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In vivo studies on the effects of immunity genes on early lytic transcription in the *Halobacterium salinarium* phage ϕH

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Summary. We have studied in vivo the effects of putative immunity genes on the expression of an early lytic gene of the *Halobacterium salinarium* phage ϕH. We transformed an *H. salinarium* host with DNA coding for a putative repressor gene, the transcript of which has been designated T6. We show that, in vivo, this gene specifically shuts off production of the early lytic transcript T4. A construct carrying the DNA transcribed as T4, but without its putative repressor binding sequences, shows T4 transcription enhanced to a level comparable to that observed in lytic growth of mutant phages capable of growing on immune *H. salinarium* strains. This transcript is insensitive to the action of the T6 product. The product of this 'unrepressed' T4 transcript is able to complement *in trans* the repressed T4 on superinfecting ϕH-sensitive phages, allowing these to grow on a strain containing the repressor gene. It has, however, no effect on the production of repressor. We also mapped the start and end points of two other transcripts, T9 and T10, which are expressed only in the lysogenic state by cells immune to superinfection by phage, cloned the coding DNA and used it to transform *H. salinarium*. This DNA, though transcribed by the transformants, has no detectable effect on the cells, which remain susceptible to phage infection.

Key words: Halophage ϕH – Immunity genes – Repressor action – *trans* complementation

Introduction

The halobacterial phage ϕH was first described by Schnabel et al. (1982a). It is capable of lytic growth, as well as establishing lysogeny, on the *H. salinarium* strain R_1. The central 12 kb region (L region) of the phage DNA is flanked on one or both sides by an insertion element, ISH*1.8*. In phages with two insertion elements, the L region can loop out and replicate in the host as an autonomous plasmid, called pϕHL. The resulting strain R_1L is immune to infection. A phage variant ϕHL, is able to overcome this immunity (Schnabel 1984). This phage has the 950 bp insertion element ISH*23* integrated in its genome. Lysogenic R_1 stains, e.g. $R_1$24, where the whole phage genome is present as an extrachromosomal element, are likewise immune against superinfection, in this case even to phage ϕHL (Schnabel and Zillig 1984).

Transcription from the plasmid pϕHL is different from that from the L region in the lytically growing phage. Two constitutive transcripts are common to both states and four transcripts are produced only in the lytic phase. In the immune state, the L plasmid codes for three immune transcripts, designated T6 (300 nucleotides), T9 (950 nucleotides) and T10 (500 nucleotides; Gropp et al. 1992). It has been suggested (Ken and Hackett 1991) from gel-shift experiments with lysogens, that T6 codes for a repressor protein, acting on the promoter for an early lytic transcript called T4. No phage growth has been observed without production of T4. ISH*23* integrates between the T4 start point and the suggested repressor binding sites. Thus, integration of ISH*23* would shift the binding sites some 1000 bp away from the promoter, rendering the repressor impotent. The start point of T6 lies back-to-back to the start of T4. The transcripts in this part of the L region are shown in Fig. 1.

Until recently, the in vivo function of these transcripts could only be conjectured. The recent development of halobacterial transformation systems (Cline and Doolittle 1987; Cline et al. 1989; Blaseio and Pfeifer 1990) has made it possible to perform in vivo studies of gene function. We cloned the DNA coding for T6 and, after determining their start and end points, for T9 and T10. We then used this DNA to transform *H. salinarium* and monitored the transformants for the effects of the putative immunity genes on infecting ϕH. In addition, we tested whether a T4 gene product supplied *in trans* in transformed repressor-containing strains is able to complement the repressed T4 product on an infecting phage.

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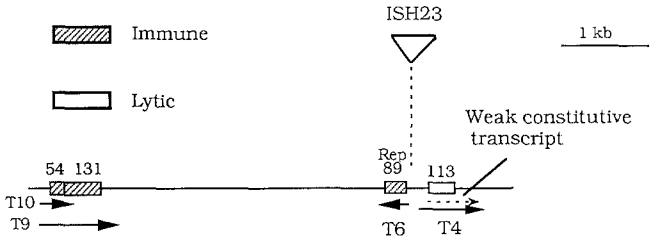

Fig. 1. Schematic map of the first 6 kb of the L region with the transcripts investigated in this work. Transcripts are shown as *arrows*, the *boxes* signify open reading frames. Only relevant transcripts are shown; other transcripts map between T6 and T9/10 as well as to the right of T4

Materials and methods

Materials. Restriction enzymes, T4 DNA polymerase, T4 DNA ligase, Klenow polymerase, T4 polynucleotide kinase were puchased either from Boehringer Mannheim or Pharmacia. *Taq* DNA polymerase was from Boehringer, *Pfu* DNA polymerase from Stratagene. α-[^{32}P]dATP, α-[^{32}P]dCTP and γ-[^{32}P]dATP were obtained from Amersham, nylon membranes for Southern transfer from Pall. The Bluescript vector is marketed by Stratagene. Sequencing was done with the dideoxy chain-termination method of Sanger et al. (1977) using the Sequenase kit from USB. The halobacterial shuttle vector pUBP2 has been described (Blaseio and Pfeifer 1990) and was a gift from F. Pfeifer. Mevilonin was a gift from A.W. Alberts (Merck, Sharp and Dohne). Standard cloning techniques were carried out as described in Sambrook et al. (1989).

Bacterial strains. All derivatives of the *H. salinarium* strain R_1 used here have been described previously (Schnabel 1984). The *H. salinarium* strain P03 used as a recipient in transformation experiments was a gift from F. Pfeifer and has been described (Blaseio and Pfeifer 1990). The phage strain used in all the infection experiments was the variant ϕH1, described previously (Schnabel et al. 1982b). The choice of strain P03 for the transformation experiments was dictated by the fact that the halobacterial replicon in the vector pUBP2 is derived from the plasmid pHH1, which is present in the ordinary ϕH host R_1 and would thus cause incompatibility problems. Strain P03 lacks pHH1 and all derivative plasmids (Blaseio and Pfeifer 1990).

Plating of phages. Phage solutions were assayed by the soft agar method (Adams 1959). The *H. salinarium* basal growth media were diluted with 1/5 volume distilled water. The plates were incubated at 42° C, and plaques typically formed after 3–4 days.

Media. Rich media, minimal media and solutions for transformation were prepared according to Cline and Doolittle (1987) and Cline et al. (1989). *H. salinarium* transformants were selected on plates containing 25 μM mevinolin.

Tranformation of halobacteria. The transformation procedure has been described (Blaseio and Pfeifer 1990) and was followed without additional modifications.

Isolation of RNA. RNA was isolated from logarithmically growing cells. RNA from ϕH-infected cells was isolated 1 h after infection. The cells were lysed, extracted with phenol and the aqueous phase centrifuged onto a 5.7 M CsCl cushion in a Beckman ultracentrifuge with an SW55 Ti rotor at 50000 rpm for 16 h. The pellet was taken up in distilled water and purified by repeated ethanol precipitation.

S1 nuclease analysis. Probes for transcriptional start points were prepared and the S1 analysis performed according to the procedures described by Reiter et al. (1990). The oligonucleotides and templates for the T6 and T4 start probes have been described (Gropp et al. 1992). The probe used to map the start point of T9/10 was generated by hybridising the γ-[^{32}P]-labelled oligonucleotide T9S (Fig. 2) to single-stranded DNA from the vector pBluescript SK$^-$ carrying the fragment BcIr, described by Schnabel (1984), and extending with Klenow polymerase. The 3′-labelled termination probe for T10 was prepared by labelling the *Bam*HI site of the fragment *Bam*H1-6 from ϕH (Schnabel et al. 1982b) with α-[^{32}P]dCTP using Klenow polymerase.

Cloning of the putative immunity genes. The start and end points of the transcript T6 have been mapped (Gropp et al. 1992). For amplification of the gene by the polymerase chain reaction (PCR), the two oligonucleotides T6PCR1 and T6PCR2 were synthesised, the one binding 80 bp upstream from the transcription start, the other 180 downstream from the end. Oligonucleotide T6PCR2 introduced a *Bam*HI restriction site; a second *Bam*HI site was already present in the sequence. A PCR reaction (Mullis and Faloona 1987; Saiki et al. 1988) was performed as described using these oligonucleotides and ϕH DNA as template; the product was cut with *Bam*HI and ligated to the plasmid pBluescript SK$^-$. After transformation into *Escherichia coli* and confirmation of the correct sequence, the T6 fragment was cut out of the vector and ligated to the halobacterial shuttle vector pUBP2 using the single *Bam*HI site of this vector. This construct was transformed into the *H. salinarium* strain P03, generating the transformant P03T6. Successful transformation and subsequent expression of the gene was monitored by S1 mapping with the probe for the T6 promoter.

The DNA transcribed as T9/10 was cloned in a similar way, using the oligonucleotides T9PCR1 and T9PCR2, binding 160 bp upstream from the T9 start and 400 bp downstream of the end point for transcription, respectively. The amplified fragment was 1400 bp long. The cleavage sites generated turned out not to facilitate cloning and were filled-in using Klenow and T4 DNA polymerases, before the construct was ligated to the pUBP2 vector at the *Hin*dIII site which had been filled-in using Klenow enzyme. The transformed P03 strain was designated p03T9.

```
          'Box A'          Start T9/10 >
GGCACGAAATTTTACTTACCGATGCGTCTATTCTATTCTGACATGATGACCCAAGACCTCGACGAGGCCG 240

ACCGTCTCGCTGCGGGAAGCGAACTCAGCAGCGAGGACGTCGACGAGGTAGCCCGCCAGATCGACCGGCA 310

                                                        OT9S
GCCACAGCGCGCGCGATGAACGACCTGGACGCGGAGGGCGAGTAAGACCTATGGCGATCGTCACCAATCG 380
                                                       [-------------------

TAGTAACATGGATCCGACGTCGACCG...    ...CTCGGTTGAATCGGTTCGCGAACTCCGCGAGCGGG 520
----------------------------...    ...--------(ORF54)-------------------]

   End T10
    ||||
AATGACGTTTTTCATCGACACGAACATCCTCATCGCAGCGACCGTCGGTGAACCGGGGCGCGGTGAGGTG 590
 [----------------------------------(ORF131)--------------------------

          End T10                                          End T9
            ||||                                    ||||||      ||||||
GCGCGCGATCTCCTTGATGCCGATTA...   ...CCTCGAGTCCTAAATCGTCTCGAAGTGGTCGTACGA 940
----------------------------...   ...-----------]

            End T9
            ||||||
GCGTTCGGTCGTGAGAATCGACCGATGACGAAGCCGGTTCCGGACGTCGTACAGCGTGTTTCCTTCTTCG 1010
```

Fig. 2. Sequence of the region for transcripts T9 and T10. The oligonucleotide OT9S used for S1 mapping is given in *underlined italics*. The box A region is *underlined*; the T9/10 start point is in *bold*. *Vertical lines* mark the regions of termination, *underlined* sequences upstream of termination sites are possible stem-loop structures. The two open reading frames are shown as *dashed lines* under the sequence

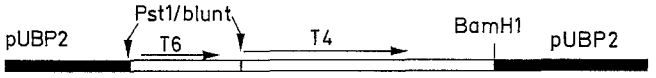

Fig. 3. Schematic linear representation of construct T4LT6 showing the orientation and direction of transcription from the cloned DNA. Only a part of the pUBP2 DNA is shown

Cloning of the repressor-insensitive T4 start site. The DNA transcribed as T4 in the phage ϕHL was amplified using the oligonucleotides T4L1 and T4L2 and ϕHL DNA as template. T4L1 binds 80 bp upstream of the transcription start and the oligonucleotide T4L2 600 bp downstream of the end point. Both oligonucleotides introduce *Bam*HI sites. The amplified fragment (T4L) was cut with *Bam*HI, the ends filled-in and the fragment ligated into the *Sma*I site of the plasmid pBluescript SK$^-$ and the correct sequence confirmed. T4L was then cut out with *Bam*HI and *Pst*I and ligated into the similarly cut shuttle vector pUBP2. The Bluescript clone with the DNA coding for T6 described above was cut with *Bam*HI, the insert recovered, its ends filled-in with Klenow polymerase and ligated into the blunt-ended *Pst*I sites of the T4L construct. The orientation and expression of the respective transcripts was confirmed by DNA sequencing and S1 mapping. (The oligonucleotides for sequencing in pUBP2 were designed from sequences generated by sequencing from the T6 construct into the vector.) The construct is shown in Fig. 3. *H. salinarium* strain P03 was transformed with the construct and the resulting strain designated T4LT6.

Results

Mapping of the transcript(s) T9/10

S1 nuclease mapping of the transcription start points for the immunity transcripts T9/10 (Fig. 4) showed only one start point. The transcription start (Fig. 2) is the G at position 210 in the published pϕHL sequence (Gropp et al. 1992). At 24 bp upstream of the transcription start is the putative promoter Box A sequence, TTTACT. This shows similarity to the consensus sequence for the Box A elements in archeal promoters (Reiter et al. 1988a, 1990; Thomm and Wich 1988).

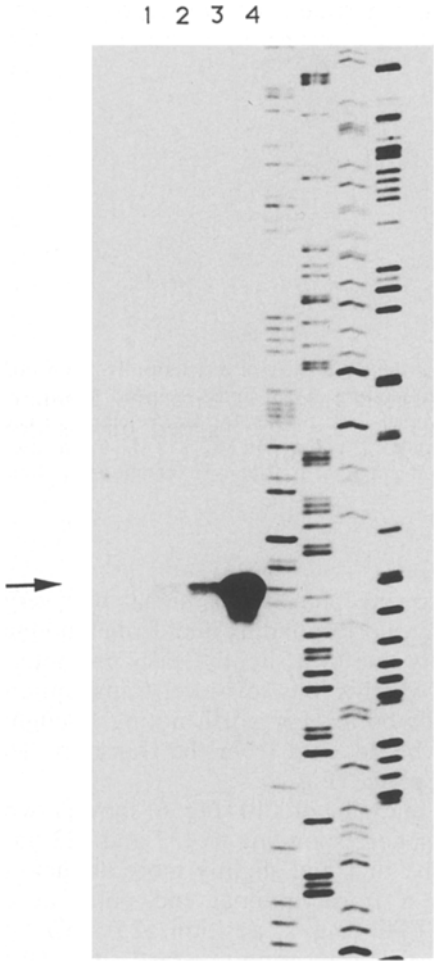

Fig. 4. S1 nuclease mapping of the start point for the immune transcripts T9/10. The sequencing ladder was generated using the same oligonucleotide as the S1 probe. The order of the sequence reactions is GATC. Lane 1, uninfected control R_1 cells; 2, R_1 1 h after ϕH infection; 3, R_1L; 4, $R_1$24

Secondly, as seen in Fig. 4, the much greater sensitivity of S1 mapping relative to Northern blots rendered visible a weak signal from the T9/10 promoter in the

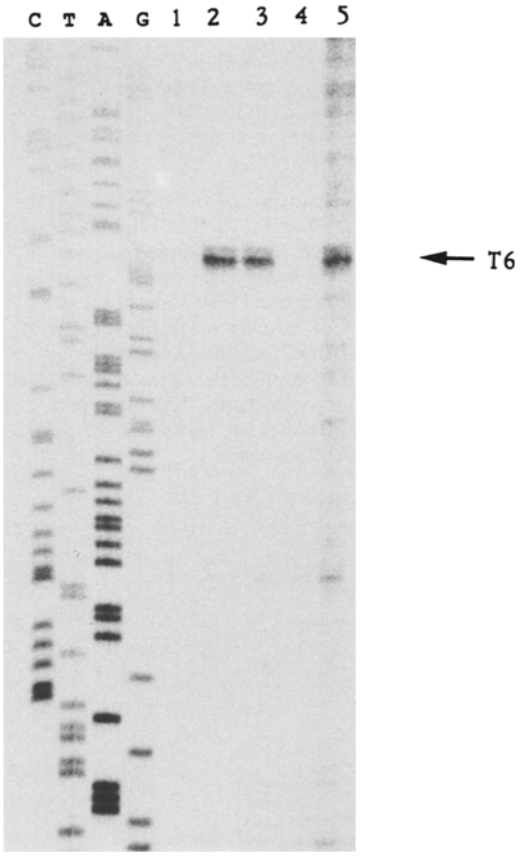

Fig. 5. S1 monitoring of the expression of transcript T6 from P03 transformants. The sequence is M13 single-stranded DNA sequenced with the universal −40 primer for size reference. Lane 1, uninfected control cells; 2, clone T4LT6; 3, T4LT6 1 h after infection with ϕH; 4, strain R_1 1 h after ϕH infection; 5, clone P03T6

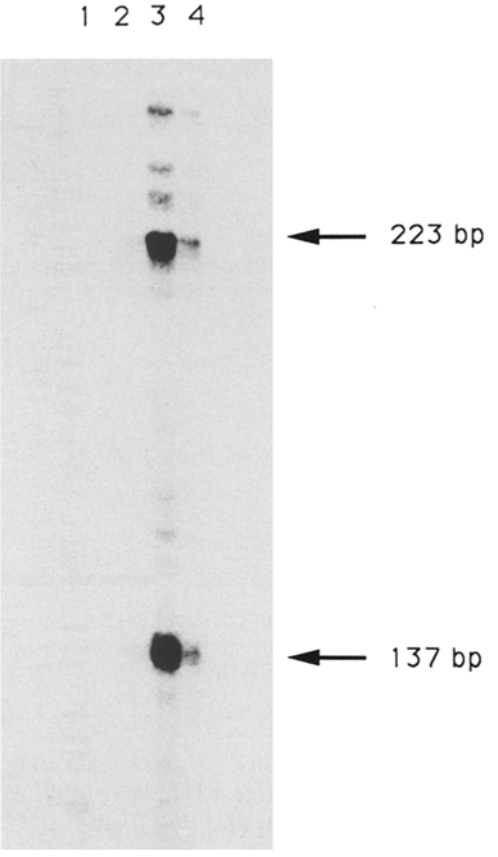

Fig. 6. Termination mapping of transcript T10. The sequence ladder is M13 single-stranded DNA sequenced with the −40 universal primer. Lane 1, uninfected control R_1, 2, R_1 1 h after infection with ϕH; 3, $R_1$24; 4, R_1L

case of lytically growing phage. This signal was very much weaker than the corresponding ones from immune strains. It is possibly due to a slightly leaky promoter. We did not check whether this low-level transcription produces T9, T10 or both. It is worth noting, though, that no signal can be detected from the transcript T6 in lytically growing phage (Fig. 5).

The termination mapping of T10 (Fig. 6) showed two protected fragments, corresponding to 137 and 223 nucleotides in size. The first and slightly more abundant fragment indicates a transcriptional end point at a stretch of CGTTTTTC (Fig. 2, position 525), 290 bp downstream from the transcription start site. This stretch of thymidines flanked by cytosines is similar to suggested termination signals in Archaea (Reiter et al. 1988b), though it should be noted that the termination point is actually upstream of this motif. The second, weaker termination site would be 370 bp from the start (Fig. 2, position 603). It has the sequence TCTCCTT, which has less similarity to suggested consensus sequences. Here, at least, the termination site is at the end of the motif.

Termination at the first site would give transcript T10 a size of 290 bp, which is less than that estimated from *Northern* blots (Gropp et al. 1989). The second site would also give a smaller transcript than previously estimated. The existence of two termination sites for T1 has not been reported previously. The open reading frame ORF 54 encoded in the T9/T10 DNA is included in both the smaller and larger transcripts. The ORF 131 starts immediately after the end of the smaller ORF.

Mapping of the T9 termination points did not yield distinct signals (data not shown). Upstream of the region of strongest termination (Fig. 2, position 926) it is possible to construct stem-loop structures. The transcription seems to trail off over a region of at least 100 bp. The two regions of T10 termination show stem-loops as well.

Effect of transcript T9/10

Infection of strain P03T9 with ϕH failed to show any effect on the lytic transcripts. The T4 signal was readily discernible. The phage formed plaques on P03T9 with the same frequency as on *H. salinarium* P03 cells.

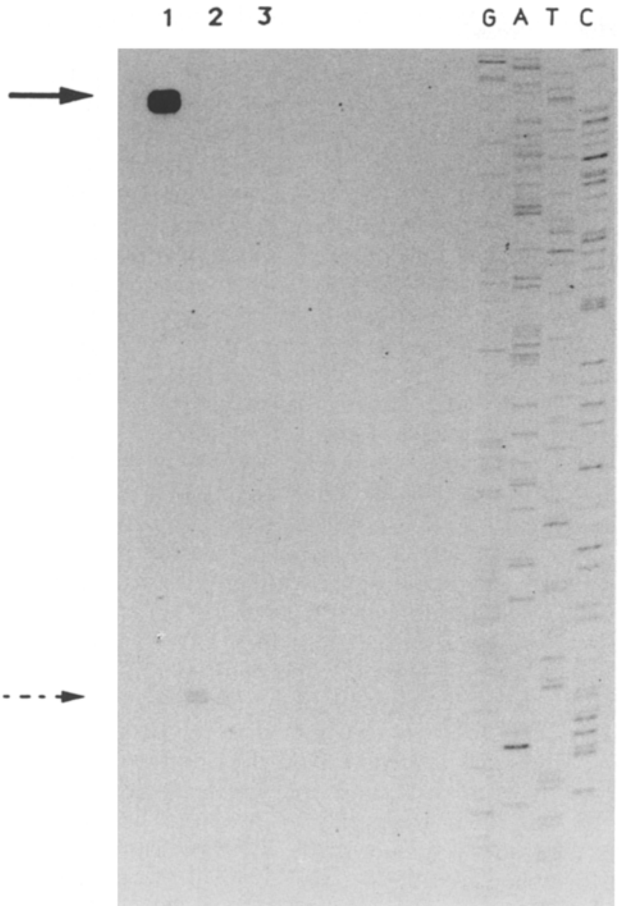

Fig. 7. S1 mapping showing the in vivo action of the cloned *rep* gene. The sequence is M13 single-stranded DNA sequenced with the −40 universal primer for refernce. The *bold arrow* points to transcript T4; the *dashed arrow* points to the faint constitutive transcript present in all three lanes (may not show up in reproduction). Lane 1, P03 1 h after ϕH infection; 2, P03T6 1 h after ϕH infection; 3, R_1L

Effect of the T6 transcript on the lytic transcript T4

Strain P03T6, actively expressing the T6 gene, was infected with phage ϕH and monitored for expression of the early lytic transcript T4. As shown in Fig. 7, the presence of T6 in the cells completely shuts off transcription of T4. This complete repression is also observed in strains R_1L and R_124, where ϕH cannot grow. We will refer to the T6-encoded repressor gene as *rep*.

As Fig. 7 shows, a weak S1 protection signal can be detected 165 bp downstream from the T4 start. This signal has been described previously (Gropp 1990) and is present in immune *H. salinarium* strains and lytically growing phage alike, and is apparently not correlated with the growth ability of the phage. The fact that this signal is present in the P03T6 strain infected with ϕH is proof of the successful entry of the phage DNA into the cells. Thus, the *rep* gene functions by shutting off T4; it does not prevent attachment or DNA injection.

Efficiency of immunity conferred by rep

To test the repressor gene for efficiency in conferring immunity, P03T6 cells infected with phage ϕH1 were plated and the phage titre compared with that of the same phage on *H. salinarium* P03. In this test the ϕH titre on the untransformed strain was 3×10^9 pfu/ml. The phage titre on the repressor-containing strain was 10^7. Thus the repressor gives an immunity which is overcome by phage with a frequency of 10^{-2}. This degree of immunity is two orders of magnitude less efficient than that of strain R_1L relative to R_1. However, since the phage plates less efficiently on *H. salinarium* strain P03 than on R_1, direct comparison between the two systems, P03/P03T6 and R_1/R_1L, is not possible. Twenty plaques were picked from phage plates with strain P03T6 and the DNA restriction patterns of the phages analysed. None of the phages analysed contained the ISH*23* element that is responsible for the ability of phage ϕHL to overcome the immunity of R_1L.

Effect of removing the T4 repressor binding sites

In order to verify the identity of the suggested repressor binding sites in the lytic transcript T4, as well as to check whether T4 was able to complement infecting phage *in trans*, we amplified the T4 gene from the phage variant ϕHL1. This variant has the insertion element ISH*23* integrated between the repressor binding sites and the start of T4. A comparison of the DNA sequence around the T4 start region in the phages ϕH and ϕHL is shown in Fig. 8.

The construct T4LT6 carries a pUBP2 vector into which the DNA transcribed in ϕHL1 as T4, but lacking the suggested repressor-binding sequences, has been inserted. The first 50 bases upstream from the ISH*23* integration site are derived from the ISH*23* element, thus mimicking the situation in phage ϕHL. The T6 gene was grafted onto this construct. If T6 codes for a repressor, cells transformed with the resulting construct should produce T4 and T6 simultaneously. If, further, the T4 gene product is able to act *in trans*, we would expect the transformed strain, carrying T6 and a functional T4,

```
                Repressor binding sites                                     Start T4
                /                     \                Box A                    |
CATTACGACAATACTTCTCGACTTAAGGAAGTGGGCTTCTCGGATTTAGGAATAGATATAAGTTAGACCCCTCGTAAAGTCCAGACTG    ϕH1
CGATCCTTGAGACTGCGCTATGACTCACCAACAATCTCCGCCACAAGAGCGATAGATATAAGTTAGACCCCTCGTAAAGTCCAGACTG    ϕHL1
                ISH23                            Target sequence ISH23
```

Fig. 8. Comparison of the sequences upstream from the T4 start from phage ϕH and Rep-insensitive ϕHL. In the ϕHL sequence, the first 50 bp shown are part of the ISH*23* element

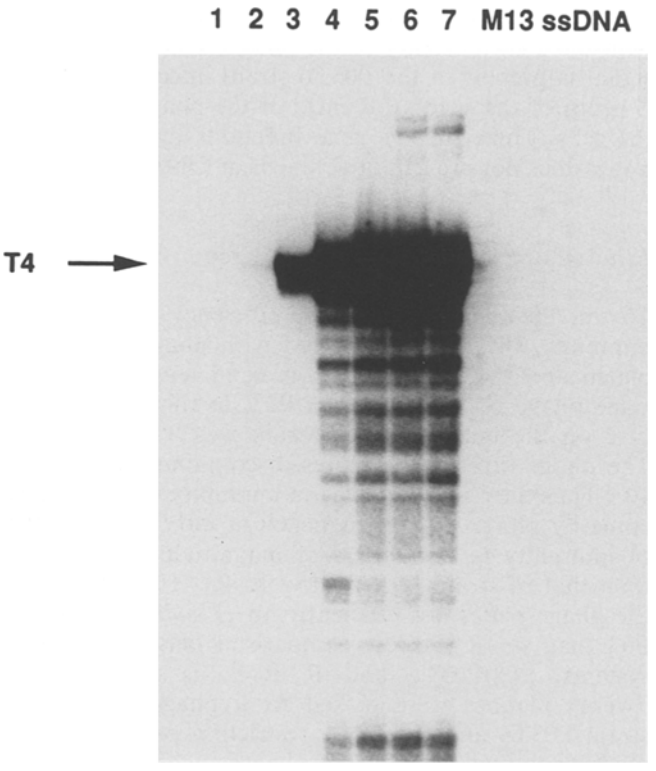

Fig. 9. Production of T4 from lytic infection and clones. The blot is somewhat overexposed in order to show the faint signal after 30 min. Lane 1, uninfected R_1 control cells; 2, ϕH-infected R_1 30 min after infection; 3, ϕH-infected R_1 1 h after infection; 4, ϕH-infected R_1 3 h after infection; 5, ϕH-infected R_1 5 h after infection; 6, clone T4LT6; 7, clone T4LT6 1 h after ϕH infection

to be sensitive to phage infection, even though T4 transcription from the incoming phage would be switched off.

As shown in Fig. 5, T6 is produced by the cells, but as seen in Fig. 9, it has no effect on the production of T4. Indeed, the overall level of T4 is raised in a similar way to that observed in the phage ϕHL, where the whole ISH*23* is present. Removal of the repressor binding sites thus leads to enhanced levels of T4. The normal phage ϕH produces similar amounts of T4 but this level is reached only 5 h following infection, probably due to a gene dosage effect.

When the strain carrying T4LT6 was infected with ϕH, the phage titre observed was 10^9 pfu/ml, compared with 3×10^9 on P03 and 10^7 pfu/ml on P03T6. Thus *rep*-repressed phage growth can be restored by *trans* complementation of the T4 product.

Discussion

Due to the long generation time of its host, genetic work with halophage ϕH is time consuming and only a small number of phage mutants have been isolated so far. Thus mapped transcripts abound, but described genes are scarce. The recent development of techniques for genetic manipulation of halobacteria has enabled us specifically to test in vivo the role of different phage transcripts.

As previously shown in vitro, the region upstream from the lytic transcript T4 of ϕH contains sequences to which a protein from lysates of immune strains of *H. salinarium* can bind (Ken and Hackett 1991). By transforming the gene for transcript T6 into a phage-sensitive host, we demonstrate here that the host becomes about 100 times more resistant to infection and that T4 transcription is turned off so effectively as not to be seen in an S1 nuclease protection experiment. Thus the transcript T6 codes for a repressor, analogous to the cI repressor in phage λ. The fact that the two promoters are located back-to-back further extends the analogy (Ptashne 1986; Gropp et al. 1992).

The action of the *rep* product seems confined to the T4 promoter. At 165 bp downstream of the T4 start point, a weak signal is observed in S1 protection assays (Gropp et al. 1989). This signal is present in lytically growing phages and lysogens alike. This second start site lies only 8 bp upstream from the ATG start of the first major open reading frame in the DNA producing T4, which spans 339 bp (Gropp et al. 1992). However, a halobacterial consensus Shine-Dalgarno sequence, AGGAGG, can be found immediately upstream from this second start site and would thus not be present in the weakly expressed transcript, which would therefore not be translated when the T4 promoter is shut off. Thus the leader sequence of the T4 transcript is important for phage growth.

That the repressor action of T6 is not the only function that contributes to immunity is further indicated by the fact that *H. salinarium* strain R_1 harbouring the plasmid pϕHL acquires an immunity which is of the order of two magnitudes more efficient than that of the *H. salinarium* P03T6 transformants. Direct comparison is not possible, since ϕH grows less well on P03 than on R_1, but since none of the phages able to grow on P03T6 had an integrated ISH*23* element, their viability was probably due to incomplete cellular immunity and not to the phage being able to overcome the *rep* action. The restriction pattern of these phages showed them all to lack the ISH*23* element.

The postulated mechanism for the ability of phage ϕHL to overcome *rep* repression – the element integration of the ISH*23* between the repressor binding sites and the transcription start – is further supported by the effects of expression of the construct T4LT6. Integration of the last 50 bp of ISH*23* between the putative repressor binding sites and the T4 start has the same effect as the integration of the entire ISH element. The enhanced production of T4 seen in phage ϕHL (Gropp et al. 1989) is seen in the case of T4LT6 as well and is obviously due to the lack of repressor binding sites. T4 is not the first transcript detectable upon infection; the lytic transcript T1 is seen within 10 min (P. Stolt, unpublished observation) and T4 is not present in detectable amounts until 30 min after infection. This could be due to competition between *rep* and T4 in the earliest stages of infection.

T4 is necessary for lytic growth. As we show here, its product (from T4LT6) is able to function *in trans*, complementing the repressed T4 on an incoming sensitive phage. The nature of the product of the T4 transcript is unclear. It does not switch off transcription from the *rep* promoter, though the transcript T6 is not seen under normal lytic ϕH growth (Fig. 5). On the T4LT6 construct, T6 and T4 are oriented in tandem (Fig. 3), whereas in the phage genome their start points lie back-to-back. Thus, it may be that T4 and T6 production are mutually exclusive in the phage. The major open reading frame on the T4 DNA would code for a protein of 113 amino acids.

The transcripts T9 (900 nucleotides) and T10 (350 nucleotides) have a common start point, but the termination analysis shows that there are in fact two termination signals for T10, producing two transcripts. T9 is generated by readthrough past these termination sequences and is produced in similar amounts to the two T10 transcripts. This generation of several transcripts from one common start point seems to be a common property of ϕH gene expression, since the lytic transcripts T1, T2 and T3 are produced in a similar way (Gropp et al. 1992). If the analogy holds, T9 might be expendable; the phage variant ϕH8 lacking T2 and T3 is perfectly viable. Similar transcriptional readthrough has been observed in the *Sulfolobus* virus SSV1 (Reiter et al. 1987). The start of T9/10 is only 160 bp from the circularisation point of the L plasmid, as defined by Schnabel et al. (1984).

The identified terminator sequences show similarity to the suggested termination signals in *Archaea* (Reiter et al. 1988b). The first termination point does, however, lie before the consensus motif, which suggest coincidental. Termination in halobacteria is a little investigated feature and reliable data on consensus sequences will have to await the definition of further functional terminators. The fact that strong signals were observed at all for T10 suggests that the size of the transcript is important. The existence of two transcriptional end points has been previously reported for the halobacterial *bop* gene (Das Sarma et al. 1984), where the major transcript has a ragged 3′ end extending over some 45 nucleotides and a more defined minor transcripts ends 170 nucleotides further downstream. The stem-loop structures seen before the two termination regions in the T10 transcript are a feature also observed in other halobacterial genes (DasSarma et al. 1983; Blanck and Oesterhelt 1987; Lechner and Sumper 1987), though the significance of such structures in termination sequences has not been proved. The lack of defined termination points for T9 is in line with observations on other transcripts in ϕH and halobacteria (DasSarma et al. 1984; C. Englert, personal communication; P. Stolt, unpublished data).

The DNA transcribed into T9/10 has few convincing open reading frames. The putative ORF 54 ends at the first termination site. The only ORF of any considerable length in the T9 region spans 131 amino acids and starts at the last base of ORF 54. This is also the first end point of the transcript T10.

Cells transformed with the DNA encoding T9/10 do not show any immunity towards ϕH infection, nor did we find that these putative genes had any other effect on phage gene expression. They may code for a co-repressor or modulator protein, acting in concert with the *rep* gene product to enhance the effect of the repressor. Such systems have been described, e.g. in phage P1 (Vellemann et al. 1990). The lower efficiency of immunity observed in P03/P03T6 compared with R_1/R_1L could be due to the lack of such a co-repressor in P03T6. The 131 amino acid ORF in the T9 gene would code for a protein that is predicted by computer analysis to have a helix-turn-helix structure, a feature that is common among DNA-binding proteins. Such a structure has been proposed for the *rep* gene product as well (Ken and Hackett 1991), though computer prognoses on the basis of primary structures alone are uncertain. Because of the limited availability of vectors and the difficulties in transforming *H. salinarium*, the possible cooperative effect of T9 on T6 has not yet been tested in vivo. The fact that there is a slight residual activity of the T9/10 promoter in the lytic phase indicates that the effect of the gene alone is not strong enough for its product to hamper lytic growth when *rep* is not present and T9/10 transcripts are produced only in small amounts.

Still another level of immunity is indicated by the fact that no phage able to grow on the lysogenic strains $R_1 3$ or $R_1 24$ has been isolated. Thus, an undefined gene situated outside the L region must be responsible for this immunity. Cloning and transformation of *H. salinarium* with various candidate regions, followed by screening for immune transformants, is now a practical, previously unavailable, approach to finding these immunity genes.

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