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 σ54-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/C-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 σ70-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 σ54 subunit (RNAP-σ54) (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.
(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^70-dependent promoter, thereby increasing the efficiency of productive contacts between regulator and RNAP-σ^70 (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^70-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^70 (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^70, 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, 1996b).

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### Table 1. Plasmids used in this work

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant genotype or characteristics*</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHYBP100</td>
<td>Ap^r, CoE1, p17Blue(R) T-vector carrying a 0.7 kb PCR fragment containing the bbpR–bbpC intergenic region</td>
<td>Jaspers et al. (2000)</td>
</tr>
<tr>
<td>pHYBP103</td>
<td>Ap^r, CoE1, pJAMA8 carrying the 0.7 kb Spbi–XbaI fragment of pHYBP100; contains bbpC::luxAB fusion</td>
<td>Jaspers et al. (2000)</td>
</tr>
<tr>
<td>pHYBP109</td>
<td>Ap^r, CoE1, pJAMA8 carrying bbpR under the control of its native promoter (P_{bbpR})</td>
<td>Jaspers et al. (2000)</td>
</tr>
<tr>
<td>pHYBP113</td>
<td>Ap^r, CoE1, pJAMA8 carrying the 0.7 kb Spbi fragment of pHYBP100; contains bbpR::luxAB fusion</td>
<td>This work</td>
</tr>
<tr>
<td>pHYBP117</td>
<td>Ap^r, Km^r, R6K, RP4oriT (Mob), PCK218 carrying the 3.1 kb NorI fragment of pHYBP113; delivery vector for mini-Tn5 (bbpR::luxAB res–npt–res)</td>
<td>This work</td>
</tr>
<tr>
<td>pHYBP124</td>
<td>Cm^r, p15A, pACYC184 (Chang &amp; Cohen, 1978) carrying bbpR under the control of its native promoter (P_{bbpR})</td>
<td>Jaspers et al. (2001)</td>
</tr>
<tr>
<td>pHYBP125</td>
<td>Cm^r, p15A, pACYC184 (Chang &amp; Cohen, 1978) carrying bbpRA under the control of its native promoter (P_{bbpR})</td>
<td>Jaspers et al. (2001)</td>
</tr>
<tr>
<td>pHYBP134</td>
<td>Ap^r, CoE1, pHYBP103 with a deleted 0.15 kb (Spbi–BamHI) DNA fragment; contains bbpC::luxAB fusion lacking UAS C-3 and UAS C-4</td>
<td>This work</td>
</tr>
<tr>
<td>pHYBP135</td>
<td>Ap^r, CoE1, pHYBP103 with a deleted 0.28 kb (NcoI–BamHI) DNA fragment; contains bbpC::luxAB fusion lacking UAS C-1 and UAS C-2</td>
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</tr>
<tr>
<td>pHYBP136</td>
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<tr>
<td>pHYBP137</td>
<td>Ap^r, CoE1, pHYBP135 with a 4 bp insertion at the NcoI site; contains bbpC::luxAB fusion lacking UAS C-1 and UAS C-2</td>
<td>This work</td>
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<tr>
<td>pHYBP138</td>
<td>Ap^r, CoE1, pHYBP137 with a 4 bp insertion at the BamHI site; contains bbpC::luxAB fusion lacking UAS C-1 and UAS C-2</td>
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</tr>
<tr>
<td>pHYBP139</td>
<td>Ap^r, CoE1, pHYBP113 with a deleted 0.15 kb (Spbi–BamHI) DNA fragment; contains bbpR::luxAB fusion lacking UAS C-3 and UAS C-4</td>
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</tr>
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<td>Ap^r, CoE1, pHYBP113 with a deleted 0.41 kb SmaI–(NcoI) DNA fragment; contains bbpR::luxAB fusion lacking all UASs</td>
<td>This work</td>
</tr>
<tr>
<td>pJAMA8</td>
<td>Ap^r, CoE1; luxAB-based promoter-probe vector</td>
<td>Jaspers et al. (2000)</td>
</tr>
</tbody>
</table>

* UASs, 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.
Transcription activation of the *hbpCA* and *hbpD* genes by the HbpR protein is mediated from two different ς^HbpR^)-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_{hbpR} 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 bisaromatic 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 allow 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. azelaiica’ HBPI is able to use 2-HBP and 2,2'-diHBP as a sole source of carbon and energy (Kohler et al., 1988). ‘P. azelaiica’ HBPI104 (Jaspers et al., 2000) and HBPI117 originate from strain HBPI and contain luxAB-based transcriptional fusions with the upstream regions of hbpC and *hbpR*, respectively, integrated on the chromosome in monopolarity. *Escherichia coli* DH5α (Sambrook et al., 1989) was used as host strain in routine cloning experiments. *E. coli* HB101(pRK2013) (Boyer & Roulland-Dussoix, 1969; Figurski & Helinski, 1979) was used as a helper strain to mobilize plasmids from *E. coli* CC118pir in triparental matings. The plasmids used in this study are listed in Table 1.

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**Fig. 1.** Summary of the HbpR-controlled *hbp* pathway in ‘P. azelaiica’ HBPI 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 between *hbpC* and *hbpD* indicate predicted IHF-binding sites. The scissors mark a putative cleavage site in the *hbpCA* intergenic region (enlarged) fused to the promoterless luxAB genes of plasmid pJAMA8 (Jaspers et al., 2001). Hairpin-like structures indicate putative rho-independent transcriptional terminators. Plasmids constructs relevant for this study are shown below. All plasmids contain inserts derived from the *hbpC*-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*-intergenic region is indicated by boxes, as follows: UAS C_{i} through C_{5}, 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, SphI; B, BamHI; Nc, Ncol; X, XbaI; Sm, SmaI; Ns, Nsil; C, ClaI. 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.
**Fig. 2.** (a) Sequence of the hbpR–hbpC intergenic region and indication of all relevant elements. The basepair numbering corresponds to the sequence of hbpRCAD in GenBank (accession number U73900). Angled arrows indicate the transcriptional start sites (›1) and the direction of transcription for the hbpC and hbpR genes. The positions of the four UASs C-4 through C-1 are indicated, as well as palindromic structures within them (arrows underneath) and an alignment with the consensus sequence for XylR/DmpR-type UASs (Perez-Martín & de Lorenzo, 1996b). DNA regions similar to the consensus IHF-binding site (5′-WATCAANNNNTTR-3′; W = A or T, R = A or G, N = any nucleotide) as proposed by Friedman (1988) are shown as dashed boxes. The numbers below putative UASs and IHF sites indicate the relative positions with respect to the transcriptional start site of the hbpC gene (compare to Fig. 1). The N-terminal parts of HbpR and HbpC and of the small cryptic ORF (crpRh) located upstream of the hbpR gene are indicated with one-letter amino acid codes below the corresponding sequences. Identical amino acid residues between HbpR and CrpRh are encircled. Black and white triangles indicate the borders (B, beginning; E, end) of the two homologous DNA regions (panel b).
Media and growth conditions. E. coli strains were grown at 37 °C on Luria–Bertani (LB) medium (Sambrook et al., 1989). ‘P. aeruginosa’ 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. aeruginosa’); rifampin, 50 µg ml⁻¹ (E. coli). ‘P. aeruginosa’ 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 (Ravatn 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 constructed to test the contribution of the different pairs of hbpR–hbpC

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

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

Single chromosomal insertion of an hbpR::luxAB fusion in ‘P. aeruginosa’. By using the unique NolI sites at the flanks, the hbpR::luxAB fusion in plasmid pHYBP113 was recovered and exchanged with the 3.2 kb NolI fragment present in the Tn5 delivery vector PK218 (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. aeruginosa’ HBP1, in a triparental mating procedure, as described previously (Jaspers et al., 2000). Selection for ‘P. aeruginosa’ 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. aeruginosa’ exconjugants (data not shown). The resulting strain is referred to as HBP117.

Luciferase assays. Induction experiments with luxAB-harbouiring E. coli and ‘P. aeruginosa’ 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-decanol 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. aeruginosa’ HBP1, 30 min after induction with 2-HBP, as described elsewhere (Jaspers et al., 2001). Primer extension analysis was performed with primer PE_hbpR (5’-GATT-TCATGGCGATGGTTCAGG-3’) (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-decanol from Sigma. Nutrient broth, yeast extract and trypsin 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. aeruginosa’ HBP1 from two independent
σ^4-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 crpRb: 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-
transcriptional 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 (plasmid 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-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 plasmid pHYBP113, which contained a luxAB-based transcriptional fusion with the complete hbpR–hbcC intergenic region in the direction of hbpR. Induction studies were carried out in E. coli DH5α 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 μM 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. azelaiaca’. A ‘P. azelaiaca’ 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.
Luciferase activity (10^4 RLU)

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).

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 pHYP113 that had deletions within the \( hbpR–hbpC \) intergenic region (plasmids pHYP139, 140, 141) (Fig. 1). Deletion of 0.28 kb DNA fragment between the BamHI and NcoI sites (Fig. 2), containing the promoter region, indeed reduced luciferase activity in \( E. coli \) with plasmid pHYP140 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 pHYP139) 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 pHYP141 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^70-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 Po_MHO 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 auto-regulation 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. azelaiaca’ (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 $\sigma^{54}$-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$_{hpbc}$ and P$_{hpbd}$ promoter from 'P. azelaica' HBP1 (U73900) (Jaspers et al., 2001); DmpR-controlled Po promoter (M60276) (Nordlund et al., 1990; Shingler et al., 1993); XylR-controlled Ps promoter (M10143) (Inouye et al., 1986, 1987) and Pu promoter (D63341) (Inouye et al., 1984); TouR-controlled P$_{ToMO}$ promoter (AJ005663) (Arenghi et al., 1999). (b, c) Alignments of distal (b) and proximal (c) UASs upstream from the $\sigma^{54}$-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 σ70 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 phiR system for phenol degradation of P. putida strain H (Burchhardt et al., 1997). This spontaneous promoter-up mutation in phiR was isolated upon selection for better growth on phenol and led to an increase in the expression of phiR and the phenol pathway enzymes. The duplication had created a slightly different –35 box and −35/−10 spacing for the phiR promoter at the 3′ end of the duplicated fragment. Maybe the regions upstream of hbpR, phiR 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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REFERENCES


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