GvpD-induced breakdown of the transcriptional activator GvpE of halophilic archaea requires a functional p-loop and an arginine-rich region of GvpD

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The two proteins involved in the regulation of gas vesicle formation in *Haloferax mediterranei*, mcGvpE (activator) and mcGvpD (repressive function), are able to interact *in vitro*. It was also found that the respective proteins cGvpE and cGvpD of *Halobacterium salinarum* and the heterologous pairs mcGvpD–cGvpE and cGvpD–mcGvpE were able to interact. Previously constructed mcGvpD mutants with alterations in regions affecting the repressive function of GvpD (p-loop motif or the two arginine-rich regions bR1 and bR2) were tested for their ability to interact with GvpE, and all still bound GvpE. Even a deletion of or near the p-loop motif in GvpD did not affect this ability to interact. Further deletion variants lacking larger N- or C-terminal portions of mcGvpD yielded that neither the N-terminal region with the p-loop motif nor the C-terminal portion were important for the binding of GvpE, and suggested that the central portion is involved in GvpE binding. The GvpD protein also induces a reduction in the amount of GvpE in *Haloferax volcanii* transformants expressing both genes under *fdx* promoter control on a single plasmid. Such DE^{ex} transformants contain GvpD, but no detectable GvpE, whereas large amounts of GvpE are found in ΔDE^{ex} transformants that have incurred a deletion within the *gvpD* gene. A similar reduction was observed in D^{ex} + E^{ex} transformants harbouring both reading frames under *fdx* promoter control on two different plasmids. GvpD wild-type and also GvpD mutants were tested, and a significant reduction in the amount of GvpE was obtained in the case of GvpD wild-type and the super-repressor mutant GvpD_{3-AAA}. In contrast, transformants harbouring GvpD mutants with alterations in the p-loop motif or the bR1 region still contained GvpE. Since the amount of *gvpE* transcript was not reduced, the reduction occurred at the protein level. These results underlined that a functional p-loop and the arginine-rich region bR1 of GvpD were required for the GvpD-mediated reduction in the amount of GvpE.

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INTRODUCTION

Halobacterium salinarum and *Haloferax mediterranei* produce gas vesicles that enable the cells to float towards the surface of brine. Fourteen *gvp* genes are involved in the formation of these gas-filled proteinaceous particles, arranged as two oppositely oriented clusters, *gvpACNO* and *gvpDEFGHIJKLM* (Englert *et al.*, 1992a; DasSarma *et al.*, 1994). *Hbt. salinarum* PHH1 contains two different *gvp* gene clusters (the p-vac region located on plasmid pHH1, and the c-vac region on the chromosome), but forms spindle-shaped gas vesicles only by the expression of p-vac (Offner *et al.*, 1996). The entire c-vac region is active in p-vac deletion variants, but only during the stationary growth phase (Englert *et al.*, 1992a). Both vac regions are related but

not identical to the *gvp1* (p-vac) and *gvp2* (c-vac) gene clusters of *Hbt. salinarum* NRC-1 (Ng *et al.*, 2000). A third vac region, mc-vac (mc, *mediterranei* chromosomal), is found in *Hfx. mediterranei*, leading to gas vesicles in the stationary growth phase only (Englert *et al.*, 1990).

gvp gene expression depends on the two regulatory proteins, GvpD and GvpE. The 21 kDa GvpE is a transcriptional activator and the larger GvpD protein (54–60 kDa) is required for the repression of gas vesicle formation. GvpE activates the promoters *P_A* and *P_D* located in front of *gvpA* and *gvpD* (Gregor & Pfeifer, 2001; Zimmermann & Pfeifer, 2003; Hofacker *et al.*, 2004). A scanning mutagenesis performed with the 35 bp region upstream of *P_{mcA}*-BRE in the mc-vac region determined that the sequence TGAACGG-n4-TGAACCAA adjacent to the BRE element is important for GvpE-mediated activation, and a related sequence element is found in the promoters *P_{PA}* of p-vac and

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P_{cA} of *c-vac* (Gregor & Pfeifer, 2005). The GvpE protein dimerizes in solution and resembles a basic leucine-zipper protein, as suggested by molecular modelling of the C-terminal portion and mutational analyses (Krüger *et al.*, 1998; Plößer & Pfeifer, 2002).

GvpD has a function in the repression of gas vesicle formation as demonstrated by transformation experiments with *Hfx. volcanii*. This halophilic archaeon lacks *gvp* genes and offers a clean genetic background for these investigations. Transformants containing an *mc-vac* region with an in-frame deletion in the *mc-gvpD* gene (ΔD transformants) are gas vesicle overproducers (Englert *et al.*, 1992b). The amount of gas vesicles is reduced to the wild-type level in $\Delta D + D^{ex}$ transformants, containing in addition to ΔD the *mc-gvpD* reading frame expressed under ferredoxin promoter (P_{fdx}) control in the expression vector pJAS35 (Pfeifer *et al.*, 1994). A p-loop motif (putative nucleotide-binding motif) located near the N terminus of GvpD and two arginine-rich regions (basic regions bR1 and bR2) are essential for the GvpD repressive function (Pfeifer *et al.*, 2001). Comparison of the amino acid sequences of the three different GvpD proteins derived from *p-vac*, *c-vac* and *mc-vac* indicates highly conserved C- and N-terminal domains that are separated by an unconserved region. The size of the unconserved region is 70 aa in mcGvpD of *Hfx. mediterranei*, 61 aa in pGvpD of *p-vac* and 17 aa in cGvpD derived from the *c-vac* region of *Hbt. salinarum* (Englert *et al.*, 1992a). Since the basal activity of the P_{mcA} promoter is not reduced in the presence of GvpD, GvpD appears to have an indirect effect on P_{mcA} promoter activity (Zimmermann & Pfeifer, 2003).

The two regulatory proteins of *Hfx. mediterranei*, mcGvpD and mcGvpE, are able to interact *in vitro* as demonstrated by affinity chromatography (Zimmermann & Pfeifer, 2003). This ability may be part of the regulation of *gvp* expression *in vivo*. *Hfx. volcanii* mcDE^{ex} transformants harbouring both consecutive genes expressed under P_{fdx} control in pJAS35 contain barely detectable amounts of mcGvpE, whereas this protein is present in large amounts in mcE^{ex} and mcADE^{ex} transformants (containing the same in-frame deletion in *mc-gvpD* as found in ΔD). It appears that the presence of mcGvpD leads to a reduction in the amount of mcGvpE in these transformants. It is possible that this GvpD-mediated reducing effect on mcGvpE is initiated by the GvpD–GvpE interaction.

In this report we investigated this hypothesis in further detail. At first we studied whether the two regulatory proteins of the *c-vac* region of *Hbt. salinarum*, cGvpD and cGvpE, and also the heterologous pairs mcGvpD–cGvpE and cGvpD–mcGvpE were able to interact *in vitro*. In addition, previously constructed mcGvpD mutants with alterations in the p-loop motif or in one of the two basic regions were studied with respect to their ability to interact with GvpE. Additional deletion mutants of GvpD were constructed in order to assign the region required for GvpE interaction more precisely. From these analyses it appeared that this region is located neither in the N-terminal portion,

including the p-loop motif, nor in the C-terminal domain. We further studied the influence of the various GvpD mutants on the amount of GvpE in *Hfx. volcanii* transformants. Mutations in the p-loop motif and bR1 abolished the reducing effect of GvpD on GvpE, suggesting that both regions are important for this property.

METHODS

Growth conditions and transformation of *Hfx. volcanii* WFD11. *Haloflex volcanii* WFD11 (W. F. Doolittle, Halifax, Canada) was used for transformation experiments as described previously (Pfeifer & Ghahraman, 1993). Prior to transformation, each construct was passed through the *E. coli* *dam*[−] strain GM1674 (Palmer & Marinus, 1994) to avoid a halobacterial restriction barrier (Holmes *et al.*, 1991). Transformants were selected on agar plates containing 6 µg mevinolin ml^{−1} (for selection of pWL102) and 0.2 µg novobiocin ml^{−1} (for selection of pJAS35). Mevinolin is a derivative of Lovastatin and was obtained as a generous gift of MSD Sharp and Dohme GmbH. The presence of the desired constructs in each transformant was controlled by DNA isolation and restriction analyses.

Constructs used for transformation of *Hfx. volcanii* WFD11. The construction of *mc-gvpD*_{Mut1}[−], *mc-gvpD*_{Mut6}[−], *mc-gvpD*_{AEAE}[−], *mc-gvpD*_{3-ADA}[−] and *mc-gvpD*_{3-AAA}–pJAS35 (Pfeifer *et al.*, 2001), *mc-gvpE*–pJAS35 (mcE^{ex}) (Gregor & Pfeifer, 2001), and *c-gvpE*–pJAS35 (cE^{ex}) (Krüger *et al.*, 1998) has been described previously. The sequences of the oligonucleotide primers used in PCR to construct the deletion derivatives are given in Table 1.

Construct *mc-gvpD*–pJAS35 (mcD^{ex}) (Pfeifer *et al.*, 2001) served as template for the construction of the various *mc-gvpD* deletion mutants. The fragment *mc-gvpD*–Cterm356 (or Cterm233 or Cterm119; the numbers state the number of amino acids derived from GvpD) was amplified by PCR using the oligonucleotides D–Cterm356 (or D–Cterm233 or D–Cterm119) and M13 Forward. The resulting fragments were inserted as *NcoI*–*Acc65I* fragments into pJAS35. The fragment *mc-gvpD*–Nterm318 (or Nterm362 or Nterm447) was obtained by PCR amplification using the oligonucleotides D–Nterm318 (or D–Nterm362 or D–Nterm447) and pJAS, and was inserted as an *NcoI*–*Acc65I* fragment into pJAS35. The Δ p-loop fragment was produced by three consecutive PCRs. The first two PCRs were done using mcD^{ex} as template with the oligonucleotides D–ploop_r and pJAS (or D–ploop_f and M13 Forward) as primer pairs. The resulting fragments were fused in the third PCR using M13 Forward and pJAS. Δ G–L was produced in an analogous manner using the primer pairs D–G32–L35_r and pJAS (or D–G32–L35_f and M13 Forward) for the first two PCRs.

The expression vector pWL_{fdx} contains the P_{fdx} promoter (Pfeifer *et al.*, 1993) as a 73 bp segment in plasmid pWL102 (Lam & Doolittle, 1989). For the construction of this vector, the P_{fdx} promoter was amplified as a 73 bp segment using the pJAS35 vector as template and the oligonucleotides *fdxProm*–*BspHI* and M13 Forward. The fragment was inserted in pWL102, resulting in the pWL_{fdx} vector, carrying *gvpA* as an exchangeable fragment. ORFs can be inserted as *NcoI*–*Acc65I* fragments. The constructs *mc-gvpD*–pWL_{fdx}, *mc-gvpD*_{3-AAA}–pWL_{fdx}, *mc-gvpD*_{Mut6}–pWL_{fdx} and *c-gvpE*–pWL_{fdx} were obtained using the respective *mc-gvpD*[−], *mc-gvpD*_{Mut6}[−], *mc-gvpD*_{3-AAA}[−] and *c-gvpE*–pJAS35 constructs as DNA source to receive the respective *NcoI*–*Acc65I* fragments that were cloned into pWL_{fdx}.

Expression and purification of Gvp_{his} fusion proteins from *E. coli* M15. The constructs *c-gvpD*–pQE8, *c-gvpE*–pQE8 (Krüger & Pfeifer, 1996) and *mc-gvpD*–pQE8, *mc-gvpE*–pQE8 (Zimmermann &

Table 1. Synthetic oligonucleotides used for the amplification by PCR.

Name	Sequence (5'–3')*	Position†
pJAS	CGACACCCGGCGAGAGAGGT	
M13 Forward	GTAACACGACGGCCAGTGAGC	
M13 Reverse	CACACAGGAAACAGCTATGA	
D-Nterm318	TCCC GGTACCTCAGG AAACAACGG	5279–5255
D-Nterm362	CGGTGTC GGTACCTGCCCTATT CCTC	5419–5393
D-Nterm447	GGGGGGTACCT CACTGTTT TCGG	5666–5644
D-Cterm356	CATCGCC ATGGG TGTCGTCACGTTG	4870–4894
D-Cterm233	AACGCC ATGGG CGTCGCCG	5240–5258
D-Cterm119	GAACTCGAGTCC ATGGT CGAGGGAG	5576–5599
D-ploop_f	GGTCACACGCTGCTGTTACAGATA	4405–4462 (Δ 4417–4450)
D-ploop_r	TATCGTGAACAGCAGCGTGTGACC	4462–4405 (Δ 4450–4417)
D-G32-L35_f	ACTGGCGACCCACTCGTTAACGGA	4394–4429 (Δ 4406–4417)
D-G32-L35_r	TCCGTTAACGAGTGGGTCGCCAGT	4429–4394 (Δ 4417–4406)
fdxProm- <i>Bsp</i> HI	GTTATCATGAGGCTTTCGTGG	

*Inserted start or stop codons are shown in bold and restriction sites are given in italics.

†The GenBank accession number of the mc-vac sequence is X64701.

Pfeifer, 2003) were used to produce the Gvp_{his} fusion proteins. The expression of the respective *gvp_{his}* reading frame in vector pQE8 (Qiagen) yields N-terminal hexa-histidine-tagged Gvp proteins. The procedure for the expression and isolation of the Gvp_{his} proteins has been reported previously (Zimmermann & Pfeifer, 2003). For affinity chromatography, the purified Gvp_{his} proteins were dialysed (since purification was carried out under denaturing conditions) against three buffers containing decreasing urea and increasing KCl concentrations in 10 mM Tris/HCl buffer, pH 7.2. Two different refolding procedures were used. (1) Buffer 1, 4 M urea, 0.8 M KCl; buffer 2, 2 M urea, 1.6 M KCl; buffer 3, 2.5 M KCl; used for mcGvpE_{his}, mcGvpD_{his} and cGvpE_{his}. (2) Buffer 1, 4 M urea, 0.8 M KCl, 10 % glycerol; buffer 2, 2 M urea, 1 M KCl, 10 % glycerol; buffer 3, 1 M KCl, 10 % glycerol; used for cGvpD_{his}. The latter buffers contained glycerol and KCl only up to 1 M to avoid precipitation of the cGvpD_{his} protein.

Affinity chromatography with Gvp_{his}-loaded Ni-NTA matrices and cell lysates of *Hfx. volcanii* transformants. Affinity chromatography was carried out as described previously (Zimmermann & Pfeifer, 2003) with the following modifications. Ni-NTA agarose matrix (100 μ l) was loaded with 400 μ g His-tagged Gvp proteins for 1 h at 20 °C and washed twice for 10 min with 1 ml wash buffer 1 (2.5 M KCl, 10 mM Tris/HCl, pH 7.2). The Gvp_{his}-loaded matrix was incubated for 30 min with 2 mg soluble proteins from *Hfx. volcanii* WFD11 transformants expressing the reading frame of the putative binding partner under *P_{fdx}* promoter control in pJAS35. Soluble proteins were isolated in the stationary growth phase. The matrix was washed four times for 10 min with 1 ml wash buffer 2 (wash buffer 1 + 50 mM imidazole). An unspecific binding of the putative binding partner to the Ni-NTA agarose could be avoided by the addition of imidazole to wash buffer 2. Elution of the Gvp_{his} proteins together with the potential interacting partner was carried out by incubating the matrix with 100 μ l elution buffer (0.5 M imidazole, 10 mM Tris/HCl, pH 7.2), two times for 20 min. In each case, 10 μ l of the elution fractions were analysed by Western analysis.

Preparation of crude extracts from *Hfx. volcanii* transformants and Western analysis. To determine the stability of GvpE in D^{ex} + E^{ex} transformants, samples of *Hfx. volcanii* transformants were taken during the exponential and stationary growth phase,

centrifuged at 12 000 g and resuspended in 400 μ l TE buffer containing 1 μ g DNase I ml⁻¹. The suspension was dialysed against 10 mM Tris/HCl, pH 7.2, at 4 °C overnight and centrifuged for 20 min at 12 000 g for membrane removal. The protein concentration was determined by the Bradford assay (Ausubel *et al.*, 1988) using BSA as standard. The soluble proteins (5–20 μ g) were separated on a 12 % Tricine SDS-polyacrylamide gel (Schägger & von Jagow, 1987). Western analyses were performed as described previously (Pfeifer *et al.*, 2001). The GvpD and GvpE proteins were detected by the respective antisera (Krüger & Pfeifer, 1996; Zimmermann & Pfeifer, 2003) at a dilution of 1:1000 in blocking buffer, and the reacting antibodies were detected using the ECL detection system (Amersham Life Science).

For the isolation of soluble proteins from *Hfx. volcanii* transformants for affinity chromatography, cells were harvested at OD₆₀₀ 1.2–1.5, resuspended in 0.1 vol. lysis buffer (2.5 M KCl, 50 mM MgCl₂, 1 mM EDTA, 5 % glycerol and 50 mM Tris/HCl, pH 8.0) and disrupted by sonication. Insoluble fragments and membranes were removed by centrifugation.

Isolation of RNA and transcript analysis. RNA was isolated from *Hfx. volcanii* transformants according to the single step method of Chomczynski & Sacchi (1987). Northern analysis involved electrophoresis of 5 μ g RNA on denaturing, formaldehyde-containing 1 % (w/v) agarose gels, followed by transfer to nylon membranes (Ausubel *et al.*, 1988).

Strand-specific RNA probes were synthesized using a 240 bp *Acc*65I fragment derived from mc-*gvpE* cloned in pJAS35 or a 367 bp *Xcm*I–*Eco*RI fragment derived from mc-*gvpA* cloned in pBluescript as template for the T3/T7 polymerase system. The RNA was labelled using the DIG RNA Labelling Kit from Roche (Germany). Strand-specific DNA probes were synthesized using the c-*gvpE* reading frame cloned in pBluescript-II KS + (Krüger *et al.*, 1998) as template for a PCR with the oligonucleotides E-Ex-*Hind*III and E-Mut-ZIP S (Plößer & Pfeifer, 2002) as primers resulting in a 152 bp fragment which was labelled using the DIG DNA Labelling Mix (Roche). Northern hybridization was carried out as described by Ausubel *et al.* (1988), but the hybridization solution contained 10 % (w/v) dextran sulfate (Sigma), 1 % (w/v) SDS and 0.5 % (w/v) skim milk powder.

DNA sequence analysis. The sequences of all constructs were confirmed by DNA sequencing according to the Sanger method using the Sequi-Therm EXCEL II Long-Read DNA Sequencing Kit-LC protocol (Biozym). The fragments were separated and determined by a Licor DNA sequencer.

RESULTS

Homologous and heterologous interaction of GvpD and GvpE

To determine whether the cGvpD and cGvpE proteins of *Hbt. salinarum*, and also the heterologous pairs mcGvpD–cGvpE and cGvpD–mcGvpE, were able to interact *in vitro*, protein–protein affinity chromatography was performed. One of the two partners was His-tagged and bound to Ni-NTA agarose, and this Gvp_{his} matrix was used to search for putative binding partners in lysates of *Hfx. volcanii* transformants containing the respective reading frame under P_{fdx} promoter control in pJAS35. The His-tagged proteins GvpD_{his} and GvpE_{his} were isolated from *E. coli*, since *Hfx. volcanii* did not yield sufficient amounts of Gvp_{his} protein due to the lack of a strong inducible promoter. The proteins were purified under denaturing conditions in 8 M urea, and renatured by stepwise dialysis against buffers containing decreasing urea concentrations and increasing KCl up to a concentration of 2.5 M (Zimmermann & Pfeifer, 2003). In the case of cGvpD, the concentration of KCl was only increased up to 1 M in 10 % glycerol to avoid protein precipitations. Halophilic archaea use the salt-in strategy to adapt to their high salt environment, and most proteins require high KCl concentrations for a proper function. A successful refolding of halophilic proteins under comparable conditions has been reported for a TBP protein

of *Hbt. salinarum* and the glucose dehydrogenase of *Hfx. mediterranei* (Soppa & Link, 1997; Pire *et al.*, 2001).

The Ni-NTA matrices were either loaded with cGvpE_{his}, mcGvpE_{his} (as positive control) or used as pure matrix (negative control) and incubated with cell lysates of the mcD^{ex} transformant carrying the mc-*gvpD* reading frame expressed under P_{fdx} control in pJAS35. The three matrices were washed extensively and the GvpE_{his} proteins plus putative binding partner were eluted with a buffer containing 0.5 M imidazole. After separation of the proteins by SDS-PAGE, the samples were analysed by Western analysis using an antiserum raised against mcGvpD to determine whether mcGvpD bound to the GvpE_{his} columns. The pure Ni-NTA matrix showed a minor hybridization band due to unspecific binding of GvpD (Fig. 1a). Larger amounts of the mcGvpD protein were found in samples derived from the mcE_{his} matrix, underlining the previously reported results (Zimmermann & Pfeifer, 2003). Also the cE_{his} matrix retained mcGvpD in similar amounts to the mcE_{his} matrix, demonstrating that the heterologous cGvpE protein bound mcGvpD (Fig. 1a). Similar experiments done with a lysate of a cD^{ex} transformant (containing c-*gvpD* derived from c-*vac* in pJAS35) indicated that also cGvpD bound to the mcE_{his} and cE_{his} matrices (data not shown). The reciprocal experiment using mcD_{his} and cD_{his} matrices and cell lysates of cE^{ex} (or mcE^{ex}) transformants containing cGvpE (or mcGvpE) resulted in the binding of cGvpE (or mcGvpE) to both matrices (Fig. 1b and data not shown). The cGvpE monomer as well as the cGvpE dimer (Plößer & Pfeifer, 2002) could be visualized by Western analysis. A third band appeared in the sample of mcD_{his} + cE^{ex}, possibly representing an unspecific binding. In contrast, the pure Ni-NTA matrix did not retain GvpE (Fig. 1b). The results of these experiments suggested that the two regulatory proteins

Fig. 1. Western analyses of the interaction studies using GvpD and GvpE derived from the mc-*vac* or c-*vac* region. In each case, 10 μ l of the two consecutive elution fractions (1, 2) and 20 μ g from soluble proteins of the respective *Hfx. volcanii* transformants were separated on 12 % SDS-polyacrylamide gels, blotted onto nitrocellulose membranes and incubated with an mcGvpD- (a) or cGvpE-specific antiserum (b). The sizes of the protein markers (kDa) are given on the left. Arrows mark the position of the respective Gvp protein bands. (a) Western analysis of samples derived from affinity chromatography of the soluble proteins of an mcD^{ex} transformant incubated with an mcE_{his}-Ni-NTA, a cE_{his}-Ni-NTA or a pure Ni-NTA matrix (p). (b) Western analysis of samples derived from affinity chromatography of a cE^{ex} lysate with a cD_{his}-Ni-NTA, an mcD_{his}-Ni-NTA or a pure Ni-NTA matrix (p).

cGvpD and cGvpE derived from the c-vac region of *Hbt. salinarum* were also able to interact. In addition, this was true for the heterologous pairs of the regulators (mcGvpD–cGvpE and cGvpD–mcGvpE) derived from c-vac of *Hbt. salinarum* or the mc-vac region of *Hfx. mediterranei*. Similar amounts of GvpD protein were recovered from these matrices when homologous or heterologous interaction partners were used.

Interaction studies with p-loop mutants of mcGvpD

The previously constructed p-loop mutants GvpD_{Mut1} (1 aa altered) and GvpD_{Mut6} (3 aa alterations in the p-loop; Pfeifer *et al.*, 2001), and two additional mcGvpD mutants harbouring either a deletion of the p-loop motif (Δ p-loop) or a deletion of the four adjacent amino acids (Δ G–L = Δ G32–L35) (see Fig. 2) were used to investigate whether the p-loop and its surroundings were involved in the GvpD–GvpE interaction. GvpD_{Mut1} and GvpD_{Mut6} are unable to repress the gas vesicle overproduction in *Hfx. volcanii* Δ D transformants (Pfeifer *et al.*, 2001), and the two novel GvpD mutants (Δ p-loop and Δ G–L) were also tested for this feature. The respective genes cloned in pJAS35 were used to complement Δ D transformants.

Gas vesicle production is easily seen by inspecting the phenotype of the colonies on agar plates. The gas-vesicle-negative *Hfx. volcanii* forms red and translucent colonies, whereas the gas vesicle overproducer transformants (Δ D) form pinkish opaque colonies (Fig. 3a). Δ D + D^{ex} transformants contained significantly reduced amounts of gas vesicles compared to Δ D transformants as seen by the

red transparent colonies (Δ D + D^{ex}, Fig. 3a) (Pfeifer *et al.*, 1994, and this report). The respective Δ D + D _{Δ p-loop} and Δ D + D _{Δ G–L} transformants formed pinkish colonies, indicating that both mcGvpD deletion variants (Δ p-loop and Δ G–L) were unable to reduce the amount of gas vesicles (Fig. 3a). Northern analysis indicated large amounts of mc-gvpA mRNA (encoding the major gas vesicle structural protein GvpA) in Δ D transformants, especially in stationary growth phase (Fig. 3b). The amount of this transcript was significantly reduced in the Δ D + D^{ex} transformant, demonstrating that the downregulation occurred at the level of P_{mcA} activity. Both Δ D + D _{Δ p-loop} and Δ D + D _{Δ G–L} transformants yielded large amounts of mc-gvpA mRNA, underlining their defect in the repression of P_{mcA} (Fig. 3b). Methylene-blue stained 23S rRNA indicated that similar amounts of RNA were applied to each slot (Fig. 3b, bottom). Western analysis confirmed the presence of similar amounts of GvpD in all Δ D + D^{ex} transformants, whereas the protein was absent in Δ D transformants as expected (Fig. 3c). Thus, the lack of the repressive activity in Δ D + D _{Δ p-loop} and Δ D + D _{Δ G–L} was indeed due to the respective p-loop deletions and not to an absence of GvpD. These results underlined the importance of the p-loop motif and of the surrounding amino acid sequences for the GvpD repressive function.

Interaction studies using an mcE_{his} matrix showed that all four p-loop mutants, GvpD_{Mut1}, GvpD_{Mut6}, GvpD _{Δ p-loop} and GvpD _{Δ G–L}, were able to bind mcE_{his} (Fig. 3d and data not shown), demonstrating that the region of the p-loop motif in GvpD was not involved in the GvpD–GvpE interaction. A summary of the GvpD mutants and their properties is given in Table 2.

Fig. 2. Schematic representation of mcGvpD and mutant proteins. The p-loop motif, the two basic regions bR1 + bR2 and the unconserved region are indicated. Alterations of previously constructed mcGvpD mutants are shown below (Pfeifer *et al.*, 2001) and numbers designate their position in the 545 aa of GvpD. Their ability (+ +) or inability (–) to reduce the amount of gas vesicles in Δ D + D^{ex} transformants is indicated. Lines below the map depict the various mcGvpD deletion mutants constructed in this report.

Fig. 3. Influence of different deletions within mcGvpD on the repressive function. (a) Vac phenotypes of *Hfx. volcanii* transformants on agar plates. Gas-vesicle-overproducing (Vac^{++}) colonies (ΔD) are pinkish-white and opaque, whereas gas-vesicle-negative (Vac^{-}) colonies (*Hfx. volcanii* or $\Delta D + D$) are red and translucent. The phenotypes of transformants $\Delta D + \Delta ploop$ and $\Delta D + \Delta G-L$ are shown on the right. (b) Northern analysis to detect the *mc-gvpA* transcripts in the transformants. Total RNA (5 μ g) isolated during exponential (e) and stationary (s) growth phases was separated on a 1.2 % denaturing agarose gel and hybridized with an *mc-gvpA*-specific RNA probe. Total RNA of *Hfx. volcanii* WFD11 was used as negative control. The sizes of the RNA marker (kb) are shown on the left and the *mc-gvpA* mRNA is marked on the right. Below is the methylene-blue staining of the nylon membrane used to estimate the quality and quantity of total RNA by visualizing the 23S rRNA. (c) Western analysis to confirm the presence of GvpD in *Hfx. volcanii* transformants. Soluble proteins (20 μ g) from the cytoplasmic fractions derived from the exponential (e) and stationary (s) growth phases were separated on 12 % Tricine SDS-polyacrylamide gels, blotted onto nitrocellulose membrane and incubated with an mcGvpD-specific antiserum. The protein marker sizes (kDa) are given on the left. The 60 kDa mcGvpD is marked by an arrowhead. (d) Western analysis of elution fractions from affinity chromatography of different mcGvpD mutants. A pure Ni-NTA matrix (p) was incubated with the soluble proteins of an ΔD^{ex} transformant. The mcGvpD_{his}-Ni-NTA matrices were incubated with the soluble proteins of ΔD^{ex} or ΔD^{mut} transformants. In each case, 10 μ l of the two consecutive elution fractions (1, 2) and 20 μ g soluble proteins of the *Hfx. volcanii* transformants producing mcGvpD was separated on a 12 % Tricine SDS-polyacrylamide gel. Western analysis was done using an mcGvpD-specific antiserum. The 60 kDa mcGvpD is marked by an arrowhead.

Interaction studies with bR1 and bR2 mutants and further deletion variants of mcGvpD

Similar binding experiments were performed with mcGvpD mutants that incurred alterations in the two basic regions bR1 and bR2 (see Fig. 2; Pfeifer *et al.*, 2001). The bR1 mutant GvpD_{AEAE} and the two bR2 mutants GvpD_{3-ADA} and GvpD_{3-AAA} were analysed for their ability to bind to an mcE_{his} matrix. As demonstrated previously, mutant

GvpD_{3-AAA} acts *in vivo* as a super-repressor of gas vesicle formation, whereas the other two mcGvpD mutant proteins are unable to reduce overexpression in ΔD and cannot repress GvpE-mediated activation of the *mc-gvpA* promoter P_{mca} (Pfeifer *et al.*, 2001). GvpD mutant proteins were produced in similar amounts in the respective transformants as determined by Western analysis using the mcGvpD antiserum (Fig. 4a). Binding studies were performed using mcE_{his}-Ni-NTA matrices. All mcGvpD mutant proteins

Table 2. Stability, activity in repression and interaction of GvpD mutant proteins

Mutants	Presence in <i>Hfx. volcanii</i>	Repression of GV formation	GvpE interaction	Presence of GvpE in D ^{ex} _{mut} + E ^{ex} transformants
GvpD wild-type	+	+	+	–
p-loop: Mut1	+	–	+	+
p-loop: Mut6	(+)	–	+	(±)
bR1: AEAE	+	–	+	+
bR2: 3-AAA	+	++	++	–
bR2: 3-ADA	+	–	+	+
Δp-loop	+	–	+	+
ΔG–L	+	–	+	+
Nterm318	+	–	–	+
Nterm362	+	–	–	+
Nterm447	+	–	+	+
Cterm356	+	–	+	+

were recovered in similar amounts as the mcGvpD wild-type protein or in slightly enhanced amounts in the case of the super-repressor GvpD_{3-AAA}, whereas the pure Ni-NTA agarose bound very minor amounts of mcGvpD (Fig. 4b). These results suggested that mutations in the bR1 and bR2 regions did not abolish GvpE binding.

Additional mcGvpD variants were constructed that contained larger deletions within the 545 aa mcGvpD protein to localize the interaction site more precisely (Fig. 2). The mcGvpD derivatives lacked different parts of the N- or C-terminal domains: Nterm318 consisted of the first 318 aa (36 kDa protein), Nterm362 contained the first 362 aa up to the unconserved region of mcGvpD (41 kDa) and Nterm447 possessed the first 447 aa of mcGvpD (50 kDa). Additional mcGvpD derivatives lacked N-terminal portions: Cterm356 (39 kDa) possessing the last 356 aa after a deletion of the first 189 aa, Cterm233 (26 kDa) consisted of the last 233 aa and Cterm119 (13 kDa) consisted of the conserved C terminus of mcGvpD,

including bR2 (Fig. 2). The presence of the different mcGvpD deletion derivatives was checked by Western analysis in lysates of *Hfx. volcanii* transformants containing the respective reading frames in the expression vector pJAS35. Except for the two smallest variants, Cterm233 and Cterm119, each mcGvpD deletion variant was present in sufficient amounts (data not shown). The two smallest C-terminal variants were almost not detectable by Western analysis, suggesting that both proteins were produced in minor amounts, were unstable in the transformants or were not detected very well by the antiserum (data not shown). Therefore, these variants were not useful for our analyses.

None of these four GvpD deletion derivatives (Nterm318, Nterm362, Nterm447 and Cterm356) was able to reduce gas vesicle formation in ΔD transformants (data not shown). Binding assays performed with the mcE_{his} matrix indicated that the mcGvpD variants Nterm318 and Nterm362 were not retained by mcE_{his}, implying that these portions of mcGvpD were not sufficient for GvpE binding (Fig. 5a, b).

Fig. 4. Western analyses to demonstrate the interaction of mcGvpE with different mcGvpD mutant proteins. The samples were separated on 12% Tricine SDS-polyacrylamide gels, blotted onto nitrocellulose membranes and incubated with the mcGvpD-specific antiserum. Numbers on the left indicate molecular masses in kDa. The arrowhead marks the position of the reacting mcGvpD protein. (a) Western analyses to determine the presence of the different mcGvpD mutant proteins in the cell lysates of *Hfx. volcanii* transformants used for the interaction studies. In each case, 10 (left) or 20 μg (right) soluble proteins was applied. (b) Western analyses of fractions eluted from mcE_{his}-Ni-NTA matrices (mcE_{his}) incubated with the soluble proteins of different mcD^{ex} *Hfx. volcanii* transformants or a pure Ni-NTA matrix (p) incubated with the soluble proteins of an mcD^{ex} transformant. In each case, 10 μl of the two consecutive elution fractions (1, 2) was analysed. As control, 10 μg soluble proteins of the mcD^{ex} transformant (mcD) was applied.

Fig. 5. Western analyses of the elution fractions from affinity chromatography of different mcGvpD deletion mutants: Nterm318 (a), Nterm362 (b), Nterm447 (d) and Cterm356 (d) with mcGvpE_{his}-Ni-NTA matrices. 'p' in panel (b) is a control using a pure Ni-NTA matrix. In each case, 10 µl of the two consecutive elution fractions (1, 2) or the last wash fraction (W) and 20 (b) or 5 µg (a, c, d) soluble proteins of the *Hfx. volcanii* transformants was applied. The samples were separated on 12% Tricine SDS-polyacrylamide gels, blotted onto nitrocellulose membranes and incubated with an mcGvpD-specific antiserum. Numbers on the left indicate molecular masses of the marker proteins in kDa. Arrowheads indicate the position of the expected mcGvpD variant.

The deletion variant Nterm447 (lacking the C-terminal domain) bound mcGvpE_{his} (Fig. 5c), as did the Cterm356 protein, suggesting that each of these proteins harboured the interaction site (Fig. 5d). These results implied that neither the immediate N-terminal region (lacking Cterm356), nor the C-terminal domain (lacking Nterm447) were required for GvpE binding, but rather a central portion close to the unconserved region was involved. These results are summarized in Table 2.

GvpD mutants and the lack of GvpE in D^{ex}+E^{ex} transformants

The DE^{ex} transformants harbouring mc-*gvpDE* as consecutive genes under *P_{fdx}* control contain undetectable amounts of GvpE, whereas GvpE is present in large amounts in ΔDE^{ex} or E^{ex} transformants (Zimmermann & Pfeifer, 2003). These data suggest that the presence of GvpD causes a strong reduction in the amount of GvpE in DE^{ex} transformants.

To investigate this effect of GvpD on the amount of GvpE in further detail, we tested the various mcGvpD mutants for their ability to cause a reduction in the amount or even a lack of the GvpE protein in transformants. To facilitate these analyses, we first investigated whether a strong reduction in the amount of GvpE is also seen when GvpD is produced from a different plasmid (i.e. D^{ex}+E^{ex} versus DE^{ex} transformants). For this purpose, a second expression vector (pWL_{fdx}) was constructed harbouring the ferredoxin promoter *P_{fdx}* as a 73 bp segment in plasmid pWL102 (Lam & Doolittle, 1989) (see Methods). The use of the *P_{fdx}* promoter to express both *gvp* genes should lead to similar amounts of Gvp proteins in the transformants, since both expression vectors (pJAS35 and pWL_{fdx}) are present in comparable amounts in the cell.

In the first series of experiments the mcE^{ex} construct (mc-*gvpE* reading frame expressed in pJAS35) was used to produce mcGvpE, and the pWL_{fdx} vector was used to produce mcGvpD or the respective mcGvpD mutant proteins (D_{pWL-fdx} constructs). The transformants D_{pWL-fdx} (= mcD), D_{pWL-fdx}+pJAS (= mcD+pJAS) and mcE^{ex}+D_{pWL-fdx} (= mcE^{ex}+mcD) were analysed for the presence of mcGvpD by Western analysis (Fig. 6a). In all three transformants, GvpD was detected in similar amounts in both exponential and stationary growth phases. With respect to GvpE, the presence of this protein was analysed in mcE^{ex} and mcE^{ex}+pWL_{fdx} (as controls), as well as in mcE^{ex}+mcD transformants. Similar amounts of mcGvpE were found in the two control transformants mcE^{ex} and mcE^{ex}+pWL_{fdx}, whereas only minor amounts of mcGvpE were detected in the mcE^{ex}+mcD transformants (Fig. 6b). The latter result demonstrated that the amount of mcGvpE was indeed reduced in the presence of mcGvpD and that both genes could be supplied on different vector plasmids to test for this property. The mutants mcGvpD_{Mut6} and mcGvpD_{3-AAA} were analysed in similar experiments. Both contained GvpD, but the GvpD_{Mut6} amount was slightly reduced (Fig. 6a). The transformant mcE^{ex}+D_{Mut6} yielded a slight reduction in the amount of mcGvpE, whereas GvpE was almost undetectable in the mcE^{ex}+D_{3-AAA} transformants (Fig. 6b). The latter result suggested that the presence of the super-repressor protein mcGvpD_{3-AAA} led to a significant reduction in the amount of mcGvpE. The slightly reduced amount of mcGvpE observed in the p-loop defective mcGvpD_{Mut6} could be due to the reduced amount of mcGvpD_{Mut6} observed in this transformant (Fig. 6a). However, similar experiments with the p-loop deletion mutants yielded no reduction in the amount of GvpE (see below), implying that a functional p-loop in GvpD was required to reduce the amount of GvpE in mcE^{ex}+mcD transformants.

Fig. 6. Western and Northern analyses to detect the products of $mcE^{ex}+mcD_{mut}$ (or empty pWL_{fdx}) *Hfx. volcanii* transformants. (a, b) Western analyses using an mcGvpD- (a) or mcGvpE-specific (b) antiserum. Soluble proteins (10 μ g) were isolated during exponential (e) and stationary (s) growth phases, separated on 12% SDS-polyacrylamide gels, blotted onto nitrocellulose membranes and incubated with the appropriate antiserum. Arrowheads indicate the respective reacting Gvp proteins. (c) Northern analysis to detect the mc-*gvpE* transcripts in the $mcE^{ex}+mcD_{mut}$ transformants or an $mcD+pJAS$ transformant used as negative control (neg. control). Total RNA (5 μ g) was electrophoretically separated on a 1.2% denaturing agarose gel and hybridized with an mc-*gvpE*-specific RNA probe. The sizes of the RNA markers (in kb) are shown on the left. Methylene-blue staining of the nylon membrane to estimate the quality and quantity of total RNA by visualizing the 23S rRNA is shown below.

To ensure that the effect on GvpE occurred at the protein level and not at the level of transcription, Northern analysis was performed to determine the amount of mc-*gvpE* mRNA in these transformants (Fig. 6c). Similar amounts of mc-*gvpE* transcripts (in each case more in the stationary growth phase) were seen in mcE^{ex} and $mcE^{ex}+pWL_{fdx}$ transformants, and slightly reduced but comparable amounts of transcript were seen in the $mcE^{ex}+D_{pWL-fdx}$, $mcE^{ex}+D_{Mut6}$ and $mcE^{ex}+D_{3-AAA}$ transformants. The vector pJAS35 lacks a transcriptional termination signal, leading to transcripts that are even longer than the 600 bp mc-*gvpE* transcript. No hybridization signal was detectable in the RNA of the mcE-negative control transformant (Fig. 6c). Since the $mcE^{ex}+D_{pWL-fdx}$, $mcE^{ex}+D_{Mut6}$ and $mcE^{ex}+D_{3-AAA}$ transformants yielded different amounts of GvpE protein but similar amounts of mc-*gvpE* transcript, these results implied that the reducing effect of GvpD did not occur at the level of transcription, but rather at the protein level.

All other mutant mc-*gvpD* genes were only available as pJAS35 constructs (mcD^{ex}). Because of this we analysed the ability of these mutants to reduce the amount of GvpE in a second series of transformants containing the c-*gvpE* reading frame on vector pWL_{fdx} ($mcD^{ex}+cE_{pWL-fdx}$) (Fig. 7a, b). The reducing effect of mcGvpD wild-type on

the amount of cGvpE was clearly seen compared to the $cE_{fdx}+pJAS$ transformant containing instead of mcD^{ex} the empty pJAS35 vector (Fig. 7b). All transformants harbouring the mutated mc-*gvpD* reading frames (mcD_{Mut1} , mcD_{AEEAE} , mcD_{3-ADA} , $mcD_{\Delta p-loop}$, $mcD_{\Delta G-L}$, Cterm356 and Nterm362) produced the respective mcGvpD protein in sufficient amounts (Fig. 7a). With respect to cGvpE, none of these mcGvpD mutants caused a reduction in the amount of cGvpE; this protein was detectable in samples derived from the exponential and stationary growth phases (Fig. 7b). The mcGvpD mutants Nterm318 and Nterm447 did not cause a reduction in the amount of cGvpE either (data not shown). Northern analysis to determine the amount of c-*gvpE* mRNA in each of these transformants yielded comparable amounts of the c-*gvpE* transcripts in all cases (Fig. 7c), again suggesting that the reduction in the amount of GvpE in the presence of GvpD wild-type was caused at the protein level. A summary of these results is given in Table 2.

Since the transformants containing the p-loop mutants mcGvpD $_{\Delta p-loop}$, mcGvpD $_{\Delta G-L}$ or the mutant mcGvpD $_{AEEAE}$ (alterations in the arginine-rich region bR1) contained cGvpE (Fig. 7b), these GvpD mutants were unable to initiate the lack of cGvpE. These results demonstrated that

Fig. 7. Western and Northern analyses to detect the products of $cE_{pWL-fdx} + mcD_{mut}^{ex}$ (or empty pJAS) *Hfx. volcanii* transformants. (a, b) Western analyses using mcGvpD- (a) or cGvpE-specific (b) antiserum. Soluble proteins (10 μ g) were isolated during exponential (a, b top) and stationary (b bottom) growth phases, separated on 12% Tricine SDS-polyacrylamide gels, blotted onto nitrocellulose membranes and incubated with the appropriate antiserum. Arrowheads indicate the respective reacting GvpE proteins. (c) Northern analysis to detect the *c-gvpE* transcripts in the $cE_{pWL-fdx} + mcD_{mut}^{ex}$ transformants or an $mcD + pJAS$ transformant used as negative control (neg. control). Total RNA (5 μ g) isolated during exponential growth phase was electrophoretically separated on a 1.2% denaturing agarose gel and hybridized with a *c-gvpE*-specific DNA probe. The sizes of the RNA markers (in kb) are shown on the left. Methylene-blue staining of the nylon membrane to estimate the quality and quantity of total RNA by visualizing the 23S rRNA is shown below.

an intact p-loop motif and an intact bR1 were required for this property. In the case of the bR2 region, the mcGvpD_{3-ADA} mutant did not affect the amount of cGvpE *in vivo* (Fig. 7b). This was in contrast to the result obtained with the super-repressor protein mcGvpD_{3-AAA} described above, where GvpE was not detectable (Fig. 6b). Both mcGvpD mutants differ only in the alteration of R495 (R495D versus R495A) in bR2, suggesting that a negatively charged amino acid in this position destroyed the ability of GvpD to induce a reduction in the amount of GvpE.

DISCUSSION

The two regulatory proteins mcGvpE and mcGvpD of *Hfx. mediterranei* are able to interact *in vitro* (Zimmermann & Pfeifer, 2003). Using affinity chromatography we could demonstrate that cGvpD and cGvpE of *Hbt. salinarum* also interact, as do the heterologous pairs mcGvpD–cGvpE and cGvpD–mcGvpE. The GvpD proteins of *Hbt. salinarum* and

Hfx. mediterranei exhibit an identity of 71 %, and cGvpE and mcGvpE are 41 % identical to each other (Horne *et al.*, 1991). Because of the observed heterologous interaction one could assume that the region of GvpD involved in the interaction is located in one of the conserved portions and not in the unconserved region that separates the N- and C-terminal domains of GvpD.

Several mcGvpD mutants with alterations in the p-loop motif or the two arginine-rich basic regions bR1 and bR2 (Pfeifer *et al.*, 2001) were also tested *in vitro* for their ability to bind GvpE, but none of them lost the ability to interact with mcGvpE_{his}. The region of the p-loop motif was not required for the interaction since the deletion mutants mcD_{Δploop} and mcD_{ΔG-L} were still able to bind GvpE. The same result was obtained with the mcGvpD deletion variant Cterm356 which had a deletion of the N-terminal 189 aa and still bound mcGvpE, whereas the GvpD mutants Nterm318 and Nterm362 (consisting of the first 318 or 362 aa, including the p-loop motif) were unable to bind

mcGvpE. Thus, the N-terminal portion of GvpD, including the p-loop motif, was not required for GvpE binding. The hypothesis that the conserved C-terminal domain of mcGvpD might be involved in the interaction was ruled out, since the deletion variant Nterm447 (lacking 98 aa of the C terminus) still bound mcGvpE_{his}. These results rather suggested that the interaction site was located in the central portion of mcGvpD. The unconserved region is certainly not involved since cGvpD lacks almost all of this region.

The different mcGvpD mutants were also used to test a second feature of GvpD *in vivo*, namely that the mcGvpE protein is almost undetectable in the presence of mcGvpD in mcDE^{ex} transformants (Zimmermann & Pfeifer, 2003), implying that mcGvpD is able to cause a reduction in the amount of mcGvpE in transformants. To test whether the p-loop motif, bR1 and bR2 are required for this behaviour, transformants were produced containing *gvpD* and *gvpE* on two different plasmids. The mcD_{pWL-fdx} + mcE^{ex} transformant, carrying both reading frames on two separate vector plasmids and expressed under *P_{fdx}* control, contained a significantly lower amount of mcGvpE, whereas mcGvpE was present in large amounts in pWL_{fdx} + mcE^{ex}, i.e. in the absence of mcGvpD. Also, the presence of the super-repressor protein mcGvpD_{3-AAA} resulted in barely detectable amounts of mcGvpE in mcD_{3-AAA} + mcE^{ex} transformants. We further tested the effect of mcGvpD on the heterologous cGvpE in mcD^{ex} + cE_{pWL-fdx} transformants, and the amount of cGvpE was significantly reduced as well. In contrast, the transformants harbouring cE_{pWL-fdx} plus the various mcGvpD mutants D_{Mut1}, D_{Mut6}, D_{Δp-loop}, D_{ΔG-L}, D_{ΔEAE}, D_{Cterm356} or D_{3-ADA} still contained cGvpE in detectable amounts, implying that each of these mutants lost the ability to induce a reduction in the amount of GvpE. Since the amount of *gvpE* transcripts was similar in all of the E^{ex} + D^{ex} and E^{ex} + D^{ex}_{mut} transformants, the reducing effect of GvpD on GvpE must have occurred at the protein level.

In summary, these results suggest that an intact p-loop motif and an intact arginine-rich region bR1 are important for the ability of GvpD to induce a breakdown of GvpE *in vivo*. Interestingly, all mcGvpD mutants unable to reduce the amount of mcGvpE in transformants were also unable to repress the GvpE-mediated activation of the *gvpA* promoter in ΔD transformants. The lack of the GvpE-reducing function in these 'inactive' GvpD proteins is presumably the reason for the overproduction of gas vesicles in these transformants. These results suggest that GvpD is not a gene repressor, but acts at the level of the activator inducing the degradation of this protein.

The exact mode of the GvpD–GvpE interaction is not yet known. It is possible that the interaction results in a phosphorylated or otherwise structurally altered GvpE protein that is degraded faster. However, phosphorylation of GvpE could not be detected. Interestingly, the N-terminal portion of GvpD exhibits a significant similarity to proteins like RadA or KaiC belonging to the RecA superfamily of ATPases (Leipe *et al.*, 2000; Iyer *et al.*, 2004), especially in the

amino acid sequence and spacing of the p-loop motif and the arginine-rich region bR1. The loss of the GvpD function in mutants of the p-loop and bR1 motifs demonstrated that these two regions are important features for the function of this protein. Members of the RecA superfamily of ATPases form hexameric ring structures (or other oligomeric ring structures) and use the binding of ATP in the p-loop region to stabilize this structure. Whether GvpD forms a multimer or hexamer is currently under investigation. Another interesting point is that a subfamily of the AAA + superfamily, the AAA 'unfoldases', associate with proteasomes and catalyse the energy-dependent degradation of proteins (Maupin-Furlow *et al.*, 2004). Future experiments will help to unravel the outcome of the interaction of this unusual pair of regulatory proteins.

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