Overlapping activator sequences determined for two oppositely oriented promoters in halophilic Archaea

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ABSTRACT

Transcription of the genomic region involved in gas vesicle formation in Halobacterium salinarum (p-vac) and Haloferax mediterranei (mc-vac) is driven by two divergent promoters, PA and PD, separated by only 35 nt. Both promoters are activated by the transcription activator GvpE which in the case of PmcA requires a 20-nt sequence (UAS) consisting of two conserved 8-nt sequence portions located upstream of BRE. Here, we determined the two UAS elements in the promoter region of p-vac by scanning mutageneses using constructs containing Ppd (without Ppa) fused to the bgaH reporter gene encoding an enzyme with β-galactosidase activity, or the dual reporter construct Ppd fused to bgaH and Ppa to an altered version of gvpA. The two UAS elements found exhibited a similar extension and distance to BRE as previously determined for the UAS in Pmca. Their distal 8-nt portions almost completely overlapped in the centre of Ppd-Ppa, and mutations in this region negatively affected the GvpE-mediated activation of both promoters. Any alteration of the distance between BRE and UAS resulted in the loss of the GvpE activation, as did a complete substitution of the proximal 8-nt portion, underlining that a close location of UAS and BRE was very important.

INTRODUCTION

The extremely halophilic Halobacterium salinarum and the moderately halophilic Haloferax mediterranei produce gas vesicles depending on environmental factors (1). We are interested in mechanisms of gene regulation in halophilic Archaea and use the gvp gene clusters encoding the proteins required for gas vesicle formation as model system. Gas vesicle formation involves the two gene clusters gvpACNO and gvpDEFGHIJKLM, which are oppositely oriented in the vac region (2). Two different vac regions of H. salinarum PHH1 are known, the plasmid pHH1-encoded p-vac and the chromosomal c-vac region, and in addition the chromosomal mc-vac region of H. mediterranei. The 14 gvp genes found in each of these vac regions encode slightly different variants of the two gas vesicle structural proteins GvpA and GvpC, and of additional Gvp proteins required for the assembly of this structure. GvpD and GvpE regulate the expression, with GvpE serving as transcriptional activator and GvpD being involved in the repression of gas vesicle formation (2–5). GvpD and GvpE are able to interact, and the interaction results in the breakdown of GvpE (4,6).

Transcription in Archaea is driven by a multi-subunit RNA polymerase (RNAP), and the initiation of basal transcription requires in addition to RNAP the TATA-box-binding protein TBP and the transcription factor TFB. A typical archaeal promoter consists of a TATA-box (= binding site of TBP) centred 24–28 nt upstream of the transcriptional start site, and the TFB-recognition element BRE located upstream and adjacent to the TATA-box (7,8). In all three vac-regions, the two promoters PA and PD are located upstream of the oppositely oriented gvpA and gvpD reading frames, and in the case of p-vac and mc-vac, the two BRE sequences are separated by 35 nt only (Figure 1A) (2). In contrast, the respective two promoters of c-vac are separated by 56 nt due to a 21-nt insertion upstream of Pcv-BRE. Except for Pcv, all of the PA and PD promoters are activated by GvpE (9–11). Besides studying the mRNAs by northern analysis in the original wild-type strains, these promoters have been analysed in Haloferax volcanii transformants using promoter fusions with bgaH as reporter gene encoding an enzyme with β-galactosidase activity (11–13). From the analyses of transformants containing the Pmca and Ppa-bgaH constructs it appears that cGvpE is the strongest activator followed by mcGvpE and pGvpE (11).

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Two scanning mutageneses have been performed to determine the sequence elements required for the basal transcription and for the GvpE-mediated activation in vivo. The first series of 4-nt mutations encompassed a 50-nt region upstream of the transcription start site of p-gvpA, and the results of this study clearly define the TATA-box and BRE in p-pA, but also a region around position –10 that might serve as additional contact site of TFB (9,14). Mutations close to the 5′-end of this 50-nt sequence also affected the GvpE-mediated activation in p-pA (most strongly by altering the sequence AACCA upstream of BRE), but the mutations between the TATA-box and the start site of transcription had no effect on the GvpE-mediated activation (9).

The Pmc-A promoter of mc-vac was used for the second 4-nt scanning mutagenesis encompassing the sequences upstream of the Pmc-A-BRE, and this study defined the 20-nt sequence TGAAGCGG-n4-TGAACCAA adjacent to BRE as important for the GvpE-mediated activation (Figure 1A) (13). A significant drop in the GvpE-induced activity was observed when two 8-nt portions of this element (labelled 1 and 2 in Figure 1) are mutated in steps of 4-nt, but a residual activity of 6–20% is still observed (BgaH activity quantified by ONPG assay). In silico analysis suggests similar 20-nt elements in all three P-A promoters and implies that they might serve as upstream activator sequence (UAS) for the GvpE-mediated activation (Figure 1A). The AACCA sequence mentioned above is part of the proximal 8-nt portion of this element in P-pA, and mutations up to CTGG had only a minor effect on the GvpE-induction in p-vac (9). Whether similar 20-nt UAS elements also occur in the GvpE-activated P-D promoters is not yet known and topic of the present report. In silico

**Figure 1.** Comparison of the three intergenic regions between P-pA and P-pD in different vac-regions, and alignment of the 8-nt UAS portions. (A) The vac regions are designated mc (mc-vac), p (p-vac) and c (c-vac). The TATA-box and BRE elements (in italics) are shaded in grey. The sequence determined as GvpE-responsive element (UAS) in PmcD is in bold and underlined, and the two conserved 8-nt portions are labelled 1 and 2. Related sequences in p-vac and c-vac are also underlined. (B) Divergent double-stranded P-pA–P-pD promoter sequence with the two putative UAS underlined. Arrows point to the direction of transcription. (C) Alignment of the 8-nt portions of the putative UAS elements found in the P-pA and P-pD promoters. The conserved sequences are shaded in grey. The distal (1) and the proximal (2) 8-nt portions of the UAS are indicated.

The Pmc-A promoter served as template in the third PCR with primer pair 5′-p-gvpA–XbaI plus p-gvpA–XbaI leader regions. The resulting subfragments served as templates in the third PCR with primer pair 5′-p-gvpA–XbaI plus 3′-pD2ΔL–BgaH to yield the fusion product that was used to substitute the P-pD promoter in P-pA–BgaH–pBluescriptSK+ resulting in P-A–pDbluescript. This construct served (i) as template for the amplification of the XbaI–BamHI fragment PpA–BgaH using the primers 3′-PpD(2) and 5′-PpD–HindIII (Table 1), (ii) for the construction of the initial pApD(UGA) to investigate the activities of both oppositely oriented promoters that turned out to be not useful due to the GvpA production and (iii) as template to construct pApD(UGA) to investigate the activities simultaneously in H. volcanii transformants.

**MATERIALS AND METHODS**

**Constructs used for transformation of H. volcanii WFD11**

The H. volcanii medium and growth conditions, the various E. coli constructs (gvpE fused to the ferredoxin promoter Pfix in the expression vector pJAS35), and also the P-gvpA promoter has been described (4,5,11). The construct pH1–bgaH contains the leaderless p-gvpA reading frame fused to the P-A–P-D promoter region, and bgaH fused to the transcript start site of p-gvpD (without the p-gvpD mRNA leader region) in the Escherichia coli vector pBluescriptSK+. The bgaH reading frame includes six codons for additional amino acids at the N-terminus to ensure the production of an active BgaH enzyme (11). The construction of p-A–p-D involved the amplification of the leaderless p-gvpA in two PCR reactions using p-vac as template and the primer pairs 3′-pD2ΔL–bgaH plus pA-Dlo-18, or 5′-gvpA–XbaI plus pA-Dl-18 (Table 1). The resulting subfragments served as templates in the third PCR with primer pair 5′-p-gvpA–XbaI plus 3′-pD2ΔL–bgaH to yield the fusion product that was used to substitute the P-pD promoter in P-pA–BgaH–pBluescriptSK+. The resulting construct (i) as template for the amplification of the XbaI–BamHI fragment PpA–BgaH using the primers 3′-PpD(2) and 5′-PpD–HindIII (Table 1), (ii) for the construction of the initial pApD(UGA) to investigate the activities of both oppositely oriented promoters that turned out to be not useful due to the GvpA production and (iii) as template to construct pApD(UGA) to investigate the activities simultaneously in H. volcanii transformants.
Table 1. Primers used to construct the P_{pd} and pApD promoter mutants

| Primer      | Sequence in 5'-3' direction* |
|------------|-------------------------------|
| 3’pdD2A1-bgaH | CCAACTGCGATAGAATCCTTCGC         |
| pA-Dlo-18   | TAGTGGTGGAGATGATCGGCAGCC        |
| pA-Dlu-18   | GGTGGGCGATACCTCATTACTA         |
| 5’-PpD-HindIII | GCAAGCTCTACTATGTTTCCG          |
| 5’p-gvpA-Xbal | ATAGTACTTCTAGACAAGCTTGACCTTCC  |
| 3’-PpD(2)   | CGTAAGGAGGTTGATCCGGTG          |
| 5’-PpD(3)   | GTGAAACACTACAGGTGCTTCGA        |
| 5’-PpD(4)   | TACAACAGCTTGCGTAAGGAGTG        |
| 5’-PpD-M1   | TTATGTTGACACTGTGFATGCTCTATTGA |
| 5’-PpD-M2   | GTGGTTAGACACTGCTTGTAATTCGA    |
| 5’-PpD-M3II | GTGGTTAGACACTGCTTGTAATTCGA    |
| 5’-PpD-M4   | GTGGTTAGACACTGCTTGTAATTCGA    |
| 5’-PpD-M5   | GTGGTTAGACACTGCTTGTAATTCGA    |
| 5’-PpD-M6   | GTGGTTAGACACTGCTTGTAATTCGA    |
| 5’-PpD-M7   | GTGGTTAGACACTGCTTGTAATTCGA    |
| 5’-PpD-M8   | GTGGTTAGACACTGCTTGTAATTCGA    |
| 5’-PpD-M9   | GTGGTTAGACACTGCTTGTAATTCGA    |
| 3’-PpA(WT)  | TACATCTTATTTGCAGACTGCC         |
| gvpA+UGA 5’ | CTCTGCGGAGATTAGTGTTCAG         |
| gvpA+UGA 3’ | CGATCACTACAAGTTGAGAAGTCGTG     |
| 5’-PpD+3    | CAGCTGTCAATGGTCCAAATGAGT        |
| 5’-PpD+6    | CAGCTGTCAATGGTCCAAATGAGT        |
| 5’-PpD-ΔIII | CAGCTGTCAATGGTCCAAATGAGT        |
| 5’-PpD-ΔIII | CAGCTGTCAATGGTCCAAATGAGT        |

*Substitutions marked in bold, insertions underlined, restriction sites in italics.

The P_{pd}-bgaH fragment inserted into pWL102 served as wild type in the P_{pd} mutation studies. The P_{pd}-bgaH promoter mutants P_{pd}-M1 through P_{pd}-M8 each contained a 5-nt substitution upstream of P_{pd}-BRE. All mutants were constructed by PCR using P_{pd}-bgaH in pBluescriptSK+ as template and primer 3’-PpD(4) plus the respective 5’-mutation primer harbouring the 5-nt substitutions (Table 1). The promoter mutants P_{pd}+3, P_{pd}+5, P_{pd}ΔIII and P_{pd}ΔIII with insertions or deletions upstream of BRE were constructed using P_{pd}-bgaH and primer 3’-PpD(3) plus the respective 5’-mutation primers (Table 1). All mutant-P_{pd}-bgaH fragments were transferred to pWL102 as XbaI–BamHI fragment.

Construct pApD_{UGA} (3 UGA-stop codons introduced in p-gvpA (position 28) was constructed using primer pair gvpA-UGA-5’ and gvpA-UGA-3’ for the PCR (Table 1). Promoter mutants harbouring 5-nt substitutions (pApD–M1 through M8, identical to those described for pD above) and an additional mutant pApD–M9 were constructed by PCR using pApD_{UGA} as template together with primer 3’-PpA(WT) or 3’-PpD–M9 plus the respective 5’-mutation primer 5’-PpD-M1 through M9 (Table 1). The resulting XbaI–BamHI gvpA–P_{pd}-bgaH fragments were transferred to pWL102 and used to transform H. volcanii.

The P_{mc}-bgaH mutant pA1+1 (substitution of the proximal UAS portion 2 by portion 1) and pA1+Δ2 (proximal UAS portion mutated) were produced by PCR using pA–bgaH in pBluescriptSK+ as template and primer 3’-PpA(WT) or 3’-PpD–M9 plus the respective 5’-mutation primer 5’-PpD-M1 through M9 (Table 1). The resulting XbaI–BamHI gvpA–P_{mc}-bgaH fragments were transferred to pWL102 and used to transform H. volcanii.

The P_{mc}-bgaH mutants were constructed in a similar way by PCR. Mutant mcA–InsI (TGT-insertion adjacent to BRE) was constructed using primer pair mcA–Xbal and InsForward for the first PCR, and mcA–NcoI2a and InsReverse for the second PCR (Table 2). The third PCR was done with primers mcA–Xbal and mcA–NcoI2a. The mcA–InsII mutant was produced using primers InsII-forward and InsII-reverse together with the primers mcA–Xbal and mcA–NcoI2a (Table 2). Construct mcA–Del was

| Primer      | Sequence in 5'-3' direction* |
|------------|-------------------------------|
| mcA-Xbal   | CCAACTAATCTAGATGTGGAC         |
| mcA-InsI-forward | CAACGTGACGATATGGATTTTAC     |
| mcA-InsI-reverse | TTCGATCTGGTTCACGCCAC        |
| mcA-InsII-forward | TTCGAATCGAACACACAGAGTG     |
| mcA-InsII-reverse | TTCGATCTGGTTCACGCCAC        |
| mcA-Del-forward | GTTACAACGGTTCACCTCC         |
| mcA-Del-reverse | ACAGT/GTGACACACAGAGGTGTA    |
| mcA-Sub-forward | GGTTCTCCACCTTGGCCTGATGAC    |
| mcA-Sub-reverse | GTCAATCACAGCAAGGTTAGACCT    |
| 5’-XbaI-PmcD | GCAGATTAACACTGAGTGTGGGATTTG |
| 3’-Nco-pA(1-5)-PmcD | CTGGCCATGAGCTCGGTCGCGGAT  |
| pA+10nt 5’ | CCAATGGTACGTGAGACACACCCCTAT  |
| pA+10nt 3’ | GGTGATGCGCAGTCAATGCTGACCAGC |
| 5’-pA1+1 | GCAGATTAACACTGAGTGTGGGATTTG |
| 5’-pA2+2 | GATGAAACACCTGTTGAAACACCAT  |
| 5’-pA1+Δ2 | GCTGGTGATGAGACACACCCCTATAG  |
| 5’-pAΔ1+2 | GAGTTCACTACCTGATGGAACACCAT  |
| 3’-pA1 | AGTTGATGAGACACACCCCTATAG  |
| 3’-pA2 | TCTCTGAATGATGCTTGTTCCTTAAG |

*Substitutions marked in bold, insertions underlined, slash marks position of deletion, restriction sites in italics.
produced using the primers Del-reverse and Del-forward, and construct mcA–Sub using Sub-reverse and Sub-forward. Construct PmcDL–bgaH was obtained using the mc-vac region in pBluescriptSK + as template and primer pair S'–Xba–PmcD and 3'–Nco–pA(1–5)–PmcD (Table 2). The fragment contains the FmcD promoter region plus the region encoding the mRNA leader of mc-gvpD. All these promoter fragments were fused as XbaI–NcoI fragments to the bgaH reading frame in pBluescript and transferred as XbaI–BamHI fragment to pWL102 that was used to transform H. volcanii. All promoter sequences and fusions were controlled by DNA sequencing. The accession numbers of the p-vac subfragments in Genbank are X64729 (p-gvpACNO), X55648 (p-gvpDFGHIIJLM) and X64701 of the mc-vac region.

Selection of transformants and BgaH assays

Prior to the transformation of H. volcanii each construct was passaged through the E. coli dam strain GM 1674 to avoid a halobacterial restriction barrier. Transformation was done as described (15), and transformants were selected on agar plates containing 6 µg mevinolin ml⁻¹ (pWL102) and/or 0.2 µg novobiocin ml⁻¹ (pJAS35). Mevinolin is a derivative of lovastatin that was obtained as a generous gift of MSD Sharp & Dohme GmbH (Haar, Germany). The amount and presence of the desired constructs in each transformant were controlled by the analysis of the isolated plasmids. The BgaH activity was measured by ONPG assay in cell lysates of the respective bgaH transformants (16). The protein concentration was determined by the Bradford assay (17) using BSA as standard.

Isolation of RNA and transcript analysis

RNA was isolated from H. volcanii transformants according to (18) from cultures at OD₆₀₀ = 1.8–2.0. Northern analyses involved electrophoresis of 2 or 0.02 µg RNA on denaturing, formaldehyde-containing, 1.2% (w/v) agarose gels, followed by capillary transfer to nylon membranes (17). A strand-specific RNA probe complementary to bgaH mRNA was synthesized using the 2.2-kb bgaH fragment inserted in pBluescriptSK + as template for T7 polymerase. Similarly, a strand-specific p-gvpA RNA probe was produced using p-gvpA inserted in pBluescriptSK + . The RNA was labelled using the DIG RNA Labelling Kit (Roche, Germany). Northern hybridization was carried out as described by Ausubel et al. (17), but the hybridization solution contained 10% (w/v) dextran sulphate (Sigma, Germany), 1% (w/v) SDS and 0.5% (w/v) skim milk powder.

Western analysis and DNA sequence analysis

Samples of H. volcanii and H. volcanii transformants were taken in early stationary growth (after 48–54 h of growth) and processed for protein isolation as described (6). Soluble proteins (20 µg) were separated on 12% tricine SDS–polyacrylamide gels (19). Western analyses were performed as described (17). Two different antisera raised against cGvpE (10) and mcGvpE (4) were used in a dilution of 1:1000 (mcGvpE) or 1:10000 (cGvpE antiserum) in blocking buffer, and the reacting antibodies were detected using the ECL detection system (GE Healthcare, Germany). DNA sequence determination of the fusion constructs was done according to the Sanger method using the Sequi-Therm EXCEL II Long-Read DNA Sequencing Kit-LC protocol (Biozym, Germany). The fragments were separated using a Licor DNA sequencer.

RESULTS

Stimulation of the PpD promoter activity by three different GvpE proteins

In silico analysis of the region upstream of PpD (and PmcD) indicated a sequence element related to the UAS of PmcA that could be a putative GvpE-responsive element (Figure 1C). In contrast, the PpD promoter of c-vac lacking the activation by GvpE did not show such a conserved sequence element (Figure 1C). An initial PmcD–bgaH construct (containing PmcD plus the region encoding the mRNA leader of mc-gvpD fused to bgaH) did not yield detectable BgaH activities in H. volcanii transformants. However, the PpD–bgaH construct containing the direct fusion of the bgaH reading frame to PpD (‘leaderless’) was useful. Transformants containing PpD–bgaH together with PEx (p-gvpE reading frame expressed under ferredoxin promoter control) produced well detectable amounts of BgaH (35 µU/mg protein). To determine the PpD activity in response to the two other GvpE proteins, cGvpE and mcGvpE, PpD–bgaH was tested in transformants carrying cEx or mcEx. The presence of each GvpE protein was confirmed by western analyses using an antiserum raised against cGvpE (10). The basal PpD promoter activity measured as BgaH activity in the lysate of the PpD–bgaH transformants was with 1.2 µM/mg rather low, but much larger GvpE-induced activities were observed in the lysates of transformants harbouring in addition to PpD–bgaH construct PEx (35 µM/mg), mcEx (410 µM/mg) or cEx (830 µM/mg). These results suggested that cGvpE was the strongest and pGvpE the weakest activator protein, similar to the results already described for the PmcA and PpA promoters (9,11).

Substitution mutagenesis to identify the UAS of the PpD promoter

A 5-nt scanning mutagenesis was performed with PpD–bgaH to identify the UAS element required for the GvpE-mediated activation of PpD. The mutations started upstream of the PpD–BRE and extended to the BRE of the oppositely oriented PpA promoter (Figure 2C). The PpA–TATA-box and further p-gvpA gene sequences were not present in this construct to exclude any activity derived from PpA. Eight mutants (PpD–M1 through PpD–M8) were obtained and investigated for their BgaH activities in H. volcanii transformants (Figure 2A and C). Transformants harbouring the wild-type (or mutant) PpD–bgaH construct plus the native pJAS35 (i.e. without gvpE) were used to determine the respective basal PpD promoter activities, and PpD–bgaH + PEx transformants were used to quantify the pGvpE-induced promoter activities. In each case, the presence of pGvpE was
assessed by western analysis using the antiserum raised against cGvpE, and all of them contained pGvpE in comparable amounts (Figure 2B). Similar basal promoter activities (<3.8 mU/mg protein) were determined for the wild-type $P_{pd}$ and all $P_{pd}$ mutants, indicating that none of these mutations affected the basal promoter activity. With respect to the GvpE-mediated activation, similar BgaH activities as found for wild-type (>84 mU/mg protein) were observed with the $P_{dM6}$, $P_{dM7}$ and $P_{dM8}$, all of which harboured the mutation upstream of the putative $P_{pd}$–UAS element and close to the opposite $P_{pd}$–BRE (Figure 2A and C). Mutants with the alteration closer to $P_{pd}$–BRE yielded reduced BgaH activities. The strongest reductions were found for $P_{dM5}$, $P_{dM4}$ and $P_{dM2}$, whereas only a minor reduction was observed for $P_{dM3}$ carrying the alterations in the 4-nt non-conserved sequence between the two hypothesized 8-nt UAS portions (Figure 2A and C). A minor reduction was also observed with $P_{dM1}$ (nucleotide substitutions adjacent to $P_{pd}$–BRE). These results proved the existence of the expected 20-nt $P_{pd}$–UAS consisting of two 8-nt portions. Alterations in the distal 8-nt portion of the $P_{pd}$–UAS ($P_{dM5}$, $P_{dM4}$) imposed a stronger effect on the $P_{pd}$ promoter activity than alterations in the proximal 8-nt portion ($P_{dM2}$, $P_{dM1}$) (Figure 2A). In summary, the region affecting the GvpE-mediated activation of $P_{pd}$ corresponded very well to the putative UAS element deduced from the comparison to $P_{mar}$.

**Substitution mutagenesis and simultaneous activation of $P_{pd}$ and $P_{pa}$**

A similar 5-nt scanning mutagenesis was performed using a dual reporter gene construct containing the native $P_{pa}$–$P_{pd}$ promoter region. The initial $pApD$ construct harboured this promoter region with $P_{pd}$ fused to $bgaH$ and $P_{pa}$ to the leaderless $p-gvpA$ reading frame. Transformants containing $pApD$ by itself were obtained in large numbers, but only a few colonies of the $pApD$–pEex transformants were found. All of them produced a low level of BgaH (7 mU/mg; $pApD$, Figure 3A) and contained strongly reduced amounts of the plasmids (data not shown). Most likely these transformants were unable to tolerate the large amounts of the hydrophobic GvpA protein produced after the induction by pGvpE. To circumvent this problem, three UGA stop codons were introduced in all three reading frames in the 5′ region of $gvpA$ in $pApD$ to prevent the translation of the $p-gvpA$ mRNA. The transformants containing $pApD_{UGA}$–pEex were easily obtained and produced the expected enhanced amounts of BgaH (UGA, Figure 3A). This $pApD_{UGA}$ construct was used as template for the 5-nt scanning mutagenesis encompassing the 35-nt of intervening sequences located between $P_{pa}$–BRE and $P_{pd}$–BRE. The nucleotides altered in constructs $pApD-M1$ through $pApD-M8$ were identical to the ones altered in the $pApD$–BRE, and for completion mutant $pApD-M9$ with an alteration close to $P_{pa}$–BRE was constructed (Figure 3B). All these $pApD_{UGA}$ mutant constructs were investigated in *H. volcanii* transformants in the presence or absence of pEex. The $P_{pd}$ promoter activities were determined by ONPG assays, and northern analysis was performed to determine the amounts of the $p-gvpA$ transcript indicative for the $P_{pa}$ activity. The presence of pGvpE was assessed by western analysis using the antiserum raised against cGvpE, and all of the pEex transformants contained pGvpE in comparable amounts (data not shown).

The basal $P_{pd}$ promoter activities of the $pApD_{UGA}$ wild-type and mutant constructs were 5–20 mU/mg (data

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**Figure 2.** Analysis of mutants derived from $P_{pd}$–$bgaH$. (A) BgaH activities determined for the $P_{pd}$–$bgaH$ wild type and the mutants in $P_{pd}$–$bgaH$ + pEex transformants, including standard deviations ($n = 4$). (B) Western analysis to confirm the presence of similar amounts of pGvpE in each of these transformants. The label pE depicts GvpE. (C) Sequence of the intergenic region of p-vac with the two oppositely located BRE elements and $P_{pd}$–TATA-box shaded in grey. The arrow points in the direction of $P_{pd}$ transcription. The UAS element determined for $P_{pd}$ is underlined. Dots in the lines underneath depict nucleotides identical to wild type, and the respective nucleotide substitutions are given. The basal and GvpE-induced activities quantified by BgaH assay are given on the right.
not shown). Only mutant \( pApD-M7 \) did not yield any basal \( PpD \) promoter activity, although the \( bgaH \) mRNA was observed in a comparable amount (data not shown). Since the \( pApD-M7 + pEex \) transformant did not show any BgaH activity as well, we assumed that construct \( pApD-M7 \) was defective in the production of an active BgaH (Figure 3A).

The other eight \( pApD_{UGA} \)-mutant constructs confirmed the results described above for the various \( PpD–bgaH \) mutants: \( pApD-M3 \) (intervening 4-nt sequence altered) and \( pApD-M6, M8 \) and \( M9 \) (mutations upstream of the \( PpD–UAS \)) yielded similar GvpE-induced \( PpD \) activities as found for the wild-type \( pApD_{UGA} \) (Figure 3A). Again, the strongest reductions in the \( bgaH \) activities were observed with mutants \( pApD-M5, M4 \) and \( M2 < 31 \mu U/mg \). All these results confirmed the 20-nt UAS upstream of the \( PpD–BRE \) as important for the GvpE-induced \( PpD \) activity.

Total RNA was isolated from each transformant and northern analysis carried out to assess the activity of \( PpA \) (Figure 4). The stable 320-nt \( p-gvpA \) transcript is always produced in large amounts. Thus, only 0.02 \( \mu g \) of total RNA were applied in each case for better visualization of the differences. The analysis of the basal transcription (without GvpE-stimulation) yielded similar amounts of \( p-gvpA \) mRNA except for the \( pApD-M6 \) transfectants that did not produce \( p-gvpA \) mRNA (data not shown). This mutant also lacked the GvpE-induction and was thus regarded as being defective (Figure 4). With respect to the GvpE-mediated induction of \( PpA \) in the other mutants, the transfectants \( pApD-M1, M2 \) and \( M3 \) contained similar amounts of \( p-gvpA \) mRNA as found for wild-type \( pApD_{UGA} \) indicating that the region altered in these mutants was not important for the GvpE-mediated activation of \( PpA \). These mutations were located upstream of the putative 20-nt \( PpA–UAS \) and close to \( PpD–BRE \) (Figures 3B and 4A). The strongest reduction in the amount of \( p-gvpA \) mRNA was seen with mutants \( pApD-M5 \) and \( M4 \), whereas less strongly reduced amounts of

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**Figure 3.** Analysis of \( pApD \) and of mutants derived from the dual reporter construct \( pApD_{UGA} \). (A) BgaH activities determined for the \( PpD–bgaH \) in \( pApD_{UGA} + pEex \) wild type and mutants, including the standard deviations (\( n = 4 \)). \( pApD \) designates the original construct, and UGA the construct containing the 3 UGA stop codons in \( p-gvpA \). The GvpE-induced activities quantified by BgaH assay are given on the right. (B) Sequence of the \( PpD–PpA \) promoter region (given in opposite orientation as in Figure 2) with the two BRE elements and TATA-boxes shaded in grey. The arrows point to the directions of transcription. The two UAS elements determined are underlined, and dots depict identical sequences. The mutations were identical to the ones introduced into the promoter region of \( PpD–bgaH \) (Figure 2). The mutations affecting \( PpD \) are shaded in grey, and mutations affecting \( PpA \) are shaded in yellow.

**Figure 4.** Northern analysis to determine the amount of \( p-gvpA \) mRNA in \( pApD_{UGA} + pEex \) transformants. (A) Nucleotide sequence of the \( PpD–PpA \) promoter region with the two BRE elements and TATA-boxes shaded in grey. The two UAS elements are underlined. Lines and numbers above the sequence depict the various 5-nt mutations in mutants \( pApD-M1 \) through \( M9 \). (B) Total RNA of 0.02 \( \mu g \) (bottom) derived from each transformant were separated on a formaldehyde containing, 1.2% agarose gels and hybridized a \( gvpA \)-specific probe. Amounts and quality of the RNA were checked by staining the 16S and 23S RNA (top; 2 \( \mu g \) RNA applied in this case).
BRE are still important for the initiation of basal transcription. None of these promoters was inducible by GvpE, demonstrating that the distance between UAS and BRE cannot be altered (Figure 5A). Even a 10-nt (= 1 helix turn) insertion between the UAS and BRE performed with PpA–bgaH resulted in the lack of the GvpE-mediated activation (Figure 5C).

Similar analyses were performed with the PmcA–bgaH construct containing the PmcA promoter of H. mediterranei (Figure 5B). Two 3-nt insertions enlarged the distance between UAS and BRE (mcA–InsII), or the space between the two 8-nt portions (mcA–InsI). Both mutants showed a normal basal promoter activity in PmcA–bgaH transformants, but all of them lacked the induction mediated by mcGvpE (Figure 5B). A 3-nt deletion in the spacer between the two 8-nt UAS portions (mcA–Del) led to the loss of the GvpE-induced activity (Figure 5B), similar to the results obtained with Ppd (Figure 5A). Altogether, these results demonstrated that the 4-nt distance between UAS-portions 1 and 2 and a close location of the 20-nt UAS to BRE are crucial for the GvpE-mediated activation. Mutant mcA–Sub contained a completely substituted PmcA–UAS element (Figure 5B). No effect was observed on the basal PmcA activity, but the GvpE-mediated activation was completely abolished, demonstrating that the UAS-element is indeed essential for the GvpE-mediated activation.

Alteration of the proximal or distal 8-nt portion of the UAS

Four additional mutants were constructed with PpA–bgaH to determine the effect of alterations in the two 8-nt portions 1 or 2 on the GvpE-induced expression (Figure 5C). Mutants pA1+2 and pA1l+2 contained one of the 8-nt portions completely substituted, and pA1+1 and pA2+2 contained two identical 8-nt portions of the UAS (Figure 5C). The basal Ppd Promoter activities (determined in the presence of the 'empty' vector pJAS35 in the respective transformants) were in the range of the PpA–bgaH transformant or at the detection limit (0.5 mU/mg), indicating that the basal transcription was not affected (Figure 5C). With respect to the GvpE-induction, pAA1+2 transformants yielded a BgaH activity similar to wild type, suggesting that the proximal 8-nt portion located close to BRE was sufficient for the activation in this single promoter construct. In contrast, the Ppd promoter in pA1+2 was not activated by GvpE underlining that UAS and BRE must be in close contact. The two constructs containing the identical 8-nt portions of the UAS (pA1+1 and pA2+2) yielded slightly reduced (pA2+2, 72% of the BgaH activity of wild type) or stronger reduced GvpE-induced activities (pA1+1, 40%) (Figure 5C). The latter result could be due to the fact that the UAS portion 1 is active in both orientations, and a location of this element next to BRE might disturb the direction of the GvpE-mediated activation of PpA.

These results underlined that both UAS portions were not equal in function, and that one of these UAS portions was required in close contact to BRE to yield a GvpE-stimulated promoter activity.
DISCUSSION

In this report, we investigated the sequences required for the GvpE-mediated activation of the two oppositely oriented promoters, \( P_A \) and \( P_{\alpha} \), driving the expression of the two gvp gene clusters involved in the gas vesicle formation. A scanning mutagenesis previously performed with the related \( P_{mcA} \) of the mc-vac region determined a 20-nt sequence adjacent to \( P_{mcA} \)-BRE sequence as important for the GvpE-mediated activation (13). This 20-nt sequence appears to be conserved between the three different \( P_A \) promoters (Figure 1). A putative \( P_pD^0 \)-UAS should be located at a similar position upstream and adjacent to BRE, and in silico analysis yielded related, but slightly divergent sequences for the two GvpE-activated \( P_pD \) promoters in mc-vac and p-vac.

The UAS element required for the GvpE-induction of \( P_pD \) was determined by scanning mutagenesis with \( P_{pd} \)-bgaH using eight different 5-nt mutations, and all these variants were investigated in \textit{H. volcanii} transformants in the presence (+pE\textsuperscript{ex}) or absence of pGvpE. None of these mutations abolished the basal transcription initiated at \( P_{pd} \), and the analysis of the transformants containing pE\textsuperscript{ex} in addition clearly defined the region required for the GvpE-mediated activation. As expected, this region was found close to the \( P_pD^0 \)-BRE and consisted of two 8-nt sequence portions spaced by four non-conserved nucleotides. Any mutations further upstream did not affect the GvpE-induction of \( P_pD \). Additional mutants constructed to determine the effect of distance variations showed that the spaces between UAS-BRE and also between the two 8-nt UAS portions were important. Similar results were also observed with distance variants of the \( P_{mcA} \)-UAS, where a 3-nt deletion, or several insertions between UAS and BRE resulted in the loss of the GvpE-mediated promoter activation. Thus, a very close location of UAS and BRE is required for the GvpE-mediated activation. In addition, mutants harbouring a complete substitution of the \( P_{mcA} \)-UAS lacked the activation by GvpE, indicating that the UAS sequence was essential for the GvpE-induced promoter activation. The consensus sequence YGAAAAYGA could be derived from all 8-nt portions of the UAS elements found in the various gvp promoters activated by GvpE. This sequence is specific for the gvp gene clusters and does not occur throughout the genome sequence of \textit{H. salinarum} NRC-1 (20). The close location of UAS and BRE suggests that GvpE could contact proteins of the basal transcription apparatus. It is possible that GvpE enhances the recruitment of TBP and thus activates the transcription initiation, similar to the transcriptional activator Ptr2 of \textit{Methanocaldococcus janaschii} (21,22).

Due to the 35-nt intervening sequence between the BRE sequences of \( P_D \) and \( P_A \), the distal portions of both 20-nt UAS elements almost completely overlap in the centre of the region. The dual reporter construct \( pApD_{UGA} \) was used to analyse the activity of both promoters simultaneously. The \( P_{pd} \)-UAS element determined by the scanning mutagenesis in \( pApD_{UGA} \) was identical to the one determined for \( P_{pd} \)-bgaH, and the northern analysis done with a p-gvpA-specific probe confirmed the \( P_{pa} \)-UAS element for \( P_{pa} \) that was predicted from the comparison with the \( P_{mcA} \) promoter region. These analyses also uncovered interesting features concerning the use and also the importance of the two 8-nt UAS portions. The mutations in both portions affected the GvpE-induced activation of the two promoters in slightly different ways. The activation of \( P_{pa} \) by GvpE was more severely affected when the mutation occurred in the 3’-region of the central 8-nt portion, whereas in the case of \( P_{pd} \) the alterations in the 5’-region of this UAS portion were more effective (Figure 6). The strongest effect on the GvpE-enhanced promoter activity was observed with mutants carrying 4- or 5-nt alterations in this distal 8-nt portion of the UAS element, that thus appeared to be the most important one in the dual promoter construct (Figure 6). A 4-nt sequence could be assigned in the central region where alterations most strongly affected the \( P_{pd} \), \( P_{pa} \), or \( P_{mcA} \) activities; this region is located next to centre and closer to \( P_A \) (Figure 6A and B, marked in bold). The sequence constitutes the 3’- (\( P_{pa} \)) or the 5’-portion (\( P_{pd} \)) of the respective overlapping distal 8-nt UAS portions. This region might be required for the initial binding of GvpE, followed by the occupancy of the respective proximal 8-nt portions of the UAS. In contrast, alterations in the proximal 8-nt portion were less severe on the GvpE-induced promoter activity (Figure 6B). The results obtained with the dual promoter construct were very similar to the results obtained with the two single promoter constructs \( P_{mcA} \)-bgaH (11) and \( P_{pd} \)-bgaH (this study), and demonstrated that both UAS elements including the overlapping portions are indeed functional in \( pApD_{UGA} \). Despite the fact that mutation of UAS portion 1 imposed the strongest effect on the

![Figure 6. Comparison of the various scanning mutageneses performed with the mc-vac (A) and p-vac (B) promoter regions. The columns above the sequences indicate the specific BgaH activities determined for the various mutants (13, and this study). The respective mutations are marked as lines below each column and include the numbers of the various pApD\textsubscript{UGA} mutants in (B). The 8-nt portions of each 20-nt UAS element are underlined, and the TATA-box and BRE are shaded in grey. Arrows point in the direction of transcription. The 4-nt next to the centre of the region that were the most important ones for the GvpE-mediated activation of both promoters are indicated in bold.](image-url)
promoter activation by GvpE, this distal portion of the UAS could be deleted without the loss of the GvpE-mediated activation in the single promoter construct pAA1+2. The reason for this discrepancy is still unclear. In contrast, construct pA1+2 (lacking the proximal UAS portion) did not respond to GvpE, underlining the importance of a close location of at least one UAS portion and BRE for the activation by GvpE.

The promther mutants carrying an UAS consisting of two identical portions yielded a slight reduction of the GvpE-induced activity (72%) in the case of pA2+2, whereas the pA1+1 promoter version resulted in only 40% of the GvpE-induced activity determined for wild type. The latter result implied that the possible use of UAS-portion 1 in two orientations somehow disturbs the recruitment of the basal transcription apparatus by GvpE, if this sequence is placed adjacent to BRE.

In summary, our results confirmed the almost complete overlap of the two distal portions of both UAS regulatory elements in the Pp–PpA promoter region. This arrangement appears to be similar to overlapping operator sequences in the genome of bacteriophage lambda. To our knowledge, this is the first case of such an arrangement of regulatory elements in Archaea. Another promoter region, containing two oppositely oriented promoters that are co-ordinately regulated, has been characterized for the genes encoding the selenium-free [NiFe]-hydrogenases in Methanococcus voltae, but the distance between both TATA-boxes is with 290-nt significantly larger (23). The compact arrangement of the regulatory elements in Pp–PpA might determine the amount and the time point for the activation of the both divergent gvp promoters during the gas vesicle formation, and it might be interesting to investigate the activity of both Pp and PpA promoters with the natural UAS-overlap compared to a construct harbouring two separated UAS elements with respect to the time-resolved expression during growth.

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