**In vivo** analyses of constitutive and regulated promoters in halophilic archaea

Dagmar Gregor and Felicitas Pfeifer

Institut für Mikrobiologie und Genetik, Technische Universität Darmstadt, Schnittspahnstr. 10, D-64287 Darmstadt, Germany

The two gvpA promoters P<sub>cA</sub> and P<sub>pA</sub> of Halobacterium salinarum, and the P<sub>mCA</sub> promoter of Haloferax mediterranei were investigated with respect to growth-phase-dependent expression and regulation in Haloferax volcanii transformants using the bgaH reading frame encoding BgaH, an enzyme with β-galactosidase activity, as reporter. For comparison, the P<sub>fdx</sub> promoter of the ferredoxin gene of Hbt. salinarum and the P<sub>bgaH</sub> promoter of Haloferax lucentense (formerly Haloferax alicantei) were analysed. P<sub>fdx</sub> driving the expression of a house-keeping gene, was highly active during the exponential growth phase, whereas P<sub>bgaH</sub> and the three gvpA promoters yielded the largest activities during the stationary growth phase. Compared to P<sub>fdx</sub>, the basal promoter activities of P<sub>pA</sub> and P<sub>mCA</sub> were rather low, and larger activities were only detected in the presence of the endogenous transcriptional activator protein GvpE. The P<sub>cA</sub> promoter does not yield a detectable basal promoter activity and is only active in the presence of the homologous cGvpE. To investigate whether the P<sub>gvpE</sub>-TATA box and the BRE element were the reason for the lack of the basal P<sub>cA</sub> activity, these elements and also sequences further upstream were substituted with the respective sequences of the stronger P<sub>pA</sub> promoter and investigated in Hfx. volcanii transformants. All these promoter chimeras did not yield a detectable basal promoter activity. However, whenever the P<sub>pA</sub>-BRE element was substituted for the P<sub>cA</sub>-BRE, an enhanced cGvpE-mediated activation was observed. The promoter chimeras harbouring P<sub>pA</sub>-BRE plus 5 (or more) bp further upstream also gained activation by the heterologous pGvpE and mcGvpE proteins. The sequence required for the GvpE-mediated activation was determined by a 4 bp scanning mutagenesis with the 45 bp region upstream of the P<sub>pA</sub>-BRE element was substituted for the P<sub>cA</sub>-BRE, an enhanced cGvpE-mediated activation was observed. The promoter chimeras harbouring P<sub>pA</sub>-BRE plus 5 (or more) bp further upstream also gained activation by the heterologous pGvpE and mcGvpE proteins. The sequence required for the GvpE-mediated activation was determined by a 4 bp scanning mutagenesis with the 45 bp region upstream of the P<sub>mCA</sub>-BRE. None of these alterations influenced the basal promoter activity, but the sequence TGAAACGG-n4-TGAACCAA was important for the GvpE-mediated activation of P<sub>mCA</sub>.

**INTRODUCTION**

The basal transcription machinery of archaea consists of a multi-subunit RNA polymerase, the TATA box-binding protein TBP and the transcription factor TFB (a homologue of the eukaryal TFIIB). This complex appears to be a minimal eukarya-type transcription system related to the eukaryal RNAPII system (Bell & Jackson, 1998). Most archaeal genes contain a TATA box centred 24–28 nt upstream of the transcription start site, representing a highly conserved 8 bp sequence element (TTTAWA<sub>tr</sub>, with W = A/T, R = A/G) which binds TBP. Many archaeal promoters also contain the TFB-responsive element BRE (cRNAAnT) located upstream and adjacent to the TATA box that binds TFB. BRE consists of an 8 bp purine-rich region, and as determined for hyperthermophilic archaea, determines the promoter strength and the orientation of the transcription apparatus (Kosa <i>et al</i>, 1997; Littlefield <i>et al</i>, 1999; Qureshi & Jackson, 1998; Bell <i>et al</i>, 1999a, b). In silico analyses of Halobacterium salinarum genes also suggest the presence of a BRE element in halophilic archaea (Soppa, 1999). In contrast to the one to two TBP and TFB proteins in hyperthermophilic and many methanogenic archaea, the halophiles <i>Hbt. salinarum</i> and <i>Haloferax volcanii</i> possess multiple TBP and TFB proteins that may influence gene regulation (Baliga <i>et al</i>, 2000). In vitro transcription systems have been used to study the transcription initiation and also the action of various transcription regulators in methanogenic and hyperthermophilic archaea (for example Hochheimer <i>et al</i>, 1999; Bell <i>et al</i>, 1999a; Brinkman <i>et al</i>, 2000; Enoru-Eta <i>et al</i>, 2000; Leonard <i>et al</i>, 2001; Ouhammouch <i>et al</i>, 2003). For halophilic archaea, a functional in vitro transcription system is not yet available, and thus the majority of the studies on the transcription initiation and regulator proteins of Hfx. volcanii and <i>Hbt. salinarum</i> have been done in vivo (Danner & Soppa, 1996; Patenge <i>et al</i>, 2000; Pfeifer <i>et al</i>, 2001; Zimmermann & Pfeifer, 2003; Hofacker <i>et al</i>, 2004). We are
using the various promoters of the gvp genes involved in gas vesicle formation of *Hbt. salinarum* and *Haloferax mediterranei* to study gene regulation in halophilic archaea.

The formation of gas vesicles requires the expression of the 14 genes *gvpACNO* and *gvpDEFGHIJKLM*, which are located in two opposite clusters in the vac region (Horne et al., 1991; Englert et al., 1992a). The TATA boxes of the promoters of gvpA (P<sub>A</sub>) and gvpD (P<sub>D</sub>) are separated by 50 bp in the plasmid-borne p-vac region of *Hbt. salinarum* PHH1 and the mc-vac region of *Hfx. mediterranei*. P<sub>A</sub> and P<sub>D</sub> are both activated by the endogenous activator protein GvpE (Röder & Pfeifer, 1996; Zimmermann & Pfeifer, 2003; Hofacker et al., 2004). *Hfx. mediterranei* harbours the single mc-vac region, whereas *Hbt. salinarum* PHH1 contains, in addition to p-vac, the related but distinct c-vac region. Both vac regions are similar but not identical to the gvp1 (p-vac) and gvp2 (c-vac) gene clusters of *Halobacterium* sp. NRC-1 (Ng et al., 2000). The expression of gvpACNO leads to the synthesis of the gas vesicle structural proteins GvpA and GvpC. The second operon encodes (among other proteins presumably involved in gas vesicle assembly, and minor gas vesicle structural proteins; Shukla & DasSarma, 2004) the two regulatory proteins GvpD and GvpE. Four promoters (P<sub>PcA</sub>, P<sub>PcD</sub>, P<sub>PfA</sub> and P<sub>Pfdx</sub>) drive the expression of p-vac, leading to the constitutive production of spindle-shaped gas vesicles in *Hbt. salinarum* PHH1 (Offner et al., 1996; Hofacker et al., 2004). A promoter scanning mutagenesis performed on a 50 bp region upstream of the transcriptional start site of P<sub>Pa</sub> determined that the sequences of BRE and the TATA box, as well as a sequence around position −10, influence the basal transcription. Furthermore, an adaptation of the putative BRE sequence element to the archaeal consensus BRE element sequence results in a significantly enhanced basal P<sub>Pa</sub> promoter activity. These analyses also imply that the sequence AACCA located upstream and adjacent to BRE is involved in the GvpE-mediated activation, suggesting a close contact of GvpE with the core transcription machinery (Hofacker et al., 2004).

The second vac region of *Hbt. salinarum* PHH1, c-vac, is only partly expressed in this wild-type strain (due to the minor activity of P<sub>Pd</sub>), but the c-gvpACNO genes are not expressed at all (Pfeifer et al., 1997). Gas vesicles due to c-vac are only formed in the p-vac deletion mutant *Hbt. salinarum* PHH4 (Krüger & Pfeifer, 1996). Using the gvp negative *Hfx. volcanii* as recipient strain, earlier investigations showed that a basal promoter activity of P<sub>Ca</sub> is not detectable and that this promoter is only active in the presence of cGvpE in c-gvpA/ c-gvpE* transformants that contain the c-gvpE reading frame expressed under the control of P<sub>fgc</sub> in pJAS35 in addition to c-gvpA (Krüger et al., 1998). Similar results on the P<sub>Ca</sub> activity have been obtained using the *bgaH* reading frame encoding an enzyme with β-galactosidase activity as reporter (Holmes & Dyall-Smith, 2000; Gregor & Pfeifer, 2001). Again, in contrast to P<sub>Pa</sub>, the P<sub>Pa</sub> and P<sub>mcA</sub> promoters yield basal promoter activities that are significantly enhanced in the presence of the respective homologous GvpE proteins. In contrast to P<sub>Pa</sub>, these two P<sub>A</sub> promoters are also activated by heterologous GvpE proteins. GvpE resembles eukaryotic basic leucine-zipper (bZIP) proteins and is able to dimerize in solution (Krüger et al., 1998; Ploesser & Pfeifer, 2002). More recent analyses on mcGvpE and the second regulatory protein, mcGvpD, involved in the repression of gas vesicle formation of *Hfx. mediterranei*, show that GvpE and GvpD are able to interact (Zimmermann & Pfeifer, 2003).

In the present study, using the *bgaH* reporter system, we compared the activities of the three gvpA promoters to that of the strong and constitutive P<sub>fgc</sub> promoter of the *fgc* gene encoding the (2Fe–2S) ferredoxin of *Hbt. salinarum* (Pfeifer et al., 1993) in *Hfx. volcanii* transformants. In addition, the activity of the P<sub>bgaH</sub> promoter was determined. The P<sub>bgaH</sub>-bgaH and P<sub>fgc-bgaH</sub> transformants yielded large amounts of BgaH activities, whereas the basal P<sub>Pa</sub> and P<sub>mcA</sub> promoter activities were rather low. Again, a basal P<sub>Ca</sub> activity was not detectable in P<sub>Ca-bgaH</sub> transformants. To investigate whether the P<sub>Ca</sub>-TATA box and BRE were the reason for the latter observation, promoter chimeras were constructed between P<sub>Ca</sub> and P<sub>Pa</sub> and analysed for reporter gene expression. None of these P<sub>Ca-Pa</sub> promoter variants yielded a detectable basal promoter activity, but the activity was enhanced in the presence of GvpE when the BRE element and/or the TATA box were exchanged. In addition, all three GvpE proteins were able to activate the P<sub>Ca-Pa</sub> promoter variants that contained the P<sub>Ca</sub>-BRE element (plus at least 5 nt further upstream), whereas the original P<sub>Ca</sub> promoter and the derivatives still containing P<sub>Ca</sub>-BRE were only activated by cGvpE. To determine the sequences important for the GvpE-mediated activation, a 4 bp scanning mutagenesis was done with the P<sub>mcA</sub> region separating the BRE elements of the two mc-vac promoters. The results obtained suggested that two conserved regions adjacent to BRE were involved in the GvpE-mediated activation of P<sub>mcA</sub>.

### METHODS

**Constructs used for transformation of *Hfx. volcanii* and reporter gene analysis.** The *Hfx. volcanii* growth medium and growth conditions have been described previously (Pfeifer et al., 2001). The p-vac construct containing the entire p-vac region, the c<sup>em</sup> P<sup>em</sup> and mc<sup>em</sup> constructs in pJAS35, and P<sub>PcA-A-bgaH</sub>, P<sub>Pfdx-bgaH</sub>, P<sub>Ca-bgaH</sub> and P<sub>mcA-bgaH</sub> in pWL102 have been described previously (Offner & Pfeifer, 1995; Gregor & Pfeifer, 2001). The 2203 bp *bgaH* reading frame was amplified as an *NcoI–BamHI* fragment using the primer pair *bgaH-NcoI* and *bgaH-BamHI* (Gregor & Pfeifer, 2001) and plasmid pMLH32 as template. The NcoI site overlaps the AUG start codon of *bgaH*. For the construction of P<sub>fgc-bgaH</sub>, the *bgaH* reading frame was isolated as an *NcoI–Aac65I* fragment from P<sub>fgc-bgaH</sub> by partial digestion and fused to P<sub>fgc</sub> in pJAS35.

The BRE and TATA substitutions in P<sub>BcA</sub> were introduced by recombinant PCR using complementary primers including the mutations, and the c-gvpA gene in pBSK+ as template. Two PCR reactions were performed to amplify two subfragments harbouring the inserted mutations in the overlapping end. The Ca-pa-TATA, ca-pa-BRE and Ca-pa-BRE/TATA fragments were amplified using the mutation primer Blt-null (GGGACACTCCCTGTAGTT) plus...
The 10d was similar, but involved the mutation primer pA-cA2 (ATGGTTTCACCAGTCGTTATGTCTCTCGTAATAGTT). The promoter chimera −5u was amplified using c-gvpA as template and mutation primer pA-cA-4 (GTTTCTCGGACACTCCCTCTGTTA) plus cA-Ncol. In the second PCR, primer pair pA-cA-5 (TGTCCTCGGAAAACGATGTGTATGGTCTCAGCACCCCTTTT) plus cA-Ncol were used. The full-length fragment was amplified using the products of both PCRs as template and primers cA-Xbal and cA-Ncol yielded +5u. Similarly, the +10u promoter was constructed using mutation primer pA-cA-3 (ATGGTTTCACCAGTCGTTATGTCTCTCGTAATAGTT). The promoter chimera −5u was amplified using c-gvpA as template and mutation primer pA-cA-7 (CGTTTCGGCGCTCGTAATAGTTCGCT) together with cA-Ncol (Gregor & Pfeifer, 2001) as template. The second PCR was done with the mutation primer mcA-M0 (ATGGTTTCACCAGTCGTTATGTCTCTCGTAATAGTTA) and cA-Xbal. A third PCR with both amplicons as template and primers cA-Xbal and cA-Ncol yielded +5u. The promoter chimera −10u was produced in a similar way, but involved the mutation primer mcA-M1 (GAGGATCCTGGGAAAACGATGTGTGTGTGGATCAGAACCCCTTTT) plus cA-Ncol. In the second PCR, primer pair mcA-M2 (AGGGGAGTGTCCGCATAAGCGCCGTTGTGA), and mcA-Del-Pal (CCAAACTATCTAGATGTTTGACCTCGGCTGCTGCTTCAGCAACC) plus cA-Ncol yielded +10u. The construction of −5d, the mutation primer mcA-ja-7 (GTATTCTGGGCGCTCGTAATAGTTGCT) was used together with cA-Xbal and c-gvpA as template; the second PCR involved primer mcA-ja-8 (AGGGGAGTGTCCGCATAAGCGCCGTTGTGA), and mcA-Del-Pal (CCAAACTATCTAGATGTTTGACCTCGGCTGCTGCTTCAGCAACC) plus cA-Ncol. The construction of −10d was similar, but involved the mutation primers mcA-ja-8 (AGGGGAGTGTCCGCATAAGCGCCGTTGTGA) and mcA-Del-Pal (CCAAACTATCTAGATGTTTGACCTCGGCTGCTGCTTCAGCAACC) plus mcA-m10 (AGGGGAGTGTCCGCATAAGCGCCGTTGTGA). The mcA promoter mutant mcA-Del was amplified using primer pair mcA-Ncol and mcA-Del-Pal (CCAAACTATCTAGATGTTTGACCTCGGCTGCTGCTTCAGCAACC) as template. Each of these amplified fragments was purified by gel electrophoresis, and the P_{mut} promoter fragments were obtained by XbaI/NcoI digestion and used to substitute the wild-type P_{A} promoter in the respective P_{A}-bgA fragment in vector pBSK+. In each case, the correct mutation and fusion of the mutant promoter to bgA was determined by DNA sequence analysis. Each of these mutant P_{mut}-bgA fragments was isolated as an XbaI–BamHI fragment and inserted in pWL102 for transformation of Hfx. volcanii.

**Transformation of Hfx. volcanii and BgaH assay.** Prior to the transformation of Hfx. volcanii, each construct was passaged through the E. coli dam strain GM 1674 to avoid a halobacterial restriction barrier. Transformation was done as described by Pfeifer & Ghahraman (1993). Transformants were selected on agar plates containing 6 µg lovastatin ml⁻¹ (for the selection of pWL102) and 0-2 µg novobiocin ml⁻¹ (for the selection of E″ in pAS35). Lovastatin was a generous gift of MSD Sharp & Dohme GmbH. The presence of and the amount of the desired constructs in each transformant were comparable in each case, as determined by plasmid isolation and gel electrophoresis. The BgaH activity in cell lysates of the various Hfx. volcanii P_{A}-bgA transformants was measured by ONPG assay as described by Holmes et al. (1997). Cells of 0-1–4 ml culture were resuspended in 100 µl medium, lyed with 50 µl 2% Triton X-100 and mixed with 800 µl ONPG test buffer (2-6 M NaCl, 10 µM MnCl₂, 0-1% w/v mercaptoethanol, 50 mM Tris/HCl, pH 7-2). Then 50 µl ONPG (8 mg ONPG ml⁻¹ in 0-1 M Tris/HCl, pH 7-2) was added, mixed and incubated for 5 min at room temperature. The BgaH activity was measured at 400 nm at room temperature. The activity was calculated as ΔA/Δt = ε x d x ∆c/Δt (ε for ONP is 3-3 x 10⁶ M⁻¹ cm⁻¹ and d = 1 cm). One unit of BgaH activity is the amount of enzyme that catalyses the hydrolysis of 1 mmol ONPG per minute. The protein concentration was determined by the Bradford assay (Ausbuch et al., 1988) using BSA as standard.

### RESULTS

**Activity of the three P_{A} promoters in comparison to the promoters P_{bgA} and P_{fdx}**

The three gvpA promoters (P_{A}), the native promoter of the bgA gene in pMLH32 (P_{bgA}), and also the P_{fdx} promoter of the respective oligonucleotides mcA-M7 through mcA-M11 together with mcA-Ncol and mc-gvpA as template. Each of these amplified fragments was purified by gel electrophoresis, and the P_{mut} promoter fragments were obtained by XbaI/NcoI digestion and used to substitute the wild-type P_{A} promoter in the respective P_{A}-bgA fragment in vector pBSK+. In each case, the correct mutation and fusion of the mutant promoter to bgA was determined by DNA sequence analysis. Each of these mutant P_{mut}-bgA fragments was isolated as an XbaI–BamHI fragment and inserted in pWL102 for transformation of Hfx. volcanii.

**Table 1. Oligonucleotides used to construct the 4 bp scanning mutants**

*Mutations are underlined; restriction sites introduced are given in italics.*

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>mcA-M0</td>
<td>ACGAATGATTTTTGTTACCT</td>
</tr>
<tr>
<td>mcA-M1</td>
<td>AAAATCATTGCTGCAATGTTTCAGCA</td>
</tr>
<tr>
<td>mcA-M2</td>
<td>AAAATCATTGCTGTTTCAGCACACCGTTCA</td>
</tr>
<tr>
<td>mcA-M3</td>
<td>AAAATCATTGCTGTTTCAGCACACCGTTACCT</td>
</tr>
<tr>
<td>mcA-M4</td>
<td>AAAATCATTGCTGTTTCAGACCCCGTTACA</td>
</tr>
<tr>
<td>mcA-M5</td>
<td>AAAATCATTGCTGTTGCTAGCATGTTTTACCT</td>
</tr>
<tr>
<td>mcA-M6</td>
<td>AAAATCATTGCTGTTGCTAGCATGTTTTACCT</td>
</tr>
<tr>
<td>mcA-M7</td>
<td>TCTAGATTCAGTTTACATCGAGAGTGTGTAACCCGTTGCT</td>
</tr>
<tr>
<td>mcA-M8</td>
<td>TCTAGATTCAGTTTACATCGAGAGTGTGTAACCCGTTGCT</td>
</tr>
<tr>
<td>mcA-M9</td>
<td>TCTAGATTCAGTTTACATCGAGAGTGTGTAACCCGTTGCT</td>
</tr>
<tr>
<td>mcA-M10</td>
<td>TCTAGATTCAGTTTACATCGAGAGTGTGTAACCCGTTGCT</td>
</tr>
<tr>
<td>mcA-M11</td>
<td>TCTAGATTCAGTTTACATCGAGAGTGTGTAACCCGTTGCT</td>
</tr>
</tbody>
</table>

http://mic.sgmjournals.org
the ferredoxin gene of *Hbt. salinarum* were investigated in *Hfx. volcanii* transformants using the *bgaH* reading frame encoding an enzyme with β-galactosidase activity (*BgaH*) as reporter. In each case, the promoter sequences were fused to *bgaH* at the fifth codon within the respective reading frame. The promoter sequences located upstream of the transcription start site are shown in Fig. 1(a). *Hfx. volcanii* transformants harbouring the *Pfdx-*bgaH construct yielded large amounts of *BgaH* activities, especially in samples derived from the exponential but also from the stationary growth phase (Fig. 1b). These large amounts reflected the high activity of *Pfdx*, which is typical for this house-keeping gene (Pfeifer et al., 1993). A slight reduction of the *BgaH* activity was always observed during the stationary growth phase (Fig. 1b). The transformants harbouring pMLH32 carrying the native *bgaH* gene yielded significantly reduced *BgaH* activity in the sample derived from exponential growth, but increased activities were seen in the stationary growth phase (Fig. 1b). The two *gvpA* promoters *PmcA* and *PpA* have low basal promoter activities: 1–4 mU (mg protein)^−1^, which represents 1% of the basal *PbgaH* and *Pfdx* activities (data not shown; Gregor & Pfeifer, 2001), but their activities were strongly enhanced in the presence of cGvpE (Fig. 1b). The GvpE-mediated activation of *PmcA* reached similarly large amounts of *BgaH* activities as that of the pMLH32 transformant, whereas the *PpA-*bgaH/cEex transformant with 200 mU (mg protein)^−1^ reached only 20% of the respective activities determined for the *Pfdx-*bgaH transformant during the stationary growth phase (Fig. 1b). The *BgaH* activity of the GvpE-induced *PcA* promoter was, with 10 mU (mg protein)^−1^, very low (Fig. 1b). In all cases, the strongest *BgaH* activities were seen during the stationary growth phase, which was unexpected, since the transcriptional activator GvpE was produced under *Pfdx* control in these transformants and should have been present in large amounts during the exponential growth phase. These observations implied an additional factor that was responsible for the increased promoter activity of *PmcA* and *PcA* in the stationary growth phase.

**Substitution of *PcA* promoter elements by *PpA* sequences**

A basal *PcA* promoter activity is not detectable, and the *BgaH* activity is only observed in the presence of cGvpE (Gregor & Pfeifer, 2001, and this report). To investigate whether the *PcA*-TATA box and/or the sequence of the putative *PcA*-BRE element were the reason for the undetectable basal promoter activity, these elements were exchanged with the respective sequences of the stronger *PpA* promoter (Fig. 2). The transformants harbouring these *PcA*-pA-bgaH constructs indicated that the substitution of the *PcA*-BRE and/or *PcA*-TATA box with the respective sequences of *PpA* derivatives. With respect to the GvpE-mediated activation, six- to eightfold enhanced *BgaH* activities were observed, implying that the TATA box and the BRE element (and/or the factors bound here) support the GvpE-mediated...
activation (Fig. 2). However, these BgaH activities did not reach the strong activities of the \( P_{pA-bgaHi/cEex} \) transformant, demonstrating that additional sequences of \( P_{pA} \) are responsible for the high level of basal and GvpE-induced promoter activities of \( P_{pA} \).

To determine the sequences required for the GvpE-mediated activation, additional substitutions were done with the sequence upstream of the \( P_{cA} \)-TATA box. The original \( P_{pA-cA} \) promoter chimera published by Gregor & Pfeifer (2001) contains a substitution of 21 bp upstream of the TATA box (including BRE), and still yields an undetectable basal promoter activity (Fig. 3). In the presence of cGvpE, this promoter shows an enhanced activity compared to the original \( P_{pA-cA} \). As shown earlier, this \( P_{pA-cA} \) promoter is activated by the heterologous pGvpE and mcGvpE proteins to a minor extent, whereas the original \( P_{cA} \) promoter is exclusively activated by cGvpE (Gregor & Pfeifer, 2001; Fig. 3). From these results we concluded that this 21 bp sequence (or a portion of it) is involved in the GvpE-mediated activation. In the present study we altered the size of the 21 bp \( P_{pA} \) region in \( P_{pA-cA} \) by exchanging portions of this sequence (or additional sequences) between \( P_{pA} \) and \( P_{cA} \) in steps of 5 bp (Fig. 3). Again, none of the resulting promoter derivatives (+10u, +5u, −5u, −10u, −5d, and −10d) yielded a basal promoter activity in the respective transformants. With respect to the GvpE-mediated activation, the four transformants +10u, +5u, −5u, and −10u yielded similarly large amounts of BgaH activities to those of the original \( P_{pA-cA-bgaHi/cEex} \) transformant. These four variants were also activated by the heterologous pGvpE and mcGvpE proteins to a similar degree to that found for the \( P_{pA-cA-bgaHi/cEex} \) transformant (Fig. 3). In contrast, the two transformants −5d/cEex and −10d/cEex (which still contained the \( P_{cA-BRE} \) element sequence) were exclusively activated by cGvpE, similar to the original \( P_{pA-bgaHi/cEex} \) transformant (Fig. 3). These results led to the conclusion that the \( P_{cA-BRE} \) element (+5 bp upstream) was the reason for the enhanced activation by cGvpE detected for the former four mutants, and also for the stimulation by the heterologous GvpE proteins. Again, the BRE element affected the GvpE-mediated activation.

These analyses on \( P_{cA} \) demonstrated that it is difficult to determine sequences involved in GvpE-mediated activation, since \( P_{cA} \) has an undetectable basal promoter activity. Mutations could affect the strength of the basal \( P_{cA} \) transcription (and thus also the GvpE-mediated activation). The stronger \( P_{pA} \) promoter has already been investigated by a 4 bp scanning mutagenesis in a 50 bp region located upstream of the transcription start, and from this analysis the sequence AACC located upstream of BRE appears to be involved in the GvpE-mediated activation (Hofacker et al., 2004).

<table>
<thead>
<tr>
<th>Archaean consensus</th>
<th>BRE TATA</th>
<th>cGvpE</th>
<th>pEex</th>
<th>mcGvpE</th>
</tr>
</thead>
<tbody>
<tr>
<td>( P_{pA} )</td>
<td>TTATCCGCCGTTTACTACGTTGATGGAATGTTGACAGGTTTACTACGTTGATGGAATG</td>
<td>3 ± 2</td>
<td>225 ± 13</td>
<td>177 ± 84</td>
</tr>
<tr>
<td>( P_{cA} )</td>
<td>TTATCCGCCGTTTACTACGTTGATGGAATGTTGACAGGTTTACTACGTTGATGGAATG</td>
<td>11 ± 6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>( P_{pA-cA} )</td>
<td>TTATCCGCCGTTTACTACGTTGATGGAATGTTGACAGGTTTACTACGTTGATGGAATG</td>
<td>42 ± 10</td>
<td>84 ± 3</td>
<td>6 ± 4</td>
</tr>
<tr>
<td>+10u</td>
<td>ACT-0</td>
<td>ACT-0</td>
<td>ACT-0</td>
<td>ACT-0</td>
</tr>
<tr>
<td>+5u</td>
<td>ACT-0</td>
<td>ACT-0</td>
<td>ACT-0</td>
<td>ACT-0</td>
</tr>
<tr>
<td>−5u</td>
<td>ACT-0</td>
<td>ACT-0</td>
<td>ACT-0</td>
<td>ACT-0</td>
</tr>
<tr>
<td>−10u</td>
<td>ACT-0</td>
<td>ACT-0</td>
<td>ACT-0</td>
<td>ACT-0</td>
</tr>
<tr>
<td>−5d</td>
<td>ACT-0</td>
<td>ACT-0</td>
<td>ACT-0</td>
<td>ACT-0</td>
</tr>
<tr>
<td>−10d</td>
<td>ACT-0</td>
<td>ACT-0</td>
<td>ACT-0</td>
<td>ACT-0</td>
</tr>
</tbody>
</table>

Fig. 2. Substitution of \( P_{cA-BRE} \) and TATA box by the respective \( P_{pA} \) sequences, and BgaH activities. The TATA box (bold) and the putative BRE element (italic) are shaded in grey, and the consensus sequences of both elements are given on top ( \( N = \) any base; \( R = A, G; W = A, T \) ). The sequences derived from \( P_{pA} \) are underlined. Dots in the mutant sequences indicate nucleotides that are identical to the \( P_{cA} \) sequence. Numbers on the right are specific activities of BgaH in mU (mg protein)\(^{-1} \).

Fig. 3. Promoter chimera between \( P_{pA} \) and \( P_{cA} \) sequences upstream of the TATA box, and BgaH activities. The sequences derived from the \( P_{pA} \) promoter region are underlined. Dots in the mutant sequences indicate nucleotides that are identical to \( P_{cA} \). Numbers on the right are BgaH activities determined in stationary growth in mU (mg protein)\(^{-1} \). ND, No detectable activities (<0.5 mU mg\(^{-1} \)).
Search for the sequence affecting the GvpE-mediated activation of \( P_{mcA} \)

The \( P_{mcA} \) promoter of \( Hfx. \) mediterranei offers the highest promoter activity of all \( P_A \) promoters when induced with GvpE, and also yields a measurable basal promoter activity (Fig. 1; Gregor & Pfeifer, 2001). A 4 bp scanning mutagenesis was performed with the 49 bp region separating the TATA boxes of \( P_{mcA} \) and \( P_{mcD} \), and the resulting mutant \( P_{mcA} \)-bgaH transformants were investigated for BgaH activities (Fig. 4). None of these 4 bp alterations affected the basal activity of \( P_{mcA} \) (Fig. 4b). The analysis of the GvpE-mediated activation yielded reduced activities (19–23 % of the wild-type activity) in mutants carrying the alterations adjacent to BRE (mcA-M1 through mcA-M3) and in the centre of this region (mcA-M5 through mcA-M7), where the most significant reductions (6–10 % of the wild-type activity) were observed (Fig. 4a; the \( P_{mcA} \) sequences affected are indicated in bold in Fig. 4b). These analyses suggested that the sequence TGAACCAA close to BRE, and also the sequence TGAAACGG in the centre of the intergenic region, were important for the GvpE-mediated activation of \( P_{mcA} \).

Mutant mcA-Del incurred a 3 bp deletion 6 bp upstream of BRE (Fig. 4b). This deletion did not affect the basal promoter activity of \( P_{mcA} \), but completely abolished the GvpE-mediated activation (Fig. 4b). In summary, these results demonstrated that the sequence upstream of BRE had no influence on the basal promoter activity, but the GvpE-mediated activation was negatively affected when the sequence TGAACCAA-n4-TGAAACGG was altered.

DISCUSSION

The bgaH reading frame was used as a reporter to analyse the activity of five haloarchaeal promoters, and also to define the sequences important for the GvpE-mediated activation of the three \( P_A \) promoters of \( Hbt. \) salinarum and \( Hfx. \) mediterranei. Each promoter–bgaH fusion was inserted into a low-copy-number plasmid, and the BgaH activities (i.e. \( \beta \)-galactosidase activities) were determined throughout the growth of the \( Hfx. \) volcanii transformants. The promoter \( P_{fdx} \) appeared to be the strongest one investigated, yielding large activities even during the exponential growth phase. The \( fdx \) gene is a typical house-keeping gene, and the expression pattern determined here reflects the predominant production of \( fdx \) mRNA during the exponential growth phase (Pfeifer et al., 1993). In contrast, the basal activities of \( P_{pa} \) and \( P_{mcA} \) were rather low, and reached only \( 10^{-3} \) of the \( P_{fdx} \) activity (Gregor & Pfeifer, 2001, and this report). Even in the presence of the activator protein GvpE, \( P_{pa} \) and \( P_{mcA} \) did not gain a similarly high level of activity as that of \( P_{fdx} \). In the presence of GvpE, all \( P_A \) promoters reached the greatest activity during the stationary growth phase of \( Hfx. \) volcanii. This was unexpected, since the GvpE protein was produced under \( P_{fdx} \) control in these \( P_A \)-bgaH/cEex transformants, that is, predominantly during the exponential growth phase. These results led to the assumption that an additional factor appearing in stationary growth was responsible for the enhanced expression in this growth phase. Since multiple basal transcription factors (6 TBP and 7 TFB proteins) have been found in...
Halobacterium sp. NRC-1 (Baliga et al., 2000) and several of these are also present in Hfx. volcanii, it is possible that one (or some) of them preferentially initiate(s) transcription in the stationary growth phase. To determine whether or not these five promoters investigated harbour differences in their BRE or the TATA box elements that might reflect their activities, these elements were compared to the respective conserved archaeal element sequences. The TATA box elements were well conserved in each case (4–6 out of 8 conserved nucleotides; see Fig. 1), but larger variations were observed with respect to BRE. P\textsubscript{dx}-BRE exhibits the highest similarity to the archaeal BRE consensus (4 bp of the 5 conserved bp; see Fig. 1), whereas the BRE elements of P\textsubscript{mcA}, P\textsubscript{pA} and P\textsubscript{cA} and also P\textsubscript{bgaiH} contained only a few conserved base pairs. It is possible that these differences cause different binding affinities of the TFB protein or even recruit a different TFB protein. An in vitro transcription system for halophilic archaea would be very helpful to determine the contributions of these sequence elements and of the different TFB (and TBP) proteins to the activity of each promoter, but unfortunately such a system is not yet available.

We investigated whether or not the P\textsubscript{cA}-TATA box and P\textsubscript{cA}-BRE element were the reason for the undetectable basal activity of P\textsubscript{cA} by substituting these elements with the respective sequences of P\textsubscript{pA} which exhibit the strongest basal promoter activity of the three P\textsubscript{A} promoters. Even the substitution of both P\textsubscript{cA} promoter elements by the respective P\textsubscript{pA} promoter sequences resulted in a promoter with no detectable basal promoter activity, demonstrating that both P\textsubscript{pA} elements were not sufficient to drive the basal transcription of these P\textsubscript{cA-pA} promoters. Since the exchange of an additional 21 bp of P\textsubscript{cA} sequences further upstream with the respective P\textsubscript{pA} sequences did not result in a measurable basal transcription, the sequence located between the TATA box and the transcription start must be responsible for the observed lack of basal transcription in P\textsubscript{cA}. Earlier results have shown that alterations in the respective sequences of the P\textsubscript{pA} promoter strongly affect the basal transcription (Hofacker et al., 2004). From these results we assume that the higher GC content found in P\textsubscript{cA} compared to P\textsubscript{mcA} and P\textsubscript{pA} (13 of 22 bp versus 9 of 22 bp, see Fig. 1) interferes with the open complex formation of the RNA polymerase and might be the major reason for the undetectable basal transcription in P\textsubscript{cA}.

In the presence of GvpE the promoter derivatives +10u, +5u, −5u, −10u led to enhanced BgaH activities, similar to the P\textsubscript{pA-cA}+bgaH construct described earlier (Gregor & Pfeifer, 2001). These four promoter variants were also activated by the heterologous pGvpE and mcGvpE proteins (to a minor extent), whereas the original P\textsubscript{cA}-bgaH construct is only induced by the homologous cGvpE (Gregor & Pfeifer, 2001). In contrast, mutants −5d and −10d harbouring the original P\textsubscript{cA}-BRE were only induced by cGvpE. These results led to the conclusion that the P\textsubscript{pA}-BRE element was a major reason for the enhanced and extended promoter activities observed with the four P\textsubscript{pA-cA} mutants mentioned above. The P\textsubscript{pA}-BRE could cause a stronger binding of the original TFB protein (or even bind a different TFB protein), resulting in the enhanced GvpE-mediated activation.

The P\textsubscript{mcA} promoter of Hfx. mediterranei was selected to determine the sequences required for GvpE-mediated activation. P\textsubscript{mcA} yields a basal promoter activity and also has the strongest GvpE-induction of all P\textsubscript{A} promoters. A 4 bp scanning mutagenesis was done throughout the 34 bp region separating the two BRE elements of the oppositely oriented P\textsubscript{mcA} and P\textsubscript{mcD} promoters. The region between the TATA box and the transcription start site was not included, since alterations in the related region of the P\textsubscript{pA} promoter did not affect the GvpE-mediated activation (Hofacker et al., 2004). None of these mutations in P\textsubscript{mcD-PmcA} affected the basal P\textsubscript{mcA} promoter activity. With respect to the GvpE-mediated activation, mutants carrying alterations of the sequences TGAACCAA adjacent to BRE and TGAACCGG located further upstream showed a reduced GvpE-induced promoter activity, demonstrating that both these sequences were important for the GvpE-mediated activation. These two sequences were similar. An alignment of the three P\textsubscript{D-P A} regions of p-vac, c-vac and mc-vac indicated three different conserved regions of >4 bp, two of which

![Fig. 5. Alignment of the intergenic region between P\textsubscript{A} and P\textsubscript{D} and of the sequences involved in GvpE-mediated activation. (a) The sequences derived from mc-vac (mc), p-vac (p) and c-vac (c). The TATA box and the BRE elements of the P\textsubscript{A} and P\textsubscript{D} promoters are shaded in grey. The sequences determined to be responsible for the GvpE-mediated activation of P\textsubscript{mcA} (this report) and P\textsubscript{pA} (Hofacker et al., 2004) are underlined. Nucleotides (>4) conserved in all three regions are marked in bold. (b) Alignment of the two regions determined in mc-vac with the respective sequences found in p-vac and c-vac (left), and deduced consensus sequence (right).](http://mic.sgmjournals.org)
are part of this sequence element (Fig. 5a, conserved nucleotides are marked in bold). An alignment of all 8 nt sequence elements led to the consensus sequence TGAAACGG-n4-TGAACCAA (Fig. 5b). A similar sequence element can be determined with respect to $P_{mcA}$ (which is also activated by GvpE). This sequence includes the conserved sequence ATTAC close to the BRE element of $P_{mcD}$ and $P_P$ (see Fig. 5a). These two promoters are activated by GvpE, whereas the $P_D$ promoter of the c-vac region is not responsive to GvpE-mediated activation, presumably because the $P_D$-BRE element is located 10 bp further away (Krüger & Pfeifer, 1996). However, this needs further proof. A footprint analysis would be very helpful, but the GvpE binding sites determined by these in vivo analyses cover almost the entire $P_{mcD}$-$P_{mcA}$ region. The palindrome sequence GTTG-n6-ACCA, originally hypothesized as the GvpE-binding site by Krüger & Pfeifer (1996), did not contribute to the GvpE-mediated activation. Although mutations in the ACCA portion of this sequence in the course of the 4 bp scanning mutagenesis in $P_{mcA}$ resulted in a reduced GvpE-mediated promoter activation, mutations in the GTTG portion of this palindrome had no influence on the promoter activation.

In summary, the results presented here imply that the sequence TGAAACGG-n4-TGAACCAA located close to $P_{mcA}$-BRE is involved in the GvpE-mediated activation, suggesting a close interaction between GvpE and the basal transcription machinery. An interaction between an archaeal regulator protein and TBP has been described for Ptr2 of the hyperthermophilic Methanococcus jannaschii (Ouhammouch et al., 2003). Ptr2 is a homologue of the leucine-responsive regulatory protein (Lrp) family, and footprint analyses indicate that it binds to two sites consisting of a palindrome located upstream of the TATA box (Ouhammouch et al., 2003). As demonstrated by in vitro analysis, Ptr2 appears to recruit the TBP protein and enhances transcription. In the case of GvpE, an in vitro transcription system would be extremely helpful to study the contribution of the various promoter elements, in concert with distinct TFB and/or TBP proteins, to the GvpE-mediated activation of $P_A$. However, the high salt requirement (up to 4 M KCl) of halophilic proteins, and presumably also the possession of multiple transcription factors, have so far complicated the establishment of such a system.

ACKNOWLEDGEMENTS

We thank Peter Zimmermann and Annette Hofacker for discussions, and Peter Zimmermann and Kathryn Nixdorff for critical reading of the manuscript. Lovastatin was a generous gift of MSD Sharp & Dohme GmbH (Haar, Germany). This work was financially supported by the Deutsche Forschungsgemeinschaft (PF 165/8-2).

REFERENCES


