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NOTE

The lysogenic region of virus ϕCh1: identification of a repressor-operator system and determination of its activity in halophilic *Archaea*

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Abstract ϕCh1 is a temperate virus infecting the haloalkaliphilic archaeon *Natrialba magadii*. As for all temperate viruses, a control of the lysogenic state versus the lytic life cycle is essential. Two open reading frames (ORFs) have been identified as putative repressor encoding genes: ORF48 and ORF49. The protein of ORF48 showed sequence similarities to putative repressor molecules. ORF49 was identified by the analysis of a mutant of ϕCh1: the lysogenic strain carrying mutant ϕCh1-1 showed a different lysis behavior than wild type virus ϕCh1, indicating a dysfunction in the regulation of gene expression. Here, we show that the intergenic region between ORF48 and ORF49 comprises a promoter/operator sequence that is a transcriptionally active region in the model system *Haloferax volcanii*. Transcription from this region can be repressed by the activity of the ORF48 gene product. Gp43/gp44 has an enhancing effect on this regulatory sequence. Evidence is given for a possible binding site of Rep and gp43/gp44 within the coding region of the *rep* gene.

Keywords *Natrialba* · Gene regulation · Haloalkaliphilic · Reporter gene · Halovirus

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Introduction

Archaeal enzymes have become very important tools for industrial purposes (Cowan 1992; Rodriguez-Valera 1992; Guzman-Maldonado and Paredes-Lopez 1995). Not only enzymes isolated from extreme thermophilic *Archaea* but also salt-tolerant enzymes are potentially useful for processes such as refining oil (Rodriguez-Valera 1992). A series of plasmids have been isolated from halophilic *Archaea* during the last years and have been used for cloning and homologous expression of several genes (Lam and Doolittle 1989; Cline and Doolittle 1985; Krebs et al. 1991; Pfeifer et al. 1994; Holmes et al. 1995). Nevertheless, none of these plasmids allows controlled induction of gene expression in their hosts, which would be a prerequisite for the expression of genes encoding toxic proteins. Regulatory elements of archaeal viruses such as transcriptional repressors, origins of replication or strong promoters could constitute key elements of an inducible expression system for halophilic *Archaea*.

For a series of lysogenic phages gene regulation is mediated by the action of transcriptional repressors (best documented for bacteriophage λ; Ptashne 1987). Sequence-specific DNA-binding proteins belonging to the helix-turn-helix family are typical for bacteriophage repressors (Pabo and Sauer 1984) and binding is dependent on the free surrounding ion concentration. A high cation concentration impedes the formation of a DNA-protein complex. Extreme halophilic *Archaea* live in an environment of 3 to 5 M NaCl and the adaptation to such high salt concentrations is achieved by balancing the extra-cellular ionic strength against the intracellular ion concentration (Sehgal and Gibbons 1960; Lanyi and Silverman 1979). Extreme halophiles thereby concentrate predominantly K^+ within the cell and extrude Na^+. Hence the high internal KCl concentrations of 3 to 5 M make such organisms interesting objects for studying protein–DNA interactions at high salt concentrations.

Natrialba magadii is a member of the haloalkaliphilic group of *Archaea* growing at a salt concentration of 3.5 M NaCl and at a pH of 9.5–11. *Nab. magadii* harbors a temperate phage, ϕCh1 (Witte et al. 1997). The genome of ϕCh1 contains at least one putative repressor gene (Klein et al. 2002). As reported for the halophilic virus ϕH, which is closely related to ϕCh1 (Klein et al. 2002), the ϕH repressor protein binds to a direct repeat upstream of its AUG start codon. Therefore, it has been speculated, that binding of ϕH repressor is part of an auto-regulation system (Ken and Hackett 1991). We decided to characterize the putative ϕCh1 repressor gene in order to use this regulatory element as a first component of an expression vector system for extreme haloalkaliphilic and/or halophilic *Archaea*. As reported for the related virus ϕH infecting *Hbt. salinarum* variants and mutants could be isolated by analysis of single plaques that differed in their lysis behavior from the wild type. At least some variants were expected to harbor mutations affecting regulatory genes. A mutant virus, named ϕCh1-1, was isolated and found to differ in lysis kinetics when compared to ϕCh1. The corresponding lysogenic strain, L11-1, showed an earlier onset of lysis and an altered lysis rate. Restriction analysis and subsequent cloning of the corresponding ϕCh1-1 DNA fragment determined a duplication of a 223 bp fragment within ORF49 located adjacent to the repressor-encoding ORF48. The sequence between ORF48 (*rep*) and ORF49 showed promoter activity in *Hfx. volcanii*. Evidence is given that Rep itself seems to act as a repressor and gp43 and/or gp44 as a transcriptional enhancer, which binds to the coding region of ORF48.

Materials and methods

Bacterial strains, plasmids and primers

All strains, plasmids and primers used are listed in Table 1.

Media and growth conditions

Nab. magadii was incubated as reported previously (Witte et al. 1997). *Hfx. volcanii* was grown in 18% modified growth medium (MGM) (Pfeifer et al. 2001) and transformation was performed as described in the literature (Cline and Doolittle 1985; Charlebois et al. 1987). Because of the instability of the lysogenic mutant strain *Nab. magadii* L11-1, the strain was stored as a glycerol stock at –80°C. Repeated passaging of the strain was reduced to a minimum. Plasmid maintenance was achieved by the addition of mevinolin (4 µg/ml) (Holmes and Dyall-Smith 2000). *Escherichia coli* strains were incubated in LB-medium at 37°C. Ampicillin and tetracycline were added to the LB-medium, as required.

Isolation and purification of virus particles

Virus particles and nucleic acids were isolated as described previously (Witte et al. 1997).

DNA manipulations

Most of the DNA manipulations were carried out essentially as described by Sambrook et al. (1989). Restriction enzymes and T4 DNA ligase were obtained from MBI Fermentas. Plasmid DNA was isolated according to Sambrook et al. (1989) or by using Qiagen Mini-Plasmid isolation columns. PCR was performed using *Pwo* polymerase (peqlab). PCR fragments were purified using QIAquick-spin PCR purification columns (Qiagen).

Plasmid isolation from *Hfx. volcanii* cells was performed as follows: 3 ml of the culture was collected by centrifugation and the pellet was resuspended in 50 µl 1 M NaCl. Thereafter, the protocol of Birnboim and Doly (1979) was followed.

Plasmid constructions

Transcriptional fusions with the *bgaH* gene of *Hfx. lucentense* were constructed as follows: the intergenic region between ORF48 and ORF49 was amplified by PCR with primers PR-1 and PR-2 and ϕCh1 as

Table 1 Strains, plasmids and primers

	Relevant marker	Source/reference
Strain		
Nab. magadii:		
L11	wt, ϕCh1 provirus	Witte et al. (1997)
L11-1	Harbors virus variant ϕCh1-1	This study
L13	Cured for ϕCh1	Witte et al. (1997)
Hfx. volcanii:		
WFD11	ΔpHV2	Charlebois et al. (1987)
E. coli:		
XL1-Blue	*endA1, gyrA96, hsdR17* ($r_{k-}m_{K+}$), *lac, recA1, relA1, supE44, thi*, (F́, *lacI*^{q}, *lacZ*ΔM15, *proAB*$^+$, *tet*)	Stratagene
Plasmids		
pKS_{II}^+	mcs, *bla*, ColE1, lacZα	Stratagene
pBgL′	pKS_{II}^+ containing ϕCh1-1 fragment *Bgl*II-L′	This study
pMHL32	*NovR*, *bgaH, bla*, ColE1, pHV2 ori	Holmes and Dyall-Smith (2000)
pMDS24	*bla*, ColE1, *MevR*, DHFR, pHV2 ori	Jolley et al. (1996)
pMI-1	*bla*, ColE1, *MevR*, pHV2 ori, intergenic region, *bgaH*,	This study
pMI-1/43-44	pMI-1, nu. 30091–31004 of ϕCh1 introduced into the *Xba*I site,	This study
pMI-2	*bla*, ColE1, *MevR*, pHV2 ori, *rep*, intergenic region, *bgaH*,	This study
pMI-2/43-44	pMI-2, nu. 30091–31004 of ϕCh1 introduced into the *Xba*I site,	This study
pMI-2Δ	*MevR*, *rep*, intergenic region, *bgaH*, AUG of *rep* deleted	This study
pMI-2-EΔ	*MevR*, *rep*, intergenic region, *bgaH*, nu. 30 of *rep* changed from T to C	This study
pMI-2Δ-EΔ	*MevR*, *rep*, intergenic region, *bgaH*, AUG of *rep* deleted, nu. 30 of *rep* changed from T to C	This study
pMI-2Δ/43-44	pMI-2Δ, nu. 30091–31004 of ϕCh1 introduced into the *Xba*I site	This study
pMI-2Δ-EΔ/43-44	pMI-2Δ-EΔ, nu. 30091–31004 of ϕCh1 introduced into the *Xba*I site	This study

Primers	Sequence
BgL-3	5′-CGCACGATCAGTCCTCT-3′
BgL-4	5′-ATGCATCGCGAGGTCT-3′
PR-1	5′-GACGACGAATTCGTCCGACAACACAATTCC-3′
PR-2	5′-GACGACGGATCCTCCTGGGCCTCTTTG-3′
PR-3	5′-GACGACGAATTCGATGCGATCTCCTCTGG-3′
Rep-hdd-E	5′-GACGACGAATTCATGCATCGCGAGGTCTCCTGGATGAAGCCCGCCG-3′
Rep-hdd-EΔ	5′-GACGACGAATTCATCGCGAGGTCTCCTGGATGAAGCCCGCCG-3′
43-5	5′-CAGCAGTCTAGACGTTGTGCCAGCCGT-3′
44-3	5′-CAGCAGTCTAGACAAACCACAGAACGGACG-3′
Bga-B	5′-TTCCGGATCCTATGACAGTTGGTGTCTGC-3′
Bga-X	5′-TCCGTCTAGAACTCACTCGGACG-3′
Rep-His3c	5′-CGATCGGCGAAGCTTCAGTCCTCTTCGAGAAGGTCTTC-3′
49NcoI-5	5′-CAGCCCATGGTTGCGTTCAGTTCCG-3′
49NcoI-3	5′-CAGCCCATGGTCGAGGCGTCATAAT-3′
Nb16f	5′-GGAGACCATTCCGG-3′
Nb16r	5′-GGATCCGTCTTCCAG-3′
PE-Rep1	5′-TTCCACGGTTTCGGG-3′
PE-Rep2	5′-GGCCATGAAATCCAAGA-3′

Recognition sequences for restriction endonucleases used for cloning are underlined

template DNA. The fragment was restricted with *Eco*RI and *Bam*HI and introduced into plasmid pKS_{II}^+, restricted with *Eco*RI and *Bam*HI, resulting in plasmid pKS-MI-1. In a second step, the *bgaH* gene was amplified using primers Bga-B and Bga-X with plasmid pMLH32 as template. The *Bam*HI/*Xba*I restricted fragment was introduced into the *Bam*HI/*Xba*I digested vector pKS-MI-1 resulting in pKS-MI-1-B. The cloned fragments were recovered by *Hin*dIII/*Xba*I digestion; the *Hin*dIII site was filled in using Klenow polymerase and cloned into pMDS24 restricted with *Sma*I/*Xba*I, thereby removing the rRNA promoter and the dihydrolipoamide dehydrogenase gene. The resulting plasmid was named pMI-1. All constructs used in this study were cloned as described for plasmid pMI-1. To construct the plasmid containing ORF48

(pMI-2), primers PR-2/PR-3 were used. Cleavage of plasmid pKS-MI-2 with *Awa*III and removal of the overhanging nucleotides with T4 DNA polymerase resulted in a deletion of the *rep* start codon. The fragment was religated to create pKS-MI-2Δ.

ORF43 and ORF44 were amplified with primers 43-5 and 44-3 using ϕCh1 DNA as a template (nu. 30091–31004). After restriction with *Xba*I, the fragment was introduced into plasmids pMI-1, pMI-2 and pMI-2Δ that had also been restricted with *Xba*I, resulting in plasmids pMI-1/43-44, pMI-2/43-44 and pMI-2Δ/43-44, respectively. The transcriptional direction of the fragment is the same as for the *bgaH* gene.

In order to destroy repeat DR1-1, a single nucleotide exchange (T to C at position 33983 of the ϕCh1 sequence) without changing the encoded amino acid was performed via PCR amplification of the *rep* gene with primers Rep-hdd-E and Rep-hdd-EΔ (without the AUG start codon of *rep*) carrying the mutation in combination with primer Rep-His3c and ϕCh1 DNA or pMI-2Δ as a template. Cloning of the two fragments was analogous to the construction of pMI-2. Thereby, plasmids pMI-2-EΔ, and pMI-2Δ-EΔ were created. The fragment 43-44 was cloned into the *Xba*I site of plasmid pMI-2Δ-EΔ, resulting in vector pMI-2Δ-EΔ/43-44.

ß-Galactosidase assays

ß-Galactosidase (BgaH) activities in *Hfx. volcanii* were determined as described previously (Holmes and Dyall-Smith 2000). The protein concentrations were quantified using the Bradford method (Bradford 1976).

Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR analyses were performed as described previously (Baranyi et al. 2000). Primers used for this experiment were BgL-3 and BgL-4 or 49Nco-5 and 49Nco-3 (Table 1). For control amplifications of the 16S rDNA primers Nb16f and Nb16r were used. All probes containing RNA showed a positive signal (data not shown).

Primer extension and DNA sequencing reaction

The synthetic oligonucleotide primers (PE-Rep2, Table 1), identical with the nu. 33863-33879 of the ϕCh1 sequence or PE-Rep1 (complementary to the nu. 34183-34197 of the ϕCh1 sequence) were used in this study. The primers were 5′-end-labeled with [γ-^{32}P] dATP and used in the extension reactions. 1 pmol of the primer was annealed to 20 µg total RNA in a final volume of 10 µl RT-buffer (50 mM Tris–HCl pH 8.3, 60 mM NaCl, 10 mM $MgCl_2$, 10 mM DTT). Then, 2U AMV reverse transcriptase premixed with 0.15 mM dNTPs were added to each reaction and cDNA synthesis was performed by incubation of the mix for 15 min at 42°C. Reactions were stopped by addition of the loading dye (10 M urea, 1× TBE, traces of bromphenol blue and xylene-cyanol). After heating (5 min, 95°C), aliquots were loaded onto an 8% PAA gel containing 8 M urea. For mapping the transcriptional start point the respective DNA region was sequenced by using the Reader™ DNA Sequencing Kit (MBI Fermentas). Reactions were performed as recommended by the manufacturer: 1.6 pmol denaturated DNA was used for primer annealing, labeling and termination reaction. The gels were run at 25 mA, dried under vacuum and exposed to a Molecular Dynamics Phosphoimager screen for analysis.

Hybridization techniques

Southern hybridization was performed as described previously (Klein et al. 2000).

DNA sequencing and sequence analysis

Sequencing was performed by VBC-Genomics on a LI-COR DNA sequencer (model 4200) using IR-labeled oligonucleotides (700 and 800 nm). Sequence similarity searches were performed using the BLAST 2.0 program (Altschul et al. 1997). Searches for blocks of conserved sequences were carried out using the BLOCKS software (Henikoff and Henikoff 1994). The mutated sequence of ORF49 was submitted to GenBank (AY525099).

Results

ORF48 shows similarities to known repressor molecules

We have determined the complete 58,498 bp nucleotide sequence of the temperate *Nab. magadii*-infecting virus ϕCh1 (Klein et al. 2002). One of the ORFs, ORF48, showed significant similarities to the repressor of the related virus ϕH, infecting *Hbt. salinarum* and to other putative repressors of *Halobacterium* sp. strain NRC-1 or *Haloarcula marismortui* (Fig. 1). An AA pair Ala^{73}-Gly^{74}, which is conserved among repressors of a number of bacteriophages (Raymond-Denise and Guillen 1991; Perry et al. 1985), is located in the C-terminal part of the ORF48 gene product. These

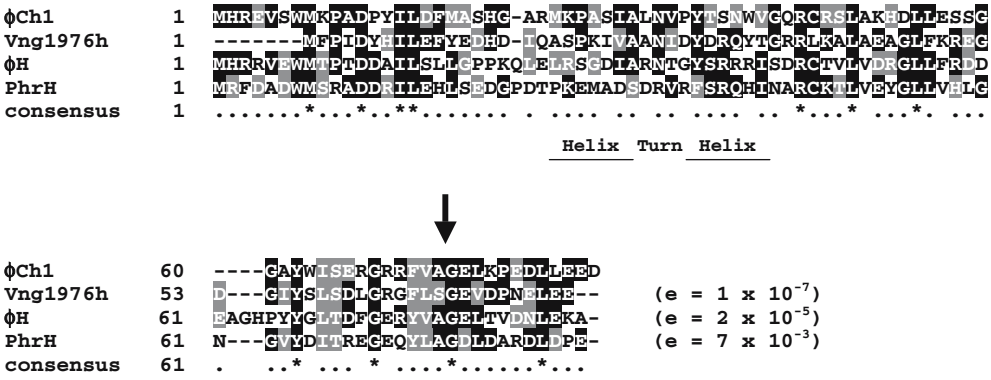

Fig. 1 Sequence similarities between the putative ϕCh1 repressor and halophilic proteins. The putative repressor of ϕCh1 was aligned to the repressor protein of ϕH (Ken and Hackett 1991, accession no. P22562), a repressor like protein of *H. marismortui* (PhrH1, Baliga et al. 2004, accession no. AAV45596) and an unknown protein (Vng1976h, accession no. AAG20151) of *Hbt. salinarum* sp. NRC-1 (Ng et al. 2000). Identical amino acid residues are shaded in *black*, conservative changes are shaded in *gray*. A consensus line is given below the sequences. The helix-turn-helix motif of the repressor of virus ϕH as well as the putative RecA mediated self-cleavage site (*arrow*) is indicated. The e-values (similarities) are given on the left with respect to the putative repressor of ϕCh1

residues have been shown to represent the site of RecA-mediated self-cleavage in the *E. coli* LexA protein as well as in λ and P22 repressors. ORF48 was therefore named *rep*. Rep belongs to the family of winged helix DNA-binding proteins (AA 15-77, e-value: 6×10^{-8}, data not shown). Such DNA-binding proteins share a related winged helix-turn-helix DNA-binding motif, where the loops are small beta-sheets. The DNA-recognition helix makes sequence-specific DNA contacts with the major groove of the DNA, while the wings make different DNA contacts, often with the minor groove or the backbone of the DNA (Gajiwala and Burley 2000). Several winged-helix proteins display an exposed patch of hydrophobic residues thought to mediate protein-protein interactions. Many different proteins with diverse biological functions contain a winged helix DNA-binding domain, including transcriptional repressors such as the biotin repressor, the LexA repressor and the arginine repressor of *E. coli* (Wilson et al. 1992).

ORF49 is involved in gene regulation of ϕCh1

In order to confirm the hypothetical function of ORF48 and/or to detect other elements involved in the regulation of virus ϕCh1 (i.e., a transcriptional repressor), cultures derived from single plaques were inspected for differences in their lysis behaviors. A virus variant termed ϕCh1-1 was isolated and characterized. This variant was identified by an increased plaque formation compared to wild type plaques. Although more than 50 different lysogenic strains obtained after infection of *Nab. magadii* L13 with ϕCh1, the phenotype described for ϕCh1-1 was observed only once. Onset of lysis in strain L11-1 harboring ϕCh1-1 is earlier than in the parental strain L11: while lysis of wild type cultures occurred typically on day 3 to 4 after incubation, the lysogenic strain harboring L11-1 started to lyse on day 2 to day 3 (Fig. 2a). The plaques produced by ϕCh1-1 were larger than those of the wild type virus (data not shown). To confirm that ϕCh1-1 is

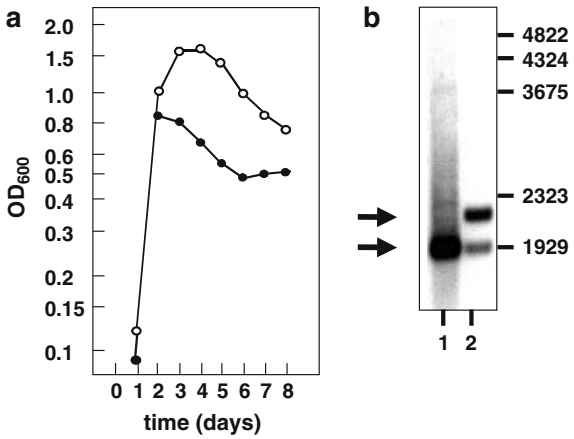

Fig. 2 The difference in lysis behavior of strains *Nab. magadii* L11 and L11-1 is the result of a short duplication within ϕCh1-1. **a** Cells were grown for 8 days with aeration in rich medium. Measuring the optical density at 600 nm monitored growth and lysis of the cells. *Open circle Nab. magadii* L11, *filled circle Nab. magadii* L11-1. **b** DNAs digested with restriction endonucleases were separated, blotted onto a nylon membrane and hybridized with a labeled *Bgl*II-L-fragment of ϕCh1. *Lane 1* ϕCh1 restricted with *Bgl*II, *lane 2* ϕCh1-1 restricted with *Bgl*II. *Arrows* point towards the fragments differing in size in ϕCh1 and ϕCh1-1, respectively. Molecular weight markers are indicated on the *right*

a variant of ϕCh1, electron micrographs were performed and no differences in the morphology could be detected. In addition, the rate of DNA methylation, the RNA content, and the protein pattern were compared (Witte et al. 1997). In all cases, no differences between the wild type and the variant virus were observed (data not shown).

However, restriction analysis of the DNAs isolated from ϕCh1 and ϕCh1-1 showed that in addition to the 1.9 kbp *Bgl*II-L fragment, a larger *Bgl*II fragment of 2.15 kbp (fragment L′) was present in ϕCh1-1. In Southern blots the labeled ϕCh1 *Bgl*II fragment L also recognized the 2.15 kbp band of ϕCh1-1 (Fig. 2b). After a few passages the larger 2.15 kbp *Bgl*II-L′ fragment tended to disappear in favor of the original 1.9 kbp *Bgl*II-L fragment, indicating that the additional sequence within this fragment is rather instable. For halophilic *Archaea*, multiple rearrangements within the chromosomal DNA as well as in the genome of virus ϕH have been reported (Pfeifer et al. 1984; Simsek et al. 1982; Schnabel et al. 1982; Ng et al. 2000). This remarkable instability of the genome has been shown to be due to the presence of a number of insertion elements within the genome of *Hbt. salinarum* and ϕH (Gropp et al. 1992; Ng et al. 2000). No reports concerning the presence of insertion sequences in *Nab. magadii* exist so far. Whether the insertion detected within the DNA of ϕCh1-1 is a result of the action of an insertion element or not is unknown. Further passaging of strain *Nab. magadii* L11-1 resulted in a complete loss of the insertion, as determined by restriction analysis. As a consequence lysis onset and the rate of lysis of the wild type were restored (data not shown), indicating that this region is relevant for the regulation of virus development in *Nab. magadii*.

Sequence analysis of the ϕCh1-1 *Bgl*II-L′-fragment revealed a duplication of 223 bp (nu. 34371–34593 of the ϕCh1 genome) inserted after nu. 34593. This duplication comprises a part of ORF49 and a small portion of its upstream region. The insertion created an additional ORF (named ORF49′) coding for a putative 13.3 kDa protein with a pI of 11.2 composed of the first 38 AA of ORF49 followed by 41 unrelated AA at the C-terminus of ORF49′. This ORF is immediately followed by the original ORF49. Both ORFs slightly overlap each other and are thus likely co-transcribed and co-translated, similar to other slightly overlapping ORFs in the ϕCh1 genome.

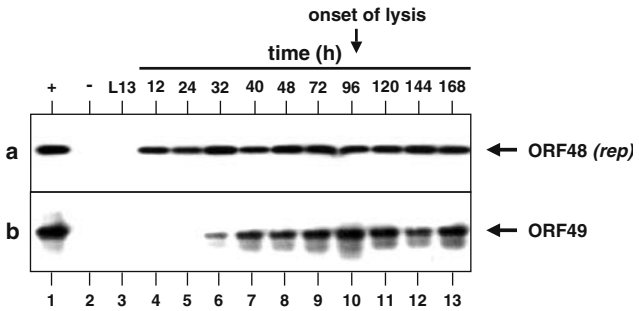

Fig. 3 Detection of ϕCh1 *rep* and ORF49 gene transcripts during the life cycle of *Nab. magadii* L11 using RT-PCR. PCR and RT-PCR products from samples taken at various time points during the life cycle of ϕCh1 in the lysogenic strain L11 were separated on 0.8% agarose gels. To confirm the fragments of the RT-PCR analysis, Southern hybridizations were performed with the *rep* or ORF49 fragment as a probe. **a** RT-PCRs of samples taken at different time points using *rep* gene specific primers BgL-3 and BgL-4. As a hybridization probe, the entire cloned *rep* gene fragment was used. (+), positive control: PCR using ϕ Ch1 genomic DNA as a template; (–), negative control: PCR without a preceding RT-reaction using total RNA isolated from *Nab. magadii* L11 120 h after inoculation; L13, RT-PCR using total RNA isolated from the virus-cured strain *Nab. magadii* L13; 12-168, RT-PCRs using samples taken at different time points (hours) after inoculation of *Nab. magadii* L11. **b** RT-PCRs using the ORF49 gene specific primers 49NcoI-5 and 49NcoI-3. As a hybridization probe, the entire cloned ORF49 gene fragment was used. *Arrows* indicate the products as well as the onset of lysis

Expression of *rep* and ORF49 during the infection cycle of ϕCh1

We have monitored the *rep* gene expression during the infection cycle of ϕCh1 in the lysogenic strain *Nab. magadii* L11 by reverse transcriptase PCR (RT-PCR). In *Nab. magadii* strain L11 *rep* was transcribed throughout the whole cycle of ϕCh1 development (Fig. 3a). Therefore, the *rep* gene of ϕCh1 is—in contrast to a series of other virus encoded transcriptional repressor genes—thought to be constitutively transcribed. Most repressor genes of temperate viruses are only expressed during the lysogenic state. Since the development of ϕCh1 virus particles spans several days mRNA stability is unlikely to be a reason for the presence of *rep* specific transcripts in the late phase of the infection cycle. As *rep* seems to be constitutively expressed, an additional mechanism responsible for gene regulation in ϕCh1 seems to exist. For *Hbt. salinarum* virus ϕH an antisense RNA mechanism has been reported to be involved in gene regulation (Stolt and Zillig 1993a, b).

In contrast to *rep*, ORF49 was not constitutively expressed in the lysogenic strain *Nab. magadii* L11. RT-PCR analysis of total RNA isolated at different time points from strain *Nab. magadii* L11 indicated, that ORF49 was expressed 32 h after inoculation of the strain for the first time. Here, a weak signal was

detected when the culture entered the logarithmic growth phase (Fig. 3b, lane 6). The intensity of the signal increased during the development of ϕCh1 (Fig. 3b, lanes 7–13). The expression profile of ORF49 would indicate that the corresponding gene product is produced in the logarithmic and/or stationary growth phase. However, the trigger of ORF49 expression is unknown so far. As a control RT-PCRs using the 16S rDNA specific primers Nb16f and Nb16r were used (data not shown).

Transcriptional start sites of *rep* and ORF49

rep and ORF49 are arranged in a head-to-head constellation as in other phage systems, such as *c*I and *cro* of phage λ (Ptashne 1987). In order to map promoters located in the ORF48-ORF49 intergentic region we isolated total RNA from *Nab. magadii* L11 and determined the transcriptional start sites within this region by primer extension analysis. The start point of the mRNA for ORF48 (*rep*) was determined as nucleotide 33944 (G) of the ϕCh1 genome, 20 nucleotides upstream of the *rep* AUG start codon. A potential promoter consensus sequence (boxA, 5′-GAATTAAATC-3′) was identified 22 nucleotides upstream of the mRNA start point (data not shown). In addition, the mRNA start point of ORF49 was determined and detected 95 nu. upstream of the ORF49 AUG start codon (Fig. 4, nu. 34385 of the ϕCh1 genome). A putative consensus promoter sequence could be identified 22 nu. upstream of the ORF49 mRNA start site (5′-GTTTTATTAC-3′) and a ribosome-binding site (GAGG) could be detected 7 nu. upstream of ORF49 (Fig. 5). In parallel, primer extension analysis was also performed with total RNA isolated from *Hfx. volcanii* (pMI-1) harboring the ORF48-ORF49 intergenic region. In this construct the original ORF49 has been replaced with the promoterless *Haloferax lucentense* ß-galactosidase gene *bgaH* (Holmes and Dyall-Smith 2000). The primer extension analysis was performed with the primer PE-Rep1 complementary to the upstream region of ORF49. This revealed the same transcriptional start point as determined with total RNA isolated from strain *Nab. magadii* L11 harboring the complete virus (Fig. 4). This indicates that the halophilic model organism *Hfx. volcanii* can be used to study ϕCh1 gene expression.

Promoter activities of the intergenic region between ORF48 (*rep*) and ORF49

The intergenic region between *rep* and ORF49 contains promoter consensus sequences typical for halophilic *Archaea* (Soppa 1999). Possible promoter sequences for *rep* and ORF49 are indicated in Fig. 5. In order to investigate the promoter activity of the intergenic region between *rep* and ORF49 as well as the influence of *rep* on transcription, transcriptional fusions with the *bgaH* gene of *Hfx. lucentense* and several fragments of the intergenic region of ϕCh1 were constructed (Fig. 6a). In Fig. 6a, a part of the ϕCh1 genome is shown (nu. 30091–34833) as well as the different constructs used in this study. In these constructs, the *bgaH* gene replaces the original ORF49. As reported by Patenge et al. (2000) as well as by Gregor and Pfeifer (2001), *bgaH* of *Hfx. lucentense* is a suitable reporter gene for investigating promoter activities within halophilic *Archaea*. Due to the lack of a transformation system for *Nab. magadii* the halophilic archaeon *Hfx. volcanii* was used as a model system. After transformation of *Hfx. volcanii* with the plasmids, expression of the *bgaH* gene was examined by determination of the ß-galactosidase activities (Fig. 6b). Analysis of BgaH activities in strain *Hfx. volcanii* (pMI-1), containing the ORF48-ORF49 intergenic region of ϕCh1, indicated a rather weak expression of *bgaH* at 37°C (22 mU/mg protein, Fig. 6b) compared to the positive control *Hfx. volcanii* (pMLH32) (463 mU/mg protein, Fig. 6b) where *bgaH* expression is under control of its own promoter. However, the

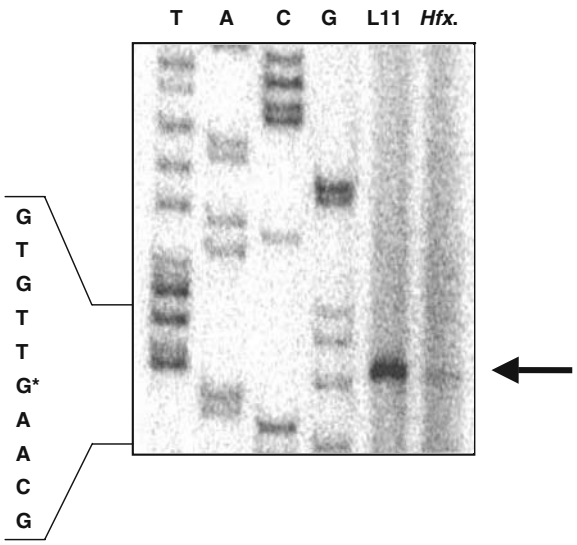

Fig. 4 Primer extension analysis of the intergenic region of ϕ Ch1 in *Nab. magadii* and *Hfx. volcanii.* Primer extension was performed using total RNA from *Nab. magadii* L11 (6 days after inoculation) and *Hfx. volcanii* (pMI-1) (taken at OD_{600} of 0.6) with primer PE-Rep1. The letters *T, A, C,* and *G* represent the dideoxynucleoside triphosphates used for the four sequencing reactions. L11 and *Hfx.* indicate the product of primer extension of samples taken from *Nab. magadii* L11 and *Hfx. volcanii* (pMI-1), respectively. The product is indicated with an *arrow*

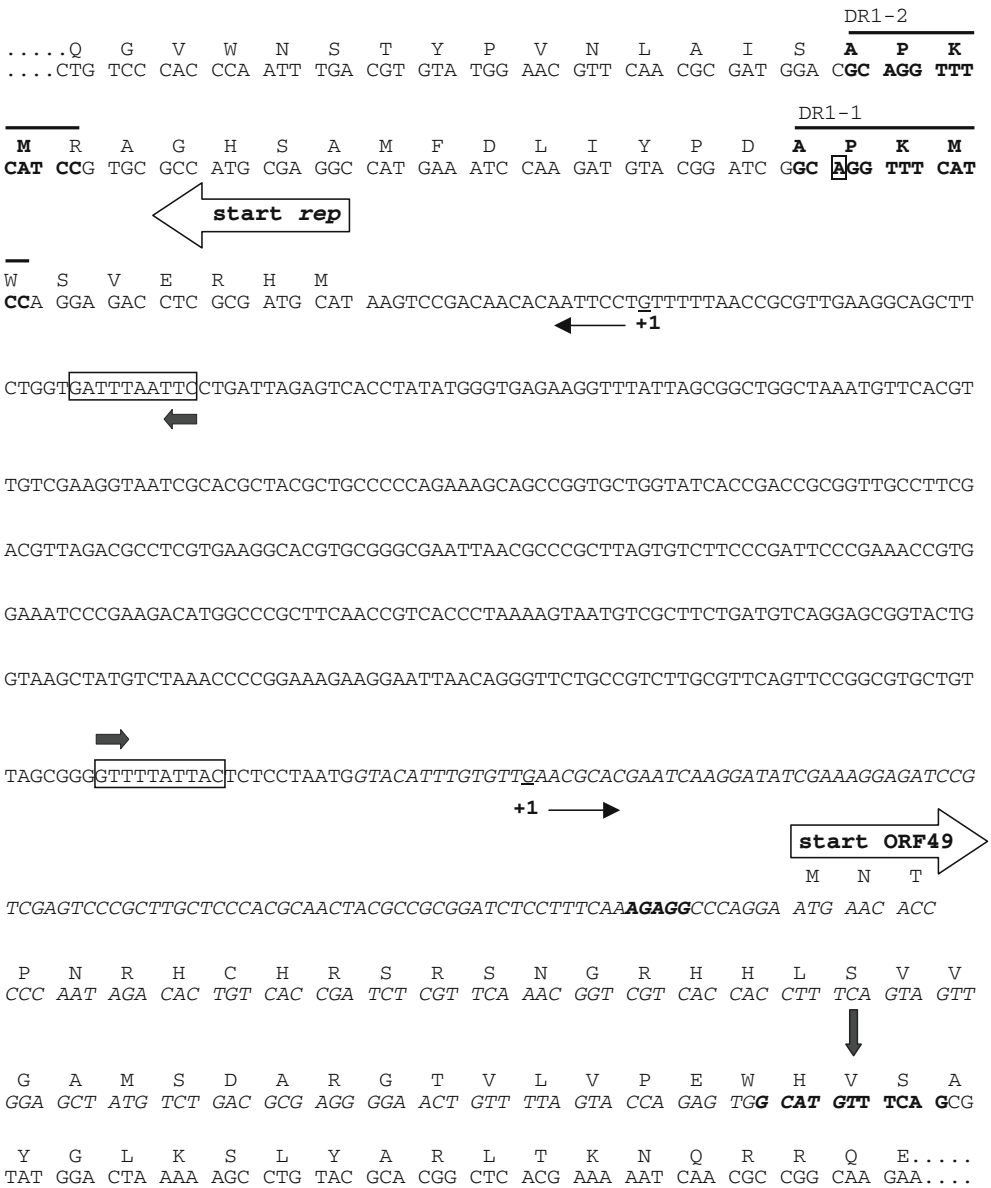

Fig. 5 Sequence of the central part of ϕCh1 containing ORF48 and ORF49. A part of the central region of ϕCh1 is given (nu. 33077–34833, accession number AF440695). The AA sequences of ORF48 and ORF49 are indicated by the *one letter code* and are given above the nucleotide sequence, regardless of the reading frames. *White arrows* indicate the transcriptional direction of *rep* and ORF49. The direct repeats DR1-1 and DR1-2 are indicated above the sequence. The position where a mutation was introduced in repeat DR1-1 is framed. Sequences with similarities to the consensus sequence of halophilic promoters are framed and 5′-ends of mRNAs determined by primer extension analyses are underlined and marked with +1 and the transcriptional direction is indicated with an *arrow*. The sequence occurring twice in ϕCh1-1 is given in *italics* and the insertion point is marked with a *vertical arrow*. A possible ribosome-binding site for ORF49 is given in *bold*

BgaH activity indicated that the intergenic region located on plasmid pMI-1 possesses promoter activity in *Hfx. volcanii*. Activities found for the intergenic region of ϕCh1 were in the same range as reported for several promoters of *Hbt. salinarum* (Patenge et al. 2000). In order to use the lysogenic region as a temperature-regulated expression system for halophilic *Archaea*, we also analyzed the effect of different growth temperatures on the system (Fig. 6b). *Hfx. volcanii* (pMI-1) showed a constant increase of BgaH activities from 6.5 to 22 and 43 mU/mg protein with decreasing temperatures (42, 37, 28°C). This temperature-dependent increase of BgaH activity could not be observed for the positive control *Hfx. volcanii* (pMLH32) (Fig. 6b) where the *bgaH* gene is expressed from its own promoter (Holmes and Dyall-Smith 2000). Therefore, the

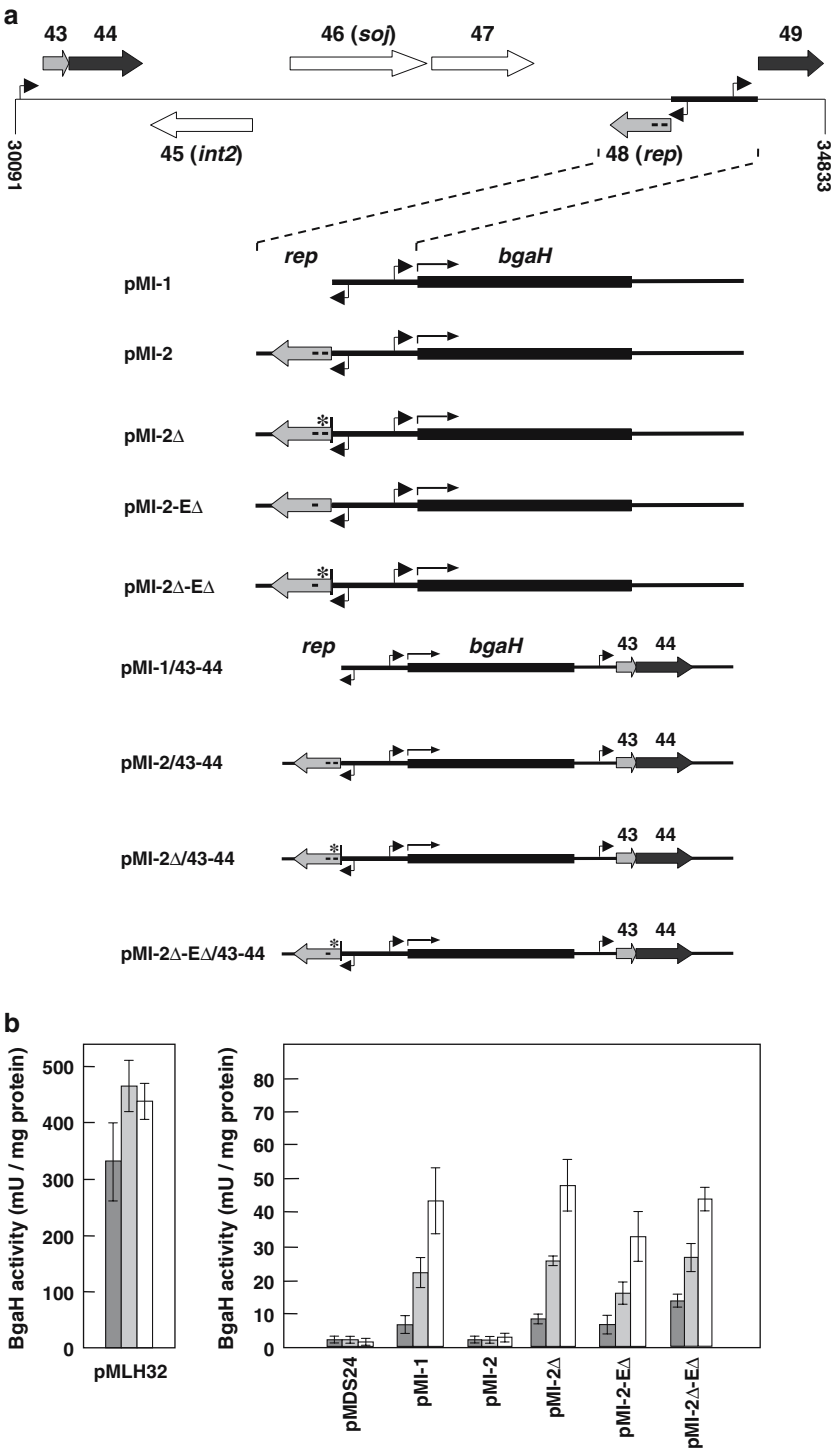

Fig. 6 Determination of promoter activities of the intergenic region between ORF48 (*rep*) and ORF49 in *Hfx. volcanii*. **a** A schematical presentation of the ORF48-intergenic region as well as the *bgaH* containing vectors is given. The halophilic *β*-galactosidase gene *bgaH* is indicated by a *black bar*. The orientation of transcription is indicated by *arrows*. *Bars* indicate the different regions cloned into shuttle vector pMDS24 (names of the corresponding plasmids are given on the *left*). The *horizontal bars within the arrow* representing ORF48 (*rep*) mark the two direct repeats. The *asterisk* and the *vertical bar* indicate the deletion of the AUG start codon of *rep*. **b** Temperature dependent *bgaH* expression. The *Hfx. volcanii* strains were grown in rich medium at different temperatures to an optical density of 0.6 and BgaH activities were determined; *dark gray bars*: 42°C, *gray bars*: 37°C, *white bars*: 28°C. The constructs used are indicated at the bottom. The values of the positive control *Hfx. volcanii* (pMLH32) are given in a distinct chart and in a different scale on the left. *Error bars* are indicated, ±1SD

higher levels of BgaH at lower temperatures could not be the result of a temperature dependent activity of BgaH. In addition, the possibility of different plasmid copy numbers at different temperatures was excluded, as the concentration of plasmid pMI-1 did not vary at different growth temperatures, indicating that no gene dosage effect had occurred (data not shown).

In order to investigate the role of ORF48 (*rep*) on transcription from the ORF49 promoter, *Hfx. volcanii* was transformed with the construct pMI-2 containing the intergenic region as well as the *rep* gene (Fig. 6a). The episomal presence of the *rep* gene completely suppressed the BgaH activity [i.e., to the basic level of 2 mU/mg protein as determined for the negative con-

trol *Hfx. volcanii* (pMDS24)] indicating that ORF48 acts as a transcriptional repressor. To verify the activity of Rep as a repressor protein for this region, the AUG start codon of the *rep* gene was deleted (plasmid pMI-2Δ, Fig. 6a). As a consequence, the activity of BgaH was restored to the same intensity as observed for construct pMI-1, containing the intergenic region but lacking the repressor gene (Fig. 6b). In order to demonstrate *rep* expression in *Hfx. volcanii* (pMI-2) and *Hfx. volcanii* (pMI-2Δ), RT-PCR with *rep*-specific primers was performed. For both strains, the specific signal expected for the *rep* transcript was obtained. For *Hfx. volcanii* (pMI-1), which carries only the intergenic region, no product was detected (data not shown). Taken together the results demonstrated that expression of *bgaH* from the ORF48–ORF49 intergenic region is under control of Rep.

For the closely related virus ϕH it was shown that the putative ϕH repressor protein binds to a direct repeat upstream of its AUG start codon. Therefore, it has been speculated that binding of the ϕH repressor is part of an auto-regulation system (Ken and Hackett 1991). This was also found for repressor molecules in other systems, e.g. for λ CI (Maurer et al. 1980). In the case of ϕCh1 direct repeats upstream of the *rep*-AUG start codon could not be detected. However, repeats can be found within its coding region (Fig. 5). These 13 bp direct repeats (DR1) are separated by a 43 bp spacer and are also evident on the AA level as peptide repeats MKPA (codons 8–11 and 26–29 of the Rep sequence). In order to prove the assumption that Rep may require these repeats for binding to the DNA, the first direct repeat was altered by changing nucleotide 33893 from an adenine residue to a cytosine (nucleotide 30 of the coding region of *rep*) without changing the AA sequence of Rep (plasmid pMI-2-EΔ Fig. 6a). This exchange within the first repeat was sufficient to partially restore the BgaH activity within *Hfx. volcanii* (pMI-2-EΔ) to two-thirds of that of *Hfx. volcanii* (pMI-1) containing only the intergenic region (Fig. 6b). The corresponding control strain *Hfx. volcanii* (pMI-2Δ-EΔ) where the start codon of Rep is also deleted did not show any significant changes compared to *Hfx. volcanii* (pMI-1) (Fig. 6b).

Effects of enhancing elements

Several fragments of the immunity conferring plasmid pϕHL (Gropp et al. 1992) when individually cloned into *Hbt. salinarum* give rise to immunity towards ϕH infection, although with different efficiencies compared to a strain containing the entire pϕHL. In particular the transcripts T9/T10 encode two ORFs of 54 and 131 codons that act co-operatively with the ϕH repressor (Stolt and Zillig 1993c). Since both ORFs exhibit high identities to ORF43 (90%) and ORF44 (94%) of ϕCh1 these ORFs were also investigated for a possible involvement in regulation of ϕCh1 gene expression. ORFs 43 and 44 are located 3512 bp (AUG of ORF43) upstream of ϕCh1 *rep* (Fig. 6a, Klein et al. 2002). Putative promoter sequences were only found upstream of ORF43. Both genes overlap with their respective start and stop codons, suggesting co-transcription and co-translation of both genes (Klein et al. 2002). ORF43-44 was cloned into the constructs used previously (Fig. 6a) and BgaH activities in *Hfx. volcanii* were determined. Strain *Hfx. volcanii* (pMI-1/43-44), containing only the intergenic region without the *rep* gene, exhibited 80% (16.8 mU/μg protein) of the BgaH activity of strain *Hfx. volcanii* (pMI-1). This indicated that gp43 and gp44 do not directly interfere with transcription from this region (Fig. 7). The relevance of ORF43-44 for expression of *bgaH* containing the intergenic region as well as the *rep* gene was investigated in strain *Hfx. volcanii* (pMI-2). As discussed above, almost no *bgaH* expression was determined (Fig. 7). Expression of ORF43-44 raised the BgaH activity to 40% (9 mU/μg protein) of that of strain *Hfx. volcanii* (pMI-1). A dramatic effect of ORF43-44 expression on BgaH activities was seen with strain *Hfx. volcanii* (pMI-2Δ/43-44) containing the intergenic region as well as the *rep* gene lacking the AUG start codon. Here, an increased BgaH activity of 260% (65 mU/μg protein) compared to *Hfx. volcanii* (pMI-2Δ) was observed. The results indicate an enhancing effect of ORF43-44 on *bgaH* expression only in the presence of the *rep* coding sequence. This suggests that the repeats occurring within the *rep* sequence may constitute the binding sites for gp43/gp44. Therefore, the results of *bgaH* expression of strain *Hfx. volcanii* (pMI-1/43-44) are in a good agreement with the suggestion that the repeats are the binding sites of gp43/gp44: here the repeat sequences are missing and no activation of the *bgaH* expression could be observed (Fig. 7). Gp43/gp44 enhanced the *bgaH* expression only when the sequence of *rep* is present (Fig. 7). *bgaH* expression was enhanced in the absence of Rep, and in the case where the repeat sequences are still present. Changing one nucleotide of repeat DR1-1 completely abolished the enhancing effect of ORF43-44 in strain *Hfx. volcanii* (pMI-2Δ-EΔ) (Fig. 7).

Discussion

In order to gain insights into the molecular mechanisms of gene regulation in halophilic *Archaea*, we

focused on a regulatory sequence of the *Nab. magadii* virus ϕCh1. By similarity searches and by analyzing the mutant strain ϕCh1-1, two genes, ORF48 (*rep)* and ORF49, were identified in this study that encode possible regulators of the ϕCh1 life cycle. Both genes are arranged head-to-head and promoter consensus sequences [A and T rich sequences typical for halophilic *Archaea* (Soppa 1999)], are present in the intergenic region between the two genes. Investigation of the ORF48-ORF49 intergenic region in *Hfx. volcanii* revealed promoter activity. Expression of the repressor gene *rep* resulted in a dramatic decrease in the expression level of the reporter gene *bgaH* when transcribed from the ORF49 promoter. Compared with the natural context, these results suggest that Rep, the gene product of ORF48, shuts down expression of ORF49 and thus probably acts as a transcriptional repressor. This is in accordance with protein structure predictions suggesting that Rep belongs to the family of winged helix repressor proteins.

The binding site for the closely related repressor of ϕH was determined by methylation interference assays and the analysis revealed a binding site of four repeats of the consensus sequence RRGAAG, with two copies on each strand. These are arranged as two pairs, each of which forms a short interrupted palindrome (Ken and Hackett 1991). These repeats are localized upstream of the ϕH *rep* gene. Due to the high similarity between ϕH and ϕCh1 (Klein et al. 2002) we looked for palindromic sequences in the ORF48-ORF49 intergenic region of ϕCh1. A most probable repeat sequence (TGYCTTC) was found, arranged in the same manner as observed for ϕH. However, the data obtained from the BgaH measurements indicated an alternative site important for the functioning of ϕCh1 Rep. A silent mutation of a direct repeat (DR1-1) within the very 5′-part of the *rep* coding region that did not change the AA sequence of Rep completely reversed Rep-mediated repression of transcription from the ORF49 promoter.

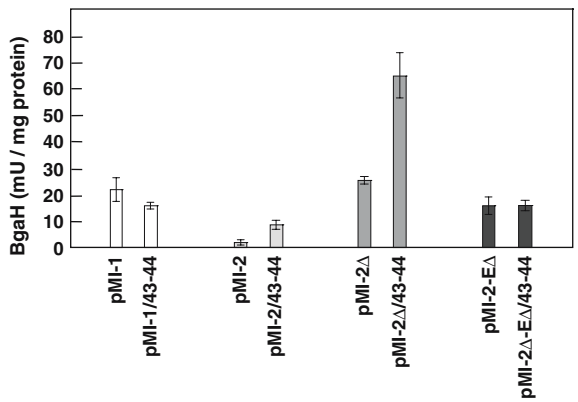

Fig. 7 Enhancing effect of ORF43-44 on transcription from the ORF49 promoter. β-Galactosidase activities were determined with *Hfx. volcanii* cells grown in 18% MGM at 37°C. The constructs used are indicated at the *bottom*. *Error bars* are indicated, ±1SD

A second gene involved in the regulation/progression of the ϕCh1 infection cycle is ORF49. In the *Nab. magadii* mutant lysogen L11-1, which exhibits an earlier onset of lysis, an insertion of 223 bp leads to a duplication of the 5′-part of ORF49 pointing to a role of ORF49 in the switch between the lysogenic and the lytic cycle and/or in the progression of the lytic cycle. Evidence for the importance of ORF49 for the lytic life cycle may also be indicated by the fact that the duplication found in ϕCh1-1 is not stable and has the tendency to revert into the wild type form within three passages of *Nab. magadii* L11-1 inoculations.

ORF49 expression is delayed in the lysogenic strain L11 compared to the expression of *rep* (Fig. 3) but steadily increases over time during the virus life cycle which finally culminates in the production of virus particles and cell lysis. Thus, it is conceivable that the gene product of ORF49 is involved in the switch between the lysogenic and the lytic life cycle or that it encodes a factor that speeds up the progression of the lytic cycle. Such a role would be in accordance with earlier observations that a transcript (T4) of the ϕH immunity conferring plasmid pϕHL that comprises an ORF (nu. 5179–5335 of pϕHL) with high sequence similarity to ORF49 is involved in the activation of the lytic cycle of ϕH (Stolt and Zillig 1994). The synthesis of a shortened variant of gp49 (comprising only one third of gp49) in addition to the original gp49 in L11-1 may possibly intensify the gp49-mediated effect on virus development and cause the earlier onset of lysis.

In our *Hfx. volcanii* model system Rep repressed transcription from the ORF49 promoter. The fact that expression of *rep* is constitutive throughout the entire ϕCh1 life cycle suggests that repression of ORF49 must be overcome with the help of other factors at some point of the viral life cycle in order to allow ORF49 expression and consequently initiation and/or progression of the lytic cycle. In this context it is interesting to note that Rep contains a predicted site for RecA-mediated self-cleavage. Such sites can be found in a group of temperate phage repressors, exemplified by the λ CI repressor, which are cleaved in lysogens upon induction of the SOS response, directly leading to the transition into the lytic phase. Thus, it is also conceivable that at some point of the ϕCh1 life cycle Rep

undergoes self-cleavage, which in turn would result in the induction of ORF49 expression.

Besides the repressor protein, the antisense RNA T_{ant} was identified as a regulatory element in ϕH (Stolt and Zillig 1993b). However, both elements do not act co-operatively. A co-operative element of $Rep_{\phi H}$ was identified within transcripts T9/T10, which encode two ORFs of 54 and 131 codons, respectively (Stolt and Zillig 1993c). The encoded ORFs show sequence similarities to ORF43-44 of ϕCh1. The gene product of ORF43, gp43, does not contain any domain of known function. However, an analysis using the Pfam database with the AA sequence of the ORF44 gene product gp44, revealed a Pin domain (AA 3-126) constituting almost the entire 131 AA protein. In addition this domain also comprises a coiled-coil domain consisting of AA 45-71. The Pin (PilT N terminus of pilus fiber) domain is a compact domain of about 100 amino acids. The domain has two nearly invariant aspartate residues and the eponymous PilT protein forms a coiled-coil with other monomer units to polymerize a pilus fiber (Wall and Kaiser 1999). The Pin domain likely plays a role in signaling (Noguchi et al. 1996) but also belongs to a larger family of predicted nucleotide-binding domains with similarities to 5′-exonucleases (Arcus et al. 2004; Marchler-Bauer et al. 2005).

In the *Hfx. volcanii* model system gp43 and/or gp44 can partially relieve Rep-mediated repression of *bgaH* transcribed from the ORF49 promoter (compare vectors pMI-2 and pMI-2/43-44 in Fig. 7). This effect does not seem to be due to a possible interaction of gp43-44 with and subsequent inhibition of Rep since only the presence of sequence of *rep* but not the protein itself is necessary for gp43-44 to enhance *bgaH* transcription from the ORF49 promoter (compare vectors pMI-2Δ and pMI-2Δ/43-44 in Fig. 7). Therefore, gp43-44 seem to interact directly or via an additional protein with the coding sequence of *rep*. For this interaction, the presence of the direct repeat 1 (DR1-1) in the 5′-part of the *rep* ORF may be important since mutation of DR1-1 decreases gp43-44-mediated enhancement of *bgaH* expression (compare vectors pMI-2Δ/43-44 and pMI-2Δ-EΔ/43-44 in Fig. 7). However, a detailed analysis of this region has to be performed. Taken together, the data implicate a model in which the 5′-part of the *rep* gene including DR1 functions as a *cis* element that positively interferes with transcription from the ORF49 promoter by allowing binding of gp43-44 which in turn enhances transcription of ORF49 by a still un-resolved mechanism. The same sequence also serves as a negative regulatory element by binding of Rep, which in turn shuts down transcription from the ORF49 promoter. Expression of ORF43-44 cannot completely overcome repression through Rep since *bgaH* expression from the ORF49 promoter is much higher in the *Hfx. volcanii* strain that harbors the *rep* coding sequence without producing Rep compared to the strain that, in addition, also produces Rep (compare vectors pMI-2/43-44 and pMI-2Δ/43-44 in Fig. 7). This may be explained by a possibly more potent repressing potency of Rep compared to a possibly rather low enhancing potency of gp43-44 or by different binding affinities of the proteins. Alternatively, it is also conceivable that the competition between Rep and gp43-44 for the binding sites within the *rep* coding region in general does not allow gp43-44-mediated enhancement of transcription that is as efficient as in the absence of Rep.

In general, the similar functional organization of the ϕCh1 viral genome [as well as that of the archaeal head-tail dsDNA viruses ψM2 and ψM100 (Pfister et al. 1998; Luo et al. 2001)] and bacteriophages demonstrates that the modular architecture of this virus group is not restricted to the members infecting bacterial cells but is conserved across the domains of life. This also includes the overall organization of the control region of ϕCh1 which resembles that of other temperate bacteriophages such as λ. This remarkable conservation argues for either extensive horizontal gene transfer between bacterial and archaeal viruses or alternatively for a common ancestor of tailed dsDNA viruses of *Archaea* and *Bacteria.*

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