Unusual location of two nearby pairs of upstream activating sequences for HbpR, the main regulatory protein for the 2-hydroxybiphenyl degradation pathway of '*Pseudomonas azelaica*' HBP1

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'*Pseudomonas azelaica*' HBP1 degrades 2-hydroxybiphenyl (2-HBP) and 2,2'-diHBP by employing a meta-cleavage pathway encoded by the *hbpCAD* genes. The regulatory gene *hbpR*, located directly upstream of the *hbpCAD* genes and oriented in the opposite direction, encodes a transcription activator protein belonging to the so-called XylR/DmpR subclass within the NtrC family. HbpR activates transcription from two separate σ^{54} -dependent promoters upstream of the *hbpC* and the *hbpD* genes, in the presence of the pathway substrates 2-HBP and 2,2'-diHBP. The DNA region upstream of the *hbpC* gene displays an unusual organization, containing two adjacent 0.3 kb regions that share 71% sequence identity. The DNA region most proximal to the *hbpC* promoter harbours one pair of putative upstream activating sequences (UASs C-1/C-2) and a small cryptic ORF that shows homology to *hbpR* itself. The second, more distal, region contains a second pair of putative UASs (UASs C-3/4) and the 5'-part of the *hbpR* gene. Transcriptional fusions in *Escherichia coli* between different deletions of the *hbpR*–*hbpC* intergenic region and the genes for bacterial luciferase revealed that most if not all of the transcriptional output from the *hbpC* promoter is mediated from the proximal UASs C-1/C-2. However, when the UASs C-1/C-2 were deleted and UASs C-3/C-4 were placed in an appropriate position with respect to the promoter region, the *hbpC* promoter was still inducible with 2-HBP, albeit at a lower level. Transcription studies in *E. coli* and '*P. azelaica*' revealed that the divergently oriented *hbpR* gene is expressed constitutively from a σ^{70} -dependent promoter situated within the cryptic ORF. The presence of UAS pair C-3/C-4 mediated a slightly higher promoter activity for transcription of *hbpR*.

Keywords: NtrC-type transcriptional activator, regulation, evolution

INTRODUCTION

The *hbp* system allows '*Pseudomonas azelaica*' HBP1 to metabolize 2-hydroxybiphenyl (2-HBP) and 2,2'-diHBP by employing a meta-cleavage pathway (Kohler *et al.*, 1988, 1993; Schmid, 1997). The *hbp* system consists of the two transcriptional units, *hbpCA* and

hbpD, encoding the enzymes for the first steps of 2-HBP degradation, plus the regulatory gene *hbpR*, which encodes the transcriptional activator (Fig. 1). HbpR belongs to the XylR/DmpR subclass of the NtrC family (Jaspers *et al.*, 2000). Members of this family activate gene expression in concert with RNA polymerase core enzyme (RNAP) containing a σ^{54} subunit (RNAP- σ^{54}) (reviewed by Kustu *et al.*, 1991; Morett & Segovia, 1993). Activation of XylR/DmpR-type transcription activators occurs through direct interaction with aromatic effector compounds, without the need for a sensor kinase protein (reviewed by Shingler, 1996). To mediate transcription activation, they bind specifically to

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Abbreviations: 2-HBP, 2-hydroxybiphenyl; UAS, upstream activating sequence; RNAP, RNA polymerase; IHF, integration host factor.

Table 1. Plasmids used in this work

Plasmid	Relevant genotype or characteristics*	Source or reference
pCK218	Ap ^r Km ^r , R6K, RP4oriT (Mob), delivery vector for mini-Tn5 (<i>luxAB res-npt-res</i>)	Kristensen <i>et al.</i> (1995)
pHYBP100	Ap ^r , ColE1, pT7Blue(R) T-vector carrying 0.7 kb PCR fragment containing the <i>bbpR</i> – <i>bbpC</i> intergenic region	Jaspers <i>et al.</i> (2000)
pHYBP103	Ap ^r , ColE1, pJAMA8 carrying the 0.7 kb <i>SphI</i> – <i>XbaI</i> fragment of pHYBP100; contains <i>bbpC'</i> :: <i>luxAB</i> fusion	Jaspers <i>et al.</i> (2000)
pHYBP109	Ap ^r , ColE1, pJAMA8 carrying <i>bbpR</i> under the control of its native promoter (<i>P</i> _{<i>bbpR</i>})	Jaspers <i>et al.</i> (2000)
pHYBP113	Ap ^r , ColE1, pJAMA8 carrying the 0.7 kb <i>SphI</i> fragment of pHYBP100; contains <i>bbpR'</i> :: <i>luxAB</i> fusion	This work
pHYBP117	Ap ^r Km ^r , R6K, RP4oriT (Mob), PCK218 carrying the 3.1 kb <i>NotI</i> fragment of pHYBP113; delivery vector for mini-Tn5 (<i>bbpR'</i> :: <i>luxAB res-npt-res</i>)	This work
pHYBP124	Cm ^r , p15A, pACYC184 (Chang & Cohen, 1978) carrying <i>bbpR</i> under the control of its native promoter (<i>P</i> _{<i>bbpR</i>})	Jaspers <i>et al.</i> (2001)
pHYBP125	Cm ^r , p15A, pACYC184 (Chang & Cohen, 1978) carrying <i>bbpRA</i> under the control of its native promoter (<i>P</i> _{<i>bbpR</i>})	Jaspers <i>et al.</i> (2001)
pHYBP134	Ap ^r , ColE1, pHYBP103 with a deleted 0.15 kb (<i>SphI</i>)–(<i>Bam</i> HI) DNA fragment; contains <i>bbpC'</i> :: <i>luxAB</i> fusion lacking UAS C-3 and UAS C-4	This work
pHYBP135	Ap ^r , ColE1, pHYBP103 with a deleted 0.28 kb (<i>NcoI</i>)–(<i>Bam</i> HI) DNA fragment; contains <i>bbpC'</i> :: <i>luxAB</i> fusion lacking UAS C-1 and UAS C-2	This work
pHYBP136	Ap ^r , ColE1, pHYBP103 with a deleted 0.41 kb (<i>SphI</i>)–(<i>NcoI</i>) DNA fragment; contains <i>bbpC'</i> :: <i>luxAB</i> fusion lacking all UASs	This work
pHYBP137	Ap ^r , ColE1, pHYBP135 with a 4 bp insertion at the <i>NcoI</i> site; contains <i>bbpC'</i> :: <i>luxAB</i> fusion lacking UAS C-1 and UAS C-2	This work
pHYBP138	Ap ^r , ColE1, pHYBP137 with a 4 bp insertion at the <i>Bam</i> HI site; contains <i>bbpC'</i> :: <i>luxAB</i> fusion lacking UAS C-1 and UAS C-2	This work
pHYBP139	Ap ^r , ColE1, pHYBP113 with a deleted 0.15 kb (<i>SphI</i>)–(<i>Bam</i> HI) DNA fragment; contains <i>bbpR'</i> :: <i>luxAB</i> fusion lacking UAS C-3 and UAS C-4	This work
pHYBP140	Ap ^r , ColE1, pHYBP113 with a deleted 0.28 kb (<i>NcoI</i>)–(<i>Bam</i> HI) DNA fragment; contains <i>bbpR'</i> :: <i>luxAB</i> fusion lacking UAS C-1 and UAS C-2	This work
pHYBP141	Ap ^r , ColE1, pHYBP113 with a deleted 0.41 kb <i>SmaI</i> –(<i>NcoI</i>) DNA fragment; contains <i>bbpR'</i> :: <i>luxAB</i> fusion lacking all UASs	This work
pJAMA8	Ap ^r , ColE1; <i>luxAB</i> -based promoter-probe vector	Jaspers <i>et al.</i> (2000)

* UAS, upstream activating sequences within the *bbpR*–*C* intergenic region (see Fig. 2); *bbpRA*, ORF generated by the introduction of a frameshift mutation at the unique *SphI* restriction site at nucleotide position 520 in *bbpR* (Jaspers *et al.*, 2000). Restriction sites in parentheses were made blunt to allow cloning.

(nearly) palindromic DNA sequences – called bacterial enhancer-like elements or upstream activating sequences (UASs) (Morett & Segovia, 1993) – located around 100–200 bp upstream of their –12/–24 target promoters. By binding to its cognate UAS, the local concentration of the regulatory protein is increased near its target σ^{54} -dependent promoter, thereby increasing the efficiency of productive contacts between regulator and RNAP- σ^{54} (Wedel *et al.*, 1990). Furthermore, binding of the regulatory proteins to two adjacent UASs brings them into the right configuration for oligomerization, a process which is a prerequisite for activation of σ^{54} -dependent promoters (Pérez-Martín & de Lorenzo, 1996a; Porter *et al.*, 1993). Other proteins and sequence elements assist in optimizing transcription activation from XylR/DmpR-regulated promoters. For example, histone-like proteins, such as integration host factor (IHF) and/or HU are necessary for establishing the optimal geometry between UAS-bound regulatory protein and RNAP- σ^{54} (reviewed by Pérez-Martín *et al.*,

1994), and between the C-terminal domain of the α -subunit of the RNAP and a further upstream-located UP-like sequence element (Bertoni *et al.*, 1998a; Carmona *et al.*, 1999; Ross *et al.*, 1993).

Different studies with the XylR-controlled *Pu* and *Ps* promoters suggested that the magnitude of transcriptional activation from these promoters is dependent on the relative positions of the different protein-binding sites (i.e. for RNAP- σ^{54} , XylR and IHF) (Abril *et al.*, 1991; Abril & Ramos, 1993; Gomada *et al.*, 1992; Inouye *et al.*, 1990; Pérez-Martín & de Lorenzo, 1996b). Changing the relative positions of the –12/–24 promoter, of the IHF-binding site and of the UASs might disturb the optimal promoter geometry and lead to a decrease in transcriptional activation. Offsetting the UASs within the *Pu* promoter by the introduction of a half integral helix–turn led to a drop in promoter output, whereas the introduction of a full integral helix–turn had no significant effect (Pérez-Martín & de

Lorenzo, 1996a, b). Apparently, offsetting the two UASs by half a helix–turn hampers cooperative interactions between the UAS-bound regulators and complicates the subsequent oligomerization and open-complex formation. Offsetting both UASs with respect to the IHF site by the introduction of a half integral helix–turn also reduced expression from the *Pu* promoter dramatically (Abril *et al.*, 1991; Abril & Ramos, 1993), probably by locating XylR and RNAP- σ^{54} to opposite sites of the DNA helix. On the other hand, moving the pair of UASs of the *Pu* promoter between 0.2 and 1.2 kb away from the $-12/-24$ promoter did not have a large effect on promoter activity. Apparently, longer distances allow random bending of the intervening DNA regardless of IHF and still enable productive contacts between UAS-bound regulators and RNAP- σ^{54} (Inouye *et al.*, 1990).

Transcription activation of the *hbpCA* and *hbpD* genes by the HbpR protein is mediated from two different σ^{54} -dependent promoters, designated P_{hbpC} and P_{hbpD} (Jaspers *et al.*, 2001) (Fig. 1). Also for the HbpR system, IHF was needed as a co-regulator, in order to obtain full activation of the P_{hbpC} and P_{hbpD} promoters (Jaspers *et al.*, 2001). Solely on the basis of the DNA sequence, regions could be identified with homology to the UASs of the XylR/DmpR type and to IHF-binding sites. It is interesting to study the HbpR-mediated regulatory system, since HbpR is the only member of the XylR/DmpR subclass described so far which is activated by biaromatic structures, such as 2-HBP and 2,2'-diHBP, and not by the classical effectors xylene and *o*-cresol (Jaspers *et al.*, 2000). Furthermore, the organization of the *hbp* genes pointed to recent evolutionary changes, which allows us to study the processes leading to the optimization of regulatory systems (Jaspers *et al.*, 2001). Here we report on the unusual location of two pairs of UASs within the *hbpR*–*hbpC* intergenic region. We addressed the questions whether both pairs of UASs are functional and necessary for transcriptional activation of the *hbpC* promoter, and whether they have any importance for transcription of the *hbpR* gene itself. These questions were studied by analysing expression from promoter/operator deletions fused to the luciferase gene and by determining the transcription start site for the *hbpR* gene.

METHODS

Bacterial strains and plasmids. '*P. azelaica*' HBP1 is able to use 2-HBP and 2,2'-diHBP as a sole source of carbon and energy (Kohler *et al.*, 1988). '*P. azelaica*' HBP104 (Jaspers *et al.*, 2000) and HBP117 originate from strain HBP1 and contain *luxAB*-based transcriptional fusions with the upstream regions of *hbpC* and *hbpR*, respectively, integrated on the chromosome in monocopy. *Escherichia coli* DH5 α (Sambrook *et al.*, 1989) was used as host strain in routine cloning experiments. *E. coli* CC118 λ *pir* (Herrero *et al.*, 1990) expresses the *pir* gene (encoding the π protein) and was used to propagate plasmids with an R6K origin of replication. *E. coli* HB101(pRK2013) (Boyer & Roulland-Dussoix, 1969; Figurski & Helinski, 1979) was used as a helper strain to mobilize plasmids from *E. coli* CC118 λ *pir* in triparental matings. The plasmids used in this study are listed in Table 1.

Fig. 1. Summary of the HbpR-controlled *hbp* pathway in '*P. azelaica*' HBP1 for 2-HBP and 2,2'-DHBP degradation. Genetic organization and pathway intermediates are shown at the top. The sizes, orientation and location of the *hbp* genes are displayed as arrows. The two promoters regulated by HbpR in the presence of 2-HBP (plus signs) are indicated above the gene organization as small arrows. White boxes between *hbpR* and *hbpC*, and *hbpA* and *hbpD*, indicate predicted IHF-binding sites. The scissors mark a putative cleavage site in the *hbpCA* cotranscribed mRNA (Jaspers *et al.*, 2001). Hairpin-like structures indicate putative *rho*-independent transcriptional terminators. Plasmid constructs relevant for this study are shown below. All plasmids contain inserts derived from the *hbpR*–*C* intergenic region (enlarged) fused to the promoterless *luxAB* genes of plasmid pJAMA8 (Jaspers *et al.*, 2000). The presence of important features for HbpR-mediated regulation within the *hbpR*–*C* intergenic region is indicated by boxes, as follows: UAS C_4 through C_1 , upstream activating sequences; IHF, putative integration host factor binding site; GG/GC, $-24/-12$ promoter of *hbpC*. Arrows indicate the position of the transcription starts for *hbpC* and *hbpR*. Numbers refer to the sequence shown in Fig. 2. Only relevant restriction sites are shown, as follows: S, *Sph*I; B, *Bam*HI; Nc, *Nco*I; X, *Xba*I; Sm, *Sma*I; Ns, *Nsi*I; C, *Clal*. Restriction sites in parentheses disappeared during cloning. The designations 'B+4' and 'Nc+4' indicate 4 bp insertions. Dotted lines represent deleted regions. Plasmid names and configurations correspond to those shown in Fig. 3 and 4.

Media and growth conditions. *E. coli* strains were grown at 37 °C on Luria–Bertani (LB) medium (Sambrook *et al.*, 1989). '*P. azelaica*' strains were routinely grown at 30 °C on *Pseudomonas* mineral medium (MM) (Gerhardt *et al.*, 1981), containing 10 mM succinate or 2.9 mM 2-HBP. When required the media were supplemented with the following antibiotics at the indicated concentrations: ampicillin, 100 µg ml⁻¹ (*E. coli*); chloramphenicol, 25 µg ml⁻¹ (*E. coli*); kanamycin, 50 µg ml⁻¹ (*E. coli* and '*P. azelaica*'); rifampin, 50 µg ml⁻¹ (*E. coli*). '*P. azelaica*' strains containing a mini-Tn5 derivative were always grown in the presence of antibiotics selecting for these traits, unless indicated otherwise.

Recombinant DNA techniques, DNA sequencing and Southern analysis. Plasmid DNA isolations, ligations, transformations and other DNA manipulations were carried out according to well-established procedures (Sambrook *et al.*, 1989). Restriction endonucleases and other DNA-modifying enzymes were obtained from Amersham, Boehringer Mannheim and New England Biolabs, and used according to the specifications of the manufacturer. DNA fragments were isolated from agarose gels using QIAquick spin columns (QIAGEN). Double-stranded template sequencing was performed on plasmids using the dideoxy chain-termination method (Sanger *et al.*, 1977) with primers that were labelled with the fluorescent dye IRD-800 at the 5'-end, as described elsewhere (Ravatt *et al.*, 1998).

Construction of *luxAB* based promoter-probe plasmids. Plasmid pHYBP103 contains a 704 bp fragment with the *hbpR*–*hbpC* intergenic region (Jaspers *et al.*, 2000) transcriptionally fused to the *luxAB* genes (Fig. 1). To obtain a promoter-probe construct for the *hbpR* promoter (*P*_{*hbpR*}) the *hbpR*–*hbpC* intergenic region was retrieved from plasmid pHYBP100 (Jaspers *et al.*, 2000) as a 0.7 kb *SphI* fragment, making use of a second *SphI* site in the multi-cloning site, and cloned into pJAMA8 digested with *SphI*. The plasmid in which *luxAB* expression was driven from *P*_{*hbpR*} was designated pHYBP113.

Deletion derivatives of pHYBP103 (*hbpC'*::*luxAB*) were constructed to test the contribution of the different pairs of UASs on transcriptional activation from *P*_{*hbpC*}. Plasmids pHYBP134 and pHYBP135 were obtained by deletion of the 0.13 kb *SphI*–*BamHI* or the 0.28 kb *NcoI*–*BamHI* DNA fragments within the *hbpR*–*hbpC* intergenic region of plasmid pHYBP103, respectively (Fig. 1). In plasmid pHYBP136 the 0.41 kb *SphI*–*NcoI* fragment of pHYBP103 was deleted (Fig. 1). To vary the relative position of the distal pair of UASs (i.e. UASs C-3/C-4) on the DNA-helix with respect to the σ^{54} promoter and the IHF-binding site, small insertions (4 and 8 bp) were introduced into plasmid pHYBP135. To do this, the plasmid was cut with *NcoI*, protruding ends filled in with Klenow DNA polymerase and religated. This resulted in a 4 bp insertion (plasmid pHYBP137) and a new *NsiI* site (Fig. 1). Subsequently, an additional 4 bp were inserted by cutting pHYBP137 with *BamHI* (adjacent to the *NsiI* site), filling in with Klenow and religating (plasmid pHYBP138) (Fig. 1).

Similarly, deletion derivatives of plasmid pHYBP113, containing an *hbpR'*::*luxAB* fusion, were constructed by deleting the 0.13 kb *SphI*–*BamHI* fragment (plasmid pHYBP139) or the 0.28 kb *NcoI*–*BamHI* fragment (plasmid pHYBP140).

Plasmid pHYBP141 was derived by deleting the 0.41 kb *SmaI*–*NcoI* fragment from pHYBP113.

Testing *hbp*::*lux* promoter-probe constructs in *E. coli*. All the different *hbp*::*lux* promoter-probe plasmids were cotransformed in *E. coli* with a compatible plasmid expressing either the *hbpR* gene (plasmid pHYBP124) or a dysfunctional *hbpR* gene (*hbpRΔ*), which carries a frameshift mutation (plasmid pHYBP125) (Jaspers *et al.*, 2001).

Single chromosomal insertion of an *hbpR'*::*luxAB* fusion in '*P. azelaica*'. By using the unique *NotI* sites at the flanks, the *hbpR'*::*luxAB* fusion in plasmid pHYBP113 was recovered and exchanged with the 3.2 kb *NotI* fragment present in the Tn5 delivery vector PCK218 (Kristensen *et al.*, 1995) to produce plasmid pHYBP117 (Table 1). By using mini-Tn5 delivery, the *hbpR'*::*luxAB* promoter-probe fusion of plasmid pHYBP117 was inserted into the chromosome of '*P. azelaica*' HBP1, in a triparental mating procedure, as described previously (Jaspers *et al.*, 2000). Selection for '*P. azelaica*' exconjugants was done on MM plates with Km and 2.9 mM 2-HBP. Proper insertion of the constructs was verified by Southern hybridization of the '*P. azelaica*' exconjugants (data not shown). The resulting strain is referred to as HBP117.

Luciferase assays. Induction experiments with *luxAB*-harbouring *E. coli* and '*P. azelaica*' strains were performed in mineral medium at 30 °C as described elsewhere (Jaspers *et al.*, 2000). Expression of luciferase was analysed by measuring bioluminescence on whole cells at a final *n*-decanal concentration of 2 mM in a MicroLumat LB 96 P luminometer (Berthold) as described previously (Sticher *et al.*, 1997).

RNA isolation and primer extension analysis. Total RNA was isolated from a carbon-limited continuous culture of '*P. azelaica*' HBP1, 30 min after induction with 2-HBP, as described elsewhere (Jaspers *et al.*, 2001). Primer extension analysis was performed with primer PE_HbpR2 (5'-GATTTCATGGCGATGGTTCAGG-3'; 5 bp downstream of the *hbpR* ATG start codon; Fig. 2a), which was labelled with the fluorescent dye IRD-800 at the 5'-end as described previously (Jaspers *et al.*, 2001).

Synthetic oligonucleotides and chemicals. Primers labelled with the fluorescent dye IRD-800 at the 5'-end were purchased from MWG-BIOTECH, all other primers from Microsynth. Ultrapure agarose, APS, TEMED, Tris and urea were purchased from Gibco-BRL Life Technologies, and Rapid Gel-XL-40% acrylamide gel solution was obtained from Amersham. IPTG and X-Gal were obtained from Biosynth and *n*-decanal from Sigma. Nutrient broth, yeast extract and tryptic casein were purchased from Biolife and ultrapure agar from Merck. Antibiotics, inorganic salts, silicon antifoam and all other organic chemicals were obtained from Fluka Chemie.

Nucleotide sequence accession number. The nucleotide sequence of the *hbpR*–*C* intergenic region can be retrieved from the GenBank database under accession no. U73900.

RESULTS

The DNA region upstream of the *hbpC* promoter contains two pairs of putative UASs

HbpR mediates transcriptional activation of the *hbp* pathway in '*P. azelaica*' HBP1 from two independent

Relevant restriction sites are indicated above the DNA sequence. The positions and directions of some of the primers used in this study are indicated with numbered black arrows. Primers 1 and 3 were used to amplify the intergenic region of plasmid pHYBP103 and its derivatives. Primer 2 was used in primer extension. (b) Alignment between the homologous regions of *crpRh* and the start of *hbpR*.

Fig. 3. Promoter activity from the different *hbpC*::*luxAB* transcriptional fusions in *E. coli*, either with a functional *hbpR* (plasmid pHYBP124) or with a disrupted *hbpR* (pHYBP125) provided *in trans* (indicated below each panel as HbpR plus or minus). Induction was performed with 0.04 mM 2-HBP (indicated as 2-HBP 'plus' below each panel) or with DMSO only (as 2-HBP 'minus'). Plasmid names and the relevant configuration details are indicated on top of each panel (also see Figs 1 and 2). Luciferase activities were measured after 3 h. The error bars indicate the standard deviation of two independent experiments each carried out in triplicate. Induction factors, i.e. the ratio between the luciferase activities in the presence and absence of 2-HBP, are boxed. RLU, relative light units. Note the different scales of luciferase activity in panels (a-b) and (c-f).

σ^{54} -dependent promoters designated P_{hbpC} and P_{hbpD} (Jaspers *et al.*, 2001) (Fig. 1). DNA sequence analysis had indicated the presence of putative IHF-binding sites and one pair of UASs upstream of each HbpR-controlled promoter (Jaspers *et al.*, 2001). A closer examination of the DNA sequence upstream of the *hbpC* promoter, however, revealed the presence of two adjacent 0.3 kb regions that shared 71% sequence identity (Fig. 2b). Included in the homologous regions were the sequences for the UAS boxes. One pair of UASs (named C-1/C-2) was located proximal to the *hbpC* promoter (−227 to −180), whereas the other pair (UASs C-3/C-4) was at position −540 through −494 with respect to the transcription start site (Fig. 2a). The two pairs of UASs displayed 14 (for C-4 and C-2) and 15 (for C-3 and C-1) identical basepairs out of a total of 16, and shared between 56 and 75% identical basepairs with the consensus binding site for XylR/DmpR (Fig. 2a).

Additionally, the homologous region harboured a small ORF (tentatively named *crpRh*: see below), oriented in the same direction as *hbpR* (see below). This ORF had a length of 123 bp and could encode a peptide of 41 amino acids. This peptide had a significant similarity to the start of *hbpR* itself (39% in 42 residues). We therefore wondered what the function of the homologous region might be for *hbpC* and *hbpR* expression.

Functional analysis of the two pairs of UASs in mediating expression from the *hbpC* promoter

To establish the *in vivo* functionality of each pair of putative UASs in transcription activation from the *hbpC* promoter, we applied a heterologous *E. coli*-based reporter system that had previously been used to study expression from this promoter (Jaspers *et al.*, 2001). Plasmids were constructed that contained transcrip-

Fig. 4. Promoter activity from the different *hbpR*::*luxAB* transcriptional fusions in *E. coli*, either with a functional *hbpR* (plasmid pHYBP124) or with a disrupted *hbpR* (pHYBP125) provided *in trans* (indicated below each panel as HbpR plus or minus). Induction was performed with 0.04 mM 2-HBP (indicated as 2-HBP 'plus' below each panel) or with DMSO only (as 2-HBP 'minus'). Plasmid names and the relevant configuration details are indicated on top of each panel (see also Figs 1 and 2). Luciferase activities were measured after 3 h. The error bars indicate the standard deviation of two independent experiments each carried out in triplicate. RLU, relative light units. Note the different scales of luciferase activity in panels (a–b) and (c–d).

tional fusions between different portions of the DNA region upstream of the *hbpC* promoter and the *luxAB* genes of *Vibrio harveyi*. These plasmids were then cotransformed into *E. coli* with a plasmid expressing either a functional HbpR (pHYBP124) or a truncated HbpRA (pHYBP125). Plasmid pHYBP103 contained the complete *hbpR*–*C* intergenic region. In the presence of a functional HbpR and after induction with 2-HBP for 3 h *E. coli* strains carrying pHYBP103 gave rise to a 23-fold increase in bioluminescence as compared to uninduced conditions (with no 2-HBP added) (Fig. 3a). *E. coli* containing plasmid pHYBP134, in which the distal pair of UASs C-3/C-4 was removed, gave rise to comparable levels of both luciferase activity and induction ratio after induction with 2-HBP as the native configuration (Fig. 3b). In contrast, deletion of the proximal pair of UASs C-1/C-2 (*E. coli* with plasmid pHYBP135) resulted in a 23-fold decrease of luciferase activity. However, expression from the *hbpC* promoter was still significantly higher under induced conditions than uninduced (2.6-fold) (Fig. 3c). Deletion of both pairs of UASs (as in pHYBP136) abolished inducible transcription activation from the *hbpC* promoter completely (Fig. 3d). In all

cases, no inducible response of the *hbpC* promoter was seen in the absence of a functional HbpR. These results indicated clearly that only the proximal pair of UASs C-1/C-2 is needed to obtain full (and wild-type) expression levels from the *hbpC* promoter (Fig. 3a, b). However, the distal UASs (C-3/C-4) seemed to be suitable in principle for HbpR-mediated activation. Their relatively poor performance in plasmid pHYBP135 might have been caused by an unsuitable relative geometry or spacing between the UASs C-3/C-4 pair compared to the –12/–24 promoter.

To determine if the geometry could have an influence on the suitability of the distal UASs to act as sites for HbpR-mediated transcription activation, we constructed derivatives of plasmid pHYBP135 which contained four (pHYBP137) and eight (pHYBP138) additional base-pairs between the UASs and the other more downstream positioned promoter elements (IHF-binding site, –12/–24 motifs) (Fig. 1, 3e, f). *E. coli* carrying pHYBP137 with the 4 bp insertion gave a luciferase activity and induction ratio that were 1.5- and 2.3-fold higher, respectively, than those observed for pHYBP135 (Fig. 3e). Plasmid pHYBP138 with the 8 bp insertion resulted in a further increase of luciferase activity (2.8-fold) and induction ratio (3.7-fold) compared to pHYBP135 (Fig. 3f). These results demonstrated that the C-3/C-4 pair of UASs are indeed capable of mediating transcription activation from the *hbpC*-promoter upon induction with 2-HBP and in the presence of HbpR. The absolute luciferase response from the C-3/C-4 pair of UASs under these geometrical conditions, however, was still 9.3-fold lower than found for the UASs C-1/C-2 activated promoter (compare induced levels in Fig. 3b and 3f).

In vivo expression of the *hbpR* regulatory gene

We then determined which of the elements in the *hbpR*–*C* intergenic region were important for expression of HbpR itself. To study the expression of the *hbpR* gene we used plasmid pHYBP113, which contained a *luxAB*-based transcriptional fusion with the complete *hbpR*–*hbpC* intergenic region in the direction of *hbpR*. Induction studies were carried out in *E. coli* DH5 α in a similar way to before. In the absence of 2-HBP, the expression from the *hbpR* promoter was 4.3-fold higher than the basal expression observed previously from the *hbpC* promoter (Figs 3a and 4a). However, in the presence of 40 μ M 2-HBP, activation from the *hbpR* promoter did not increase, whereas that from the *hbpC* promoter increased 23-fold (Figs 3a and 4a). Based upon these results in *E. coli*, we concluded that the *hbpR* gene was transcribed constitutively. The expression studies were then repeated in '*P. azelaica*'. A '*P. azelaica*' strain was constructed that contained one copy of a chromosomally integrated *hbpR*::*luxAB* transcriptional fusion (strain HBP117). Similarly as in *E. coli*, luciferase expression levels obtained under uninduced conditions from the *hbpR* promoter in strain HBP117 were about fourfold higher than those from the *hbpC* promoter

Fig. 5. HbpR-mediated *in vivo* transcriptional activation from P_{hbpR} in '*P. azelaica*' (a) compared to that from P_{hbpC} (b) in the presence of different concentrations of 2-HBP. Luciferase activities were measured 2.25 h after induction with 2-HBP. Error bars indicate the standard deviation in two independent experiments each carried out in triplicate. Fragment length and orientation of the P_{hbpR} and P_{hbpC} promoters used for driving *luxAB* expression are indicated by black solid arrows at the top. RLU, relative light units. Note the different scales of luciferase activity in panels (a) and (b).

(Fig. 5). Transcription from the *hbpR* promoter was again independent of addition of 2-HBP, whereas the response from an *hbpC*::*luxAB* fusion in '*P. azelaica*' increased 40-fold over a range of 1 μ M to 0.4 mM 2-HBP (Fig. 5b). At higher 2-HBP concentrations, however, luciferase activity decreased similarly for both strains. This was probably not due to down-regulating of the *hbpR* and *hbpC* promoters *per se*, but more likely reflected the inhibitory effects of 2-HBP on the cellular metabolism and/or on the luciferase activity (Folkert, 1996; Oelze & Kamen, 1975).

The results obtained in '*P. azelaica*' confirmed that the *hbpR* gene was constitutively expressed. The *in vivo* transcriptional start site of the *hbpR* gene was then determined by primer extension analysis with RNA isolated from a chemostat culture of strain HBP1, 30 min after induction with 0.5 mM 2-HBP. By using primer PE_HbpR2 (Fig. 2a), different extension products could be seen (Fig. 6). The longest primer extension product (marked 'A*' in Fig. 6) corresponded to a transcriptional start site at a position 271 bp upstream of the ATG start codon of the *hbpR* gene (Fig. 2a). The smallest primer extension product was 9 nt shorter. This might indicate that more than one promoter sequence is recognized in this region.

To confirm whether this region was indeed important for *hbpR* transcription *in vivo*, plasmids were derived from pHYBP113 that had deletions within the *hbpR*–*hbpC* intergenic region (plasmids pHYBP139, 140, 141) (Fig. 1). Deletion of 0.28 kb DNA fragment between the

Fig. 6. Mapping of the *in vivo* transcriptional start sites of the *hbpR* gene by primer extension analysis on RNA isolated from a glucose-grown chemostat culture of '*P. azelaica*' HBP1, 30 min after induction with 0.5 mM 2-HBP. The primer extension products were run next to sequence reactions performed with the same primer. On the left, an expanded view of the DNA sequence surrounding the putative σ^{70} -dependent promoter for the longest transcript (marked A*) is shown. Also shown is an alignment with the consensus sequence for σ^{70} -dependent promoters (Harley & Reynolds, 1987).

*Bam*HI and *Nco*I sites (Fig. 2), containing the promoter region, indeed reduced luciferase activity in *E. coli* with plasmid pHYBP140 22-fold compared to the wild-type promoter (Fig. 4c). Deletion of a 0.15 kb DNA fragment containing the pair of UASs C-3/C-4 (as in pHYBP139) also decreased luciferase activities in *E. coli*, but only twofold (Fig. 4b). Finally, deletion of a 0.43 kb DNA region completely encompassing both pairs of UASs as in plasmid pHYBP141 abolished almost all promoter activity, which corresponded to a 99-fold reduction in

luciferase activity compared to the wild-type promoter (Fig. 4d). In all cases when a truncated *hbpR* gene was provided *in trans* (on plasmid pHYBP125), luciferase activities in *E. coli* increased moderately (Fig. 4a–c).

DISCUSSION

Previously, we had determined that the HbpR protein, which is encoded by a 1710 bp ORF closely linked and oriented oppositely to the *hbpCAD* genes, is the main transcriptional activator of the *hbp* pathway (Jaspers *et al.*, 2000). HbpR is an NtrC-type monocomponent regulator and activates transcription from two separate σ^{54} -dependent promoters, P_{hbpC} and P_{hbpD} , in the presence of the pathway substrate 2-HBP (Fig. 1) (Jaspers *et al.*, 2001). Characteristic for promoters regulated by NtrC-type transcription activators are UASs, which form the actual binding sites of the regulatory protein to the DNA. We have now discovered that the *hbpR*–*hbpC* intergenic region actually contains two pairs of UASs. This is the result of a short 0.3 kb duplicated DNA region upstream of *hbpR*. Although the duplicated regions are only 71% identical, the sequences for the UASs are relatively well conserved (Fig. 2). The small ORF with homology to the 5' end of *hbpR* (tentatively named *crpRh*) which is still present in the region is probably a relict of a previous recombination event. Our data suggest that the *crpRh* ORF is not functionally transcribed, since the transcription start for *hbpR* was located within *crpRh*.

Induction experiments in *E. coli* with different deletions of the *hbpR*–*hbpC* intergenic region fused to *luxAB* showed that most if not all of the transcriptional output from the *hbpC* promoter is mediated from the proximal pair of UASs (i.e. UASs C-1/C-2). We concluded this from the observations that deleting UASs C-3/C-4 did not result in a decrease of luciferase activity or of induction ratio as compared to the native promoter (Fig. 3). However, our data also indicated that the distal pair of UASs (C-3/C-4) is principally functional for HbpR-mediated activation, since placing it at a similar position (–225 to –271) relative to the *hbpC* transcription start site as UASs C-1/C-2 (–180 to –227) resulted in transcription activation in the presence of 2-HBP. More recently, we also observed binding of an HbpR-fusion protein to DNA fragments containing either the proximal or distal pair of UASs, which indicated that these DNA sequences indeed act as HbpR-binding sites *in vitro* (D. Tropel & J. R. van der Meer, unpublished).

By aligning the UASs of the *hbp* system with those of other known XylR/DmpR-type regulators (Fig. 7), we can conclude that the distance between the centres of the binding motifs is not dramatically different among them, except for the UASs of the *hbpD* promoter. However, this larger distance between the UAS motifs in the *hbpD* promoter does not result in a much smaller promoter output compared to the *hbpC* promoter (Jaspers *et al.*, 2001). Interestingly, the UASs of the *hbp* system all have a larger positive twist between their centres (Fig. 7), but it is not clear whether this 'positive' turn is a specific

binding determinant for HbpR. Compared to the location of the UASs in the *Po*, *Pu*, *Ps* and *P_{toMO}* promoters, the UASs of the *hbpC* and *hbpD* operators are also further away from the –24/–12 sequences (Fig. 7). Based upon an alignment including the UAS-sequences in the *hbp* genes, we propose a refined consensus sequence for the distal and proximal region (Fig. 7b, c).

From our promoter deletion studies and primer extension mappings we concluded that the *hbpR* transcriptional start sites were situated within the *crpRh* ORF. The sequence upstream of the *hbpR* start site showed two hexamers (CTAACA and TGAAAA) that weakly resembled the conserved –35 (TTGACA) and –10 (TATAAT) hexamers normally found in σ^{70} -dependent promoters in *E. coli* (reviewed by Harley & Reynolds, 1987) (Fig. 6). However, the presence of multiple primer-extension products suggests that alternative promoter sequences in that region may be used, as for the *xylR* promoter (Marqués *et al.*, 1998). The location of the *hbpR* promoter in this region would imply that the HbpR-binding sites do not directly overlap with the RNA– σ^{70} polymerase-binding site. This is in contrast to the situation at the divergent *xylR* and *xylS* promoters (Inouye *et al.*, 1986, 1988; Marqués *et al.*, 1998). In this case the UASs needed for expression from the *Ps* promoter overlap with the tandemly oriented σ^{70} promoters for the *xylR* gene. Overlapping binding sites are thought to result in physical hindering of both proteins and (in the case of the *xylR* gene) in autorepression of *xylR* transcription (Bertoni *et al.*, 1997; Marqués *et al.*, 1998). Since the binding sites in the HbpR system do not overlap, no such strong autorepression of *hbpR* transcription can occur by this mechanism. We did observe a slight decrease of luciferase expression from the *hbpR* promoter in the presence of HbpR (Fig. 4a), but since this effect was also visible in promoters deleted for UASs C-3/C-4 (Fig. 4b), it seems unlikely that this is autoregulation caused by HbpR proteins occupying the UASs C-3/C-4 and hindering transcription elongation. Also in contrast to the XylR situation, where the presence of effectors increased the autorepression of *xylR* transcription (Bertoni *et al.*, 1998b; Marqués *et al.*, 1998), the *hbpR*':*luxAB* fusions essentially were not responsive to the presence of 2-HBP, both in *E. coli* (Fig. 4) and in '*P. azelaica*' (Fig. 5).

Is there any advantage in keeping the unusual organization of the *hbpR*–*hbpC* intergenic region? Keeping two pairs of UASs does not seem to increase transcription from the *hbpC* promoter since the distal pair of UASs can be deleted without affecting expression of the *hbpC* promoter. On the other hand, any recombinatorial deletion between the homologous regions would result in loss of the *hbpR* transcription start site and place the UASs at a position rather close to the start of *hbpR* (46 bp, whereas the distances between the distal UAS and the start of *dmpR* or *xylR* are 218 and 120 bp, respectively). Therefore, there seems to be a selective disadvantage for recombined structures in this region. We speculate that the unusual organization of pairs of

Fig. 7. (a) Comparison of the *hbpC* and *hbpD* operator regions with other σ^{54} -dependent promoters controlled by XylR/DmpR-type regulators. The putative UASs were identified based on homology to the consensus sequence for XylR/DmpR-type UASs (5'-TTGATCAATTGATCAA-3') (Pérez-Martín & de Lorenzo, 1996b). Angled arrows indicate the transcriptional start sites (+1) and the direction of transcription for the operons that are expressed from these promoters, with the first gene transcribed indicated in parentheses. Predicted relative orientations (assuming non-bent DNA) between the centre of the proximal UAS and the distal UAS are indicated in parentheses. The sequences of the different promoters are as follows (GenBank accession numbers in parentheses): *P_{hbpC}* and *P_{hbpD}* promoter from '*P. azelaica*' HBP1 (U73900) (Jaspers *et al.*, 2001); DmpR-controlled *P_o* promoter (M60276) (Nordlund *et al.*, 1990; Shingler *et al.*, 1993); XylR-controlled *P_s* promoter (M10143) (Inouye *et al.*, 1986, 1987) and *P_u* promoter (D63341) (Inouye *et al.*, 1984); TouR-controlled *P_{ToMO}* promoter (AJ005663) (Arengi *et al.*, 1999). (b, c) Alignments of distal (b) and proximal (c) UASs upstream from the σ^{54} -dependent promoters. The numbering corresponds to the relative position towards the transcriptional start site (+1) in the respective promoters. Conserved residues in all promoters are indicated against a black background, whereas residues conserved in at least five of seven UASs appear boxed and are indicated at the bottom.

UASs in the *hbpR*-C intergenic region was the result of a DNA duplication. The original *hbpR* configuration would have had the C-3/C-4 pair of UASs relatively close to the HbpR translation start, which resulted in poor expression, since no proper σ^{70} promoter sequence was available. A short duplication now placed the UASs further away (the present C-1/C-2 pair) and provided a promoter within the duplicated region. Interestingly, this scenario is not so far-fetched. A similar 5' duplication, although larger (0.9 kb) in size, was isolated in *E. coli* carrying a plasmid with the *phlR* system for phenol degradation of *P. putida* strain H (Burchhardt *et al.*, 1997). This spontaneous promoter-up mutation in *phlR* was isolated upon selection for better growth on phenol and led to an increase in the expression of *phlR* and the phenol pathway enzymes. The duplication had created a slightly different -35 box and -35/-10 spacing for the *phlR* promoter at the 3' end of the duplicated fragment. Maybe the regions upstream of *hbpR*, *phlR* and other homologous systems are prone to duplication, which might help to overcome limiting amounts of enzymes on new potential substrates that are inefficient effectors.

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