restricted pBR322.

*BclI* and *SphI* sites were introduced into the *psp* promoter region by site-directed mutagenesis. The *BclI* site was created by a G to C transition at position -122 with respect to the start of transcription, and the *SphI* site was generated by a T to C transition at position -76 (Fig. 1). The annealing and synthesis reactions of the mutagenesis were performed as previously described (42) using single strands of p $\Delta$ 5'1 (18) as the template, and oligonucleotides JABR8 (5'-GCCATGATCAAATTCGCC) or JABR9 (5'-CCTGGCATGCTGCTGTTG). Following the synthesis reactions, the plasmid DNA was transformed into either



**Figure 1.** Sequence of the *psp* promoter region. The  $\sigma^{54}$  recognition sequence is in bold type. The 13 bp IHF consensus binding sites are underlined. The bar marks the region protected by IHF from DNase I digestion. Point mutations introduced by site-directed mutagenesis are indicated above the sequence. The transcription start site is marked by an arrow, and the sequence is numbered relative to this site.

K1108 or HB101 F<sup>+</sup>, and the transformant colonies were pooled. Plasmid DNA was prepared from the transformants and cut with either *BclI* or *SphI*. The linear DNA was gel purified, ligated, and transformed into the same strains. Plasmid DNA from individual transformant colonies was then screened for the presence of the *BclI* (pJLB14) or *SphI* (pJLB17) sites.

Deletions were made in the *psp* promoter by removing promoter fragments from pJLB14 or pJLB17, and using these fragments to replace the *psp* promoter on pPS-1 [the complete *psp* operon on Bluescript (18)]. Hence the 229 bp *BclI*-*BsmI* fragment of pJLB14, or the 180 bp *SphI*-*BsmI* fragment of pJLB17, were ligated to pPS-1 restricted with *BamHI* and *BsmI*. These ligations generated pJLB15 and pJLB18, which contain deletions of all promoter sequences upstream from positions -125 and -76 respectively. The promoter regions of these plasmids were sequenced to confirm that no additional mutations were present. To subclone these deletion constructs onto pBR322, the ~2.1 kb *SacI*-*KpnI* fragments of pJLB15 and pJLB18 were each ligated to *EcoRV*-restricted pBR322, creating pJLB16 (deletion ending at position -125) and pJLB19 (deletion ending at position -76). Both pJLB16 and pJLB19 contain all *psp* coding sequences.

pJLB11 contains a deletion of all *psp* promoter sequences upstream from position -55 (relative to the transcription start site), and was constructed by ligating closed pPS-1 digested with *BglII* and *BamHI*. To subclone this *psp* deletion construct onto pBR322 (creating pJLB13), the 2 kb *SacI*-*KpnI* fragment of pJLB11 was ligated to pBR322 restricted with *EcoRV*. Like pJLB16 and pJLB19, pJLB11 contains all *psp* coding sequences.

To move the activation sequence of the *psp* promoter farther upstream, a 1.1 kb *BglII*-*BamHI* fragment from the *kan<sup>R</sup>* cassette of pSKS101 (43) was ligated into the *BglII* site of pBRPS-1, creating pLW42. The activation sequence was deleted and replaced with the 1.1 kb *kan* fragment (pLW43) by ligating the *kan* fragment to pBRPS-1 restricted with *BglII* and *BamHI*. For both pLW42 and pLW43, the *kan* coding sequence is in the same orientation as the *psp* coding sequence; the *kan* promoter is not present on the 1.1 kb fragment.

### Gel retardation assays using crude protein extracts

Bacterial protein extracts were prepared from 25 ml cultures ( $5 \times 10^8$  cells/ml). The cells were centrifuged and resuspended in 0.5 ml of cold 10% sucrose, 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA and 5 mM DTT. Lysozyme was added to 300  $\mu$ g/ml, and the preparations were incubated on ice 10 min. The samples

were flash frozen in a dry ice-ethanol bath, thawed quickly at 37°C, and centrifuged at 20 000 r.p.m. in a type 40 rotor at 4°C for 60 min. The extracts were aliquoted, flash frozen, and stored at -70°C.

*psp* promoter DNA fragments were end-labelled with [ $\gamma$ -<sup>32</sup>P]ATP and T4 polynucleotide kinase as described (41). The DNA was separated from unincorporated label on a Sephadex G-50 spin column (41).

The gel retardation assays were performed essentially as described in Fried and Crothers (45). The <sup>32</sup>P-labelled promoter fragments (typically ~3 fmol and ~50 000 c.p.m.) were incubated at room temperature with the extract in 80 mM KCl, 20 mM Tris-HCl pH 8.0, 0.25 mM EDTA, 5 mM DTT, 0.2 mg/ml bovine serum albumin and 5% glycerol (total volume 20  $\mu$ l). Cold-specific or non-specific competitor DNA was added in 100-fold excess over the <sup>32</sup>P-labelled DNA; the non-specific competitor was pBS digested with *Hae*III. The reactions were loaded onto 5% polyacrylamide gels (4.94% acrylamide/0.06% bisacrylamide) and electrophoresed at room temperature at 6.5 V/cm. The gels and running buffer were 1  $\times$  TBE (0.09 M Tris, 0.09 M boric acid, 2.8 mM EDTA), and the running buffer also contained 1 mM DTT. The gels were pre-run for 1 h prior to loading.

#### Gel retardation assays with purified IHF

These assays were performed essentially as described by Tsui and Freundlich (46). Approximately 0.1 pmol of an end-labelled *Taq*I-*Bsm*I fragment (position -174 to +79) was incubated with IHF at 22°C for 30 min in the following buffer: 50 mM Tris-HCl (pH 6.5), 70 mM KCl, 1 mM EDTA, 7 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 5% (v/v) glycerol, 1 mM  $\beta$ -mercaptoethanol. Poly(dI,dC) (Promega Biotec, Madison, WI) at 9  $\mu$ g/ml was used to prevent non-specific binding of IHF to the DNA. The mixture was loaded onto a 5% polyacrylamide gel (29:1 acrylamide:bisacrylamide) prepared in buffer containing 6.7 mM Tris-HCl (pH 8.0), 3.3 mM sodium acetate and 1 mM EDTA. An IHF-specific 48 bp DNA fragment was used as a competitor. This fragment was constructed to give the maximum score for IHF binding as calculated in Goodrich *et al.* (47). Purified IHF was kindly supplied by Howard Nash.

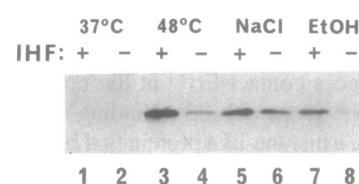
#### DNase I footprinting

These assays were performed essentially as described by Galas and Schmitz (48). Approximately 0.1 pmol of the end-labelled *Taq*I-*Bsm*I fragment was incubated in the absence or presence of increasing concentrations of IHF at 28°C in the same buffer used in the gel retardation assays. After 20 min, DNase I was added to 1.7 ng/ml, and the incubation was continued for 2 min at 22°C. The reaction was terminated with 150  $\mu$ l of stop solution (1.5 M ammonium acetate, 37.5 mM EDTA, 50  $\mu$ g/ml yeast tRNA). The reaction products were analyzed by electrophoresis on an 8% polyacrylamide gel (19:1 acrylamide:bisacrylamide) containing 8 M urea.

## RESULTS

### IHF enhances *psp* expression

The consensus binding sequence for IHF is 5'-A/TAT-CAAN<sub>4</sub>TTA/G (47,49), and two such consensus sites are present in the *psp* promoter region between positions -60 and -33 with



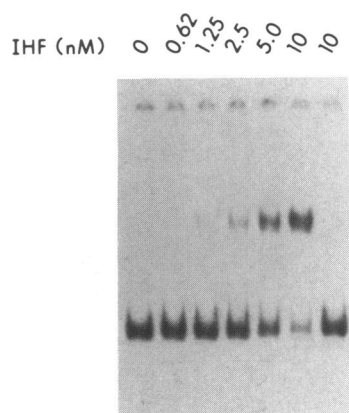
**Figure 2.** IHF stimulates *psp* expression. Strains K361 (*himAD*<sup>+</sup>; odd-numbered lanes) and K1173 (*himAD*<sup>-</sup>; even-numbered lanes) were grown at 37°C, exposed to various agents, and pulse-labelled with <sup>35</sup>S-methionine. <sup>35</sup>S-labelled proteins were immunoprecipitated with antiserum to PspA and analyzed by SDS-PAGE. Lanes 1 and 2, no treatment; lanes 3 and 4, 48°C, 5 min; lanes 5 and 6, 0.6 M NaCl, 20 min; lanes 7 and 8, 8% ethanol, 30 min.

respect to the transcription start site (Fig. 1). IHF is a heterodimer of the products of the *himA* and *himD* genes. *psp* expression in a *himAD* double mutant strain (K1173) was compared with its wild-type parent (K361) following several inducing treatments. The *himA* allele of K1173 contains both a deletion and a Tn10 insertion (50). The *himD* allele contains a deletion of amino acids 3-46 and an insertion of the chloramphenicol resistance gene (51). Bacteria were pulse-labelled with <sup>35</sup>S-methionine, and *psp* expression was assayed by the immunoprecipitation of PspA. As shown in Figure 2, the IHF mutations significantly reduced PspA synthesis during heat shock, and nearly eliminated expression in response to ethanol treatment. During osmotic shock, *psp* expression underwent only a limited reduction in the absence of IHF, and remained strongly inducible. Thus IHF is necessary for high level *psp* expression in response to certain agents.

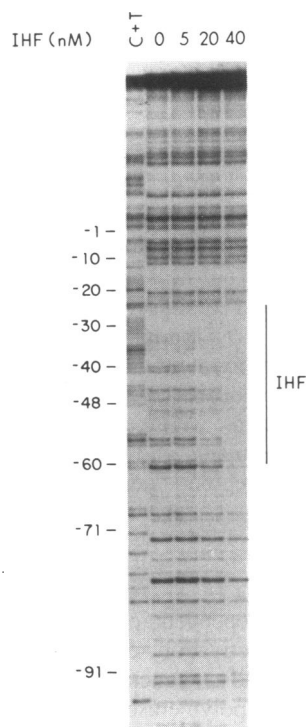
The ability of IHF to bind to the *psp* promoter was analyzed *in vitro* by gel retardation assays and DNase I footprinting. As shown in Figure 3, IHF exhibits a strong affinity for a *psp* promoter fragment. A single retarded DNA-protein complex was progressively formed as the concentration of IHF was increased from 0.62 to 10 nM, with 50% binding achieved at ~5 nM. In order to localize IHF binding within the promoter region, DNase I footprinting experiments were performed using the same DNA fragment. IHF protected a single site from approximately position -60 to -25 (Fig. 4); one IHF molecule normally protects ~35 bp from DNase I digestion (52). IHF binds to similar upstream positions in several other  $\sigma$ <sup>54</sup>-dependent promoters (31,53).

### The *psp* promoter contains an upstream activation sequence with enhancer-like properties

To identify sequence elements necessary for *psp* expression, the operon was placed on a multicopy plasmid, and deletions were introduced into the promoter region. The deletions removed all nucleotides upstream from positions -125 (pJLB16), -76 (pJLB19) or -55 (pJLB13) with respect to the start of transcription. To construct pJLB16 and pJLB19, new restriction sites were created in the promoter region by introducing point mutations (Fig. 1). The promoter deletion constructs, as well as control plasmids containing the site-directed point mutations and no deletions, were assayed for phage shock protein synthesis in response to heat shock and f1 infection. As shown in Figure 5, *psp* expression was not affected by the deletion of the region upstream from position -125 (lanes 3 and 8), but the deletion of the segment upstream from position -76 virtually abolished *psp* induction (lanes 4 and 9). The site-directed point mutations did not alter *psp*



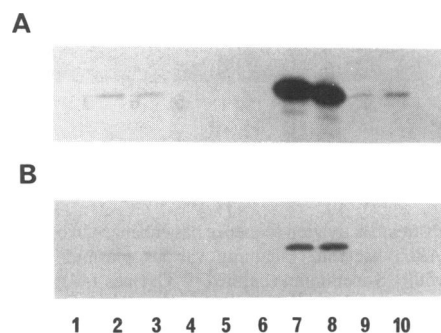
**Figure 3.** Gel retardation analysis of IHF binding. An end-labelled *psp* promoter fragment was incubated with increasing IHF concentrations as indicated, and DNA-protein complexes were separated on a polyacrylamide gel as described in the Materials and Methods. In the last lane, 2.5  $\mu$ M of IHF-specific competitor DNA was added.



**Figure 4.** DNase I footprinting of IHF bound to the *psp* promoter. The DNA fragment used in this experiment was the same as in Figure 3. The sequence is numbered as shown in Figure 1. The extent of protection by IHF is indicated by the line. Lane C+T shows the products of a C+T sequencing reaction.

inducibility (data not shown). Thus the region between positions  $-125$  and  $-76$  contains a sequence required for *psp* activation.

As stated in the Introduction, several upstream activation sites (UASs) of  $\sigma^{54}$ -dependent promoters were shown to have the properties of eukaryotic enhancers, in that they can stimulate transcription irrespective of their distance and orientation from the promoter (26–30). Many  $\sigma^{54}$  promoters contain IHF sites



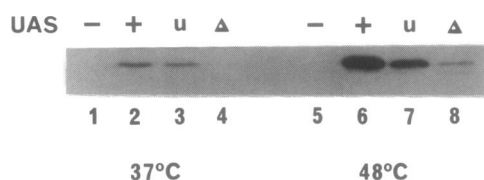
**Figure 5.** Deletion analysis of the *psp* promoter. Strain L24 containing various plasmid constructs was grown at 37°C (A and B, lanes 1–5), and either heat-shocked at 48°C for 5 min (A, lanes 6–10), or infected with f1 for 30 min (B, lanes 6–10). The bacteria were pulse-labelled for 1 min with  $^{35}$ S-methionine, and  $^{35}$ S-labelled proteins were immunoprecipitated with antiserum to PspA. The immunoprecipitations were analyzed by SDS-PAGE. Lanes 1 and 6, pBR322; lanes 2 and 7, pBRPS-1 (the complete *psp* operon); lanes 3 and 8, pJLB16 (promoter deletion ending at  $-125$ ); lanes 4 and 9, pJLB19 (promoter deletion ending at  $-76$ ); lanes 5 and 10, pJLB13 (promoter deletion ending at  $-55$ ).

between the activator and  $\sigma^{54}$  recognition sequences (23), and although IHF may normally stimulate transcription from these promoters (30,35), altering the spacing between the activator, IHF, and  $\sigma^{54}$  binding sites can result in IHF acting as an inhibitor of transcription (30,54). IHF-induced DNA bending is believed to facilitate contact between UAS-bound activator proteins and promoter-bound  $\sigma^{54}$  through the formation of DNA loops (30,35). It was proposed that when the spacing between the UAS and  $\sigma^{54}$  recognition sequence is altered, IHF may inhibit transcriptional activation by wrapping the DNA such that a loop of different size or orientation cannot form (30,54).

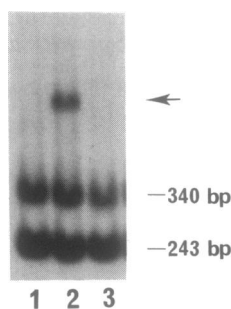
The upstream region of the *psp* promoter was tested for the enhancer-like ability to activate at a distance. Starting with the wild-type operon on a plasmid (pBRPS-1), a 1.1 kb segment of the kanamycin resistance gene was inserted into the *psp* promoter at position  $-55$  with respect to the start of transcription (pLW42). As a control, a plasmid was constructed containing both the *kan* insertion and a deletion of sequences upstream of position  $-55$  (pLW43). The constructs were assayed for PspA expression in a  $\Delta$ *himD*,  $\Delta$ *pspABC* strain (L106), and thus all PspA was produced from the plasmids. As shown in Figure 6, the upstream region of the *psp* promoter activated expression at a distance during heat shock (lane 7), although this level of PspA synthesis was not as high as that observed for the wild-type promoter (lane 6). The plasmid deleted for the upstream region was weakly heat-inducible (lane 8). Identical results were obtained for these plasmids following an osmotic shock (data not shown).

#### A DNA-binding activity dependent upon the *psp* UAS

We tested for the formation of protein complexes at the *psp* promoter using gel retardation assays (45,55). Crude protein extracts were made from bacteria containing the complete *psp* operon on a high copy plasmid (pPS-1) before and after extreme heat shock. For *psp* promoter DNA, a 583 bp *BsmI* fragment was gel purified and then re-cut with *HphI* to yield 340 and 243 bp fragments. The 243 bp *HphI*–*BsmI* fragment extends from positions  $-164$  to  $+79$  with respect to the transcription start site,



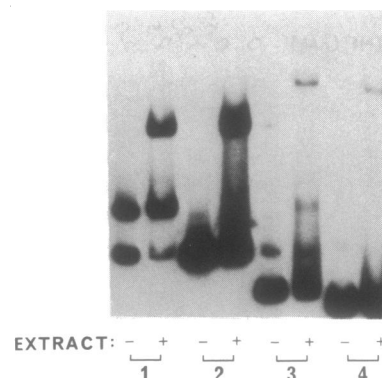
**Figure 6.** The upstream activation sequence has enhancer-like properties. L106 ( $\Delta$ *himD*,  $\Delta$ *pspABC*) bacteria containing various plasmid constructs were pulse-labelled with  $^{35}\text{S}$ -methionine before (37°C; lanes 1–4) and 5 min after a heat shock of 48°C (lanes 5–8).  $^{35}\text{S}$ -labelled proteins were immunoprecipitated using anti-PspA serum and electrophoresed. Lanes 1 and 5, pBR322 (indicated by a '-'); lanes 2 and 6, pBRPS-1 (wild-type *psp* operon; indicated by a '+'); lanes 3 and 7, pLW42 (activation sequence moved 1.1 kb upstream; indicated by a 'u'); lanes 4 and 8, pLW43 (deletion of the activation sequence and insertion of the 1.1 kb segment; indicated by a ' $\Delta$ ').



**Figure 7.** Gel electrophoretic analysis of protein bound to the *psp* promoter. HB101 F<sup>+</sup> bacteria containing either pBS or pPS-1 were grown at 37°C and heat-shocked for 5 min at 50°C. Protein extracts were prepared and incubated with  $^{32}\text{P}$ -labelled *psp* promoter DNA as described in Materials and Methods. The promoter DNA was two *BsmI*-*HphI* fragments of 340 (position -505 to -165) and 243 (position -164 to +79) bp. The reactions included either cold-specific (the *BsmI*-*HphI* *psp* promoter fragments) or non-specific (pBS digested with *HaeIII*) competitor DNA in 100-fold excess over the  $^{32}\text{P}$ -labelled DNA. Protein-DNA interactions were analyzed by polyacrylamide gel electrophoresis. All lanes show extracts from heat-shocked cells. Lane 1, pBS extract, non-specific competitor; lane 2, pPS-1 extract, non-specific competitor; lane 3, pPS-1 extract, specific competitor. Arrow marks complex with reduced mobility.

and includes the upstream activation region, IHF site,  $\sigma^{54}$  recognition sequence, and first 13 codons of *pspA*. As shown in Figure 7, the extract from heat-shocked cells carrying pPS-1 produced a mobility shift for one of the promoter fragments (lane 2). The shift occurred in the presence of a 100-fold excess of cold, non-specific competitor DNA, whereas the addition of cold, specific competitor eliminated the mobility shift (lane 3). The migration of *psp* DNA was not altered by extracts from bacteria containing only the Bluescript vector (lane 1). Extracts prepared from pPS-1-containing cells which were grown at 37°C and not heat-shocked shifted a smaller amount of the labelled DNA to the same position as the heat-shocked extracts (data not shown).

To determine which DNA fragment was present in the shifted complex, the shifted band was cut from the gel, and the DNA was electroeluted. The eluted DNA was then compared to the  $^{32}\text{P}$ -labelled 340 and 243 bp fragments on a polyacrylamide gel. The shifted complex was found to contain only the 243 bp fragment (data not shown). Thus in bacteria carrying the *psp* operon in high copy number, heat shock induces a DNA-binding activity that is specific for the *psp* promoter.



**Figure 8.** Analysis of protein-DNA complex formation using *psp* promoter deletions. Protein extracts were prepared from pPS-1-containing HB101 F<sup>+</sup> following a 5 min heat shock at 50°C, and incubated with various  $^{32}\text{P}$ -labelled *psp* promoter fragments. The reactions included a 100-fold excess of cold non-specific competitor DNA. Protein-DNA interactions were analyzed by polyacrylamide gel electrophoresis. Lanes are grouped by promoter fragment; the presence or absence of the bacterial protein extract is indicated. The endpoints of each DNA fragment are as follows: group 1, position -505 to -165, and -164 to +79 (the 340 and 243 bp fragments used in Fig. 7); group 2, position -121 to +79; group 3, position -76 to +79; group 4, position -55 to +79.

The gel retardation assays were performed on promoter fragments with or without the enhancer-like activation sequence (the 49 bp segment between positions -125 and -76). A fragment extending from position -121 to +79 (Fig. 8, group 2) formed the same slower-migrating complex as the -164 to +79 fragment described above (group 1). A fragment extending from position -76 to +79, which lacked the enhancer-like element, did not generate the same shifted complex (group 3). This DNA segment contained the IHF and E $\sigma^{54}$  binding sites, and at very low levels, formed a complex that migrated at a slower rate than the complex formed in the presence of the UAS. Thus the results of the gel retardation assays correlate with the *in vivo* expression studies of the promoter deletion mutants, and the principal complex formed with the *psp* promoter region is dependent on the enhancer-like regulatory sequence.

## DISCUSSION

Similar to other  $\sigma^{54}$ -dependent promoters, the *psp* promoter contains an upstream activation sequence that is necessary for strong *psp* expression and can function at large distances from the binding site for RNA polymerase holoenzyme. As shown in Figures 5 and 6, the operon remains very weakly inducible when the UAS is deleted. Weak expression in the absence of an activation sequence has been observed for other  $\sigma^{54}$ -dependent promoters and may result from non-specific or reduced specificity binding by an activator protein (24,26,35,56).

The UAS binds a factor (or factors) that is present in heat-shocked bacteria containing pPS-1, a high-copy plasmid that carries a 4.5 kb genomic fragment including the entire *psp* operon. The factor was not detected in gel retardation assays using crude extracts from heat-shocked bacteria carrying only the Bluescript vector (Fig. 7), and thus the presence of the insert in high copy number appears to increase the amount or activity of this factor. Even though PspA can repress *psp* expression, and PspB and PspC are positive regulators, the binding activity may not consist of any of these proteins, as  $\Delta$ *pspABC* bacteria exhibit

high level, constitutive expression of the *psp* promoter from a plasmid (11). Furthermore, we have produced the PspA, PspB and PspC proteins, both individually and in combination, from expression vectors using *lac* or T7 promoters, but extracts containing these proteins did not produce a mobility shift in gel retardation assays (57). We have recently isolated an activator protein that is required for *psp* transcription, and found that the gene encoding it is present on pPS-1 upstream from the operon (G. Jovanovic, L. W. and P. M., manuscript in preparation). It is likely that this protein represents the binding activity specific for the *psp* UAS.

IHF binds *in vitro* to a site (position -60 to -25) located between the UAS and  $\sigma^{54}$  recognition sequence. This region also contains a Dam site (position -55) that was shown by others to remain unmethylated in the chromosome (58). The absence of methylation at a Dam site is presumably due to protection by a protein or non-protein factor, and thus IHF appears to bind stably to this site *in vivo*.

IHF is required for strong *psp* expression during heat shock and ethanol treatment, but only weakly stimulates *psp* induction by osmotic shock. One possible explanation for this result is that hypertonic stress induces a structural change in the chromosome that makes *psp* activation less dependent on an IHF-generated DNA bend. Osmotic shock was shown to alter DNA topology and increase the negative supercoiling of plasmids *in vivo* (59). However, IHF can stimulate  $\sigma^{54}$ -dependent transcription from a linear template, implying that IHF facilitates transcription through a simple bend (36,54). An alternative possibility is that two different factors activate *psp* expression—one utilizing IHF to stimulate transcription, and a second capable of acting independently of IHF. We have isolated *E. coli* mutants that display a *psp* expression pattern similar to IHF mutants, but do not exhibit any other IHF phenotypes (unpublished data). Although these mutations are not yet mapped, they do not appear to lie in the IHF genes. The mutations may prevent *psp* activation via IHF, and these results lead us to favor the possibility of IHF-dependent and IHF-independent activating factors.

In previous studies, we found that high-level *psp* expression during osmotic shock, ethanol treatment and f1 infection requires PspB and PspC, but heat-induced expression occurs through a PspB- and PspC-independent mechanism (11). We report here that the dependence of *psp* activation on IHF also varies with the inducing agent, as *psp* expression in response to high temperature and ethanol is more strongly stimulated by IHF than expression during osmotic shock. There is no clear correlation between the conditions that activate via PspB and PspC, and the treatments that require IHF to induce high-level expression. The results suggest a significant complexity to *psp* regulation in which the activation mechanisms are at least partially agent-specific and distinct.

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