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# Structure specific ds/ss-RNase activity in the extreme halophile *Halobacterium salinarium*

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## ABSTRACT

**A ds/ss-RNA processing activity involved in antisense-RNA mediated gene regulation in the extremely halophilic archaebacterium *Halobacterium salinarium* was investigated *in vivo*. *H.salinarium* cells were transformed with DNA encoding an RNA species complementary to a part of the major lytic transcript, termed T4, of the *H.salinarium* phage φH. The transformants transcribing this construct, when infected by phage were able to process T4 in a similar way to the processing of the lytic transcript denoted T1, in the natural sense-antisense system. Processing of T4 was not observed under normal phage growth on wild-type cells. Thus the antisense-RNA mediated processing activity earlier reported is dependent on the presence of an RNA duplex and is not sequence specific.**

## INTRODUCTION

The temperate phage φH infects the extremely halophilic archaebacterium *Halobacterium salinarium* (formerly *H.halobium*). The central part of the phage genome, the so-called L region, can exist independently as a plasmid (pφHL), conferring to the host a certain immunity from phage infection. (For reviews see Ref. 1–3). Part of this immunity is mediated by an antisense RNA, termed $T_{ant}$, which is transcribed in the opposite direction to the phage transcript T1 (4). Both transcripts are encoded from constitutive promoters on the plasmid pφHL, but full-length T1 is seen only during lytic phage growth on non-immune cells. The antisense RNA, the first such reported in an archaebacterium, is able to form a duplex with the first 151 nt of T1, which is then processed at the ends but without rapid degradation of the products (4). This processing, of a kind which has not been previously reported, removes the Shine–Dalgarno motif from the T1 species, leaving a truncated RNA that is inaccessible to the ribosome. Consequently, the first open reading frame on the T1-encoding DNA is only expressed by lytically growing phage even though the T1 transcript itself is produced by the immune cells as well (3).

This unique RNA-processing activity was postulated to be structure specific, recognising the ends of the $T1/T_{ant}$ RNA duplex. Since we have not been able unambiguously to demonstrate this activity *in vitro* (4), the possibility remained that the RNA processing was specific for this very duplex. In order to test whether the processing enzyme would act on other ds/ss-RNA junctions as well, we transformed an *H.salinarium* strain with a DNA construct encoding an antisense RNA, complementary to a part of the φH major early lytic transcript, termed T4. T4 is necessary for lytic growth and its production can be repressed by the product of the *rep* gene, which is located on the L region (5, 6). The T4-antisense construct lacked the repressor binding sites and was constitutively expressed by the transformants. If the ds/ss-RNase activity was structure specific, T4 was expected to be processed by the transformants at the end of the dsRNA (shown schematically in Fig. 1a). Under normal phage growth on wild-type cells, T4 is not processed in this region.

## MATERIALS AND METHODS

### Materials

Restriction enzymes, T4 DNA polymerase, Klenow polymerase, T4 DNA ligase, T4 polynucleotide kinase, Taq DNA polymerase and MLV reverse transcriptase were purchased either from Boehringer Mannheim, USB or Pharmacia. Vent™ DNA polymerase was from New England Biolabs. [α-$^{32}$P]dATP, [α-32P]CTP and [γ-$^{32}$P]ATP were obtained from Amersham, nylon membranes for Southern transfer from Pall and the Bluescript vector from Stratagene. The halobacterial shuttle vector pUBP2 has been described (7). It was a gift from F.Pfeifer. Mevinolin was a gift from A.W.Alberts (Merck, Sharp and Dohne).

*Bacterial strains.* The *H.salinarium* strains used have been described previously (4, 8). The phage strain used in the infection experiments was the variant φH1, described previously (9).

### Methods

*Transformation of halobacteria.* The transformation procedure for halobacteria has been described (10–12) and was followed with the slight modifications introduced by Blaseio and Pfeifer (7).

*RNA analysis.* RNA was isolated from phage-infected cells (moi=5) one hour *post infectionem*. RNA preparation and S1 mapping was performed as earlier described (6). All oligo-

nucleotides used are depicted in Fig. 1b. The S1 probe pT4X110 was generated by asymmetric PCR using the 5′-labelled oligonucleotide OT4X110 and the ϕH restriction fragment BamHI-4 (9) as template. The termination probe pT4Xt was generated by performing a PCR reaction with the two primers OT4X110 and OT4XP, digesting the product with the restriction enzyme AvaII and labelling the 3′ end of the resulting fragment using dGTP and [α-$^{32}$P]dATP. Where nothing else is given, 5 mg RNA were used for each analysis.

For primer extension analysis of RNA, 0.2 pmol 5′-labelled oligonucleotide was used with 200 units MLV reverse transcriptase (USB) according to the manufacturer's instructions.

*Construction of strain P03-pT4I.* In order to obtain an *H.salinarium* strain producing an RNA complementary to parts of the early lytic transcript, T4, from ϕH, the oligonucleotide OT4I (see Fig. 1b) was synthesised. This oligonucleotide contains the promoter sequences for T4 transcription. The beginning and end sequences were designed to overlap with the 5′ overhang and 3′ overhang of BamHI/PstI digested pBlueScript vector DNA, where it was inserted (pBST4I).

In a PCR reaction using the primers OT4IP and OT4IX, a 200bp DNA fragment was amplified which corresponds to a segment encompassing bp 4763 to 4958 in the L region. (All numberings in the L region are according to the published sequence, EMBL/GenBank Accession Number X65098 (13). When ligated to the pBST4I construct the resulting insert codes for an RNA with the nucleotides 3−193 complementary to the nucleotides 102−292 of T4. The insert, including the promoter sequences, was cut out and blunt-end ligated to the halobacterial shuttle vector pUBP2. The resulting construct, named pT4I, was used to transform *H.salinarium*.

*Other molecular biological techniques.* DNA sequencing was done with the dideoxy chain terminating method of Sanger (14) using the Sequenase™ kit from USB. Constructions of recombinant DNA, transformations into *E.coli* DH5$_\alpha$, DNA isolations and restriction analyses were all carried out as described (15). Plating of phages was done as described (16). Sequences were analysed using the programs of UWGCG, Devereux (17) and Staden (18). Predictions of secondary structure were calculated with the programs of Zuker and Stiegler (19, 20).

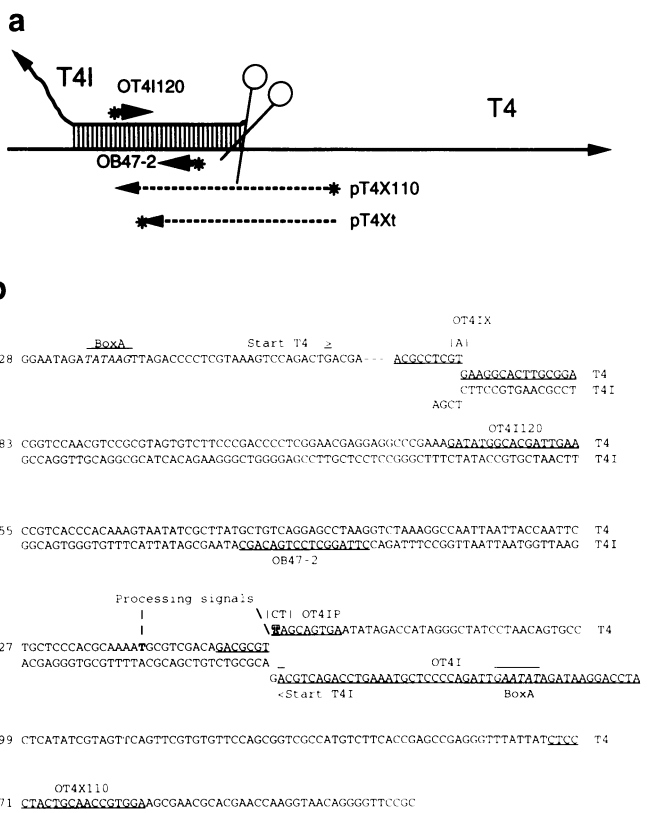


**Figure 1. a)** Schematic representation of the sense-antisense RNA pair T4/T4I, showing the location of the oligonucleotides and S1 nuclease protection probes used in the investigations. Arrows point to the 3′ ends. An asterix denotes a [$^{32}$P] label. The pair of scissors marks the expected processing site at the end of the RNA duplex. The unpaired parts of T4I are encoded by the pUBP2 vector. The termination point has not been determined. **b)** Nucleotide sequence of the DNAs encoding the transcripts T4 and T4I, showing the region of complementarity, the positions of processing in the RNA duplex and the sequences of the oligonucleotides used in constructing the vector pT4I as well as those used in the studies of transcription. The parts of the sequences producing complementary RNA species are paired. Start points of transcription are overlined; archaebacterial 'box A' motifs (29) are in overlined italics. The sequences of the oligonucleotides are underlined with exchanged bases shown above the sequence. Note that the complementary sequences for the oligonucleotides OT4IP and OT4X110 are given. The position of RNA cleavage at the end of the duplex is indicated by an outlined T at position 4959. The processing point 17 bp into the duplex is boldfaced. The numbering is according to the published sequence of the plasmid pFHL.

## RESULTS

*H.salinarium* cells carrying parts of the halophage ϕH genome producing the natural antisense RNA transcript termed $T_{ant}$, show an RNA processing activity directed towards the ends of the RNA duplex formed between $T_{ant}$ and the lytic transcript T1 (4). In order to test whether this processing activity was specific for this very RNA duplex or a general activity, recognising ds/ss-RNA regions, we constructed the vector pT4I. This contains the promoter for the major lytic transcript, called T4, which is necessary for lytic phage growth (6). The repressor binding sites present upstream of the T4 promoter (5) were absent on the construct which would be constitutively expressed in the transformants. Behind the promoter was placed in reverse a

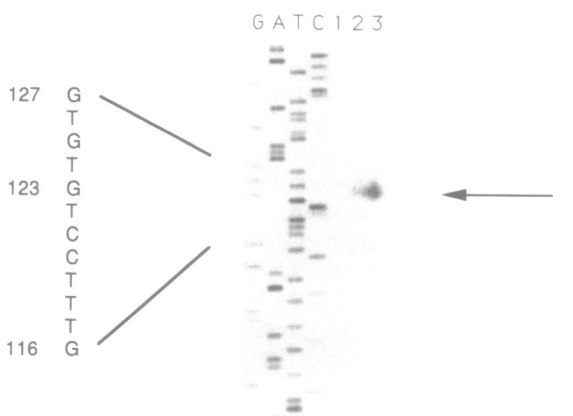


**Figure 2.** Primer extension analysis showing the of the antisense-RNA generating DNA construct pT4I. Lane 1, ϕH-infected wild type cells. 2 and 3, transformant P03-pT4I. In lane 3, 10 μg RNA were used. The sequence is M13mp18 sequenced with the 40 universal primer as size marker. The numbering refers to the size of the DNA fragments.

section from the DNA encoding T4. *H.salinarium* cells transformed with this construct were expected to produce an antisense RNA complementary to parts of T4. Upon phage infection, this antisense RNA would pair with a part of the lytic T4 and, if the RNase activity was structure specific, mediate processing of T4 at the end of the RNA duplex (see Fig. 1a).

The successful introduction of the pT4I construct into the cells was confirmed in a primer extension experiment (Fig. 2, lanes 2 and 3). The transformants (strain P03-pT4I) constitutively express the antisense transcript, T4I. Notably, though the promoter region upstream of the translational start point was unchanged from that of the T4 promoter (Fig. 3), and the only change was the switching from AC to CA two bp downstream of the T4 start point, the start point of transcription in the transformants was shifted two bases downstream of that of the wild type T4. Also, the pT4I promoter seemed weaker than the wild type T4 promoter, though no quantitative study was undertaken.

Wild-type cells infected with $\phi$H gave no signal in the primer extension experiment (Fig. 2, lane 1).

We next infected the transformants with $\phi$H and investigated whether the T4 transcript was processed in these cells, using 5′-labelled and 3′-labelled probes in S1 nuclease protection assays. The 5′-labelled probe pT4X110 showed a protected fragment corresponding to the expected RNA processing event at the start of the duplex region (Fig 4a, lane 3). This band was not present in $\phi$H-infected wild-type cells (Fig. 4a, lane 2).

Termination experiments with the 3′-labelled probe pT4Xt yielded a 174-nt fragment corresponding to processing at the end of the dsRNA as well as a 156-nt protected fragment corresponding to processing of the T4 RNA at a point 17 bp into the duplex region (Fig 4b, lane 3).

Longer exposure of the films from the S1 mapping with the probe pT4X110 revealed a weak band at a position corresponding to the processing 17 bp into the duplex (not shown).

The positions of the RNA processing in the antisense-RNA producing cells detected are shown in Fig. 1b. The signal indicating processing at the end of the duplex corresponds to the processing signal seen in the naturally occurring T1/$T_{ant}$ RNA processing. Thus, the RNase activity indeed recognises ds/ssRNA junctions and is not limited to the T1/$T_{ant}$ duplex.

The S1 nuclease protection analysis with the 5′-labelled probe pT4X110 also showed a 410-nt signal corresponding to a full-length unprocessed T4 species in the transformants as well as in the wild-type strain (not shown). This indicates that the processing mediated by the pT4I-encoded antisense RNA is not complete.

When infected with $\phi$H, the strain P03-pT4I showed no acquired immunity; the phage plated on the transformants with the same titre as on wild-type P03 cells.

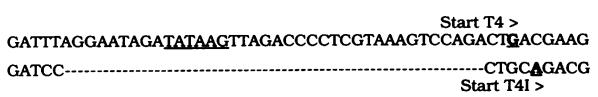


**Figure 3.** Comparison of the sequences from the wild-type promoter region for transcript T4 and the promoter on the construct pT4I showing the different start points of transcription. The nucleotides where transcription starts are underlined and boldfaced. The dashed line represents nucleotides that are identical in the two sequences.

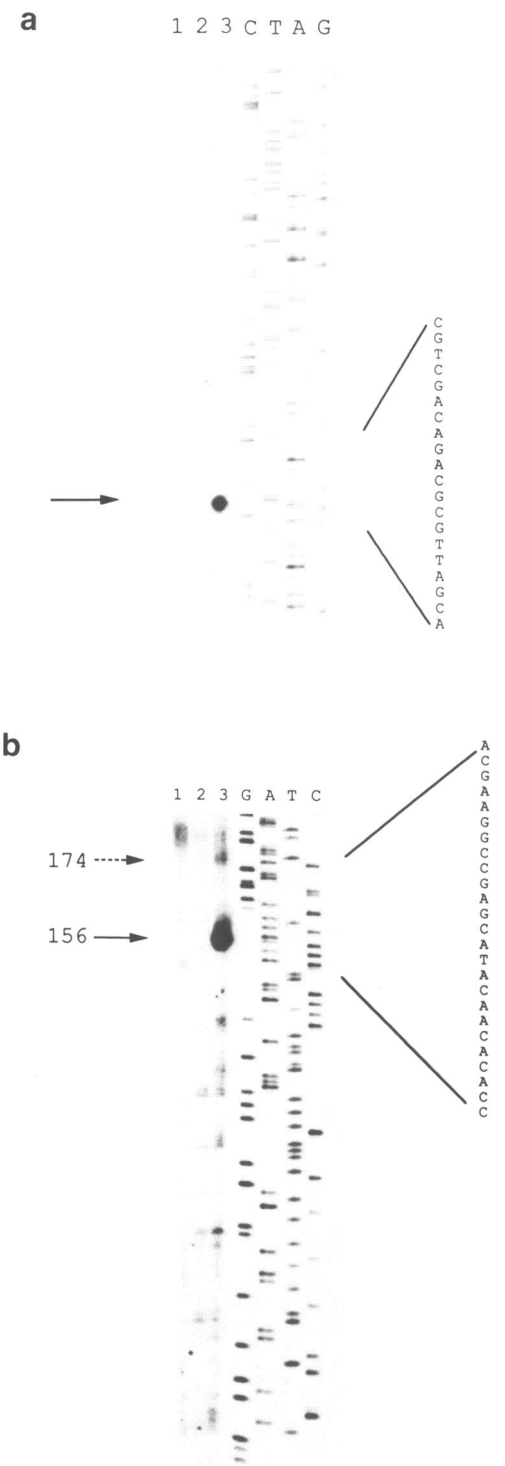


**Figure 4.** Processing signals from the end regions of the T4/T4I dsRNA. **a)** S1 mapping using the 5′-labelled probe pT4X110. Lane 1, uninfected control cells. 2, $\phi$H-infected wild-type cells. 3, $\phi$H-infected transformants strain P03-pT4I. The arrow points to the signal arising from the processing event at the end of the dsRNA. The sequencing ladder is the BamHI-4 DNA fragment from the plasmid p$\phi$HL (9) sequenced with the same oligonucleotide used in generating the S1 probe. **b)** Termination mapping using the 3′-labelled probe pT4Xt. Lane 1, uninfected control cells. 2, $\phi$H-infected wild-type cells. 3, transformants P03-pT4I infected with $\phi$H. The sequence is M13mp18 sequenced with the 40 universal primer as size marker. The dashed arrow points to the 174-nt protected fragment arising from the processing event at the end of the RNA duplex. The second signal indicates a processing event 17 bp into the duplex.

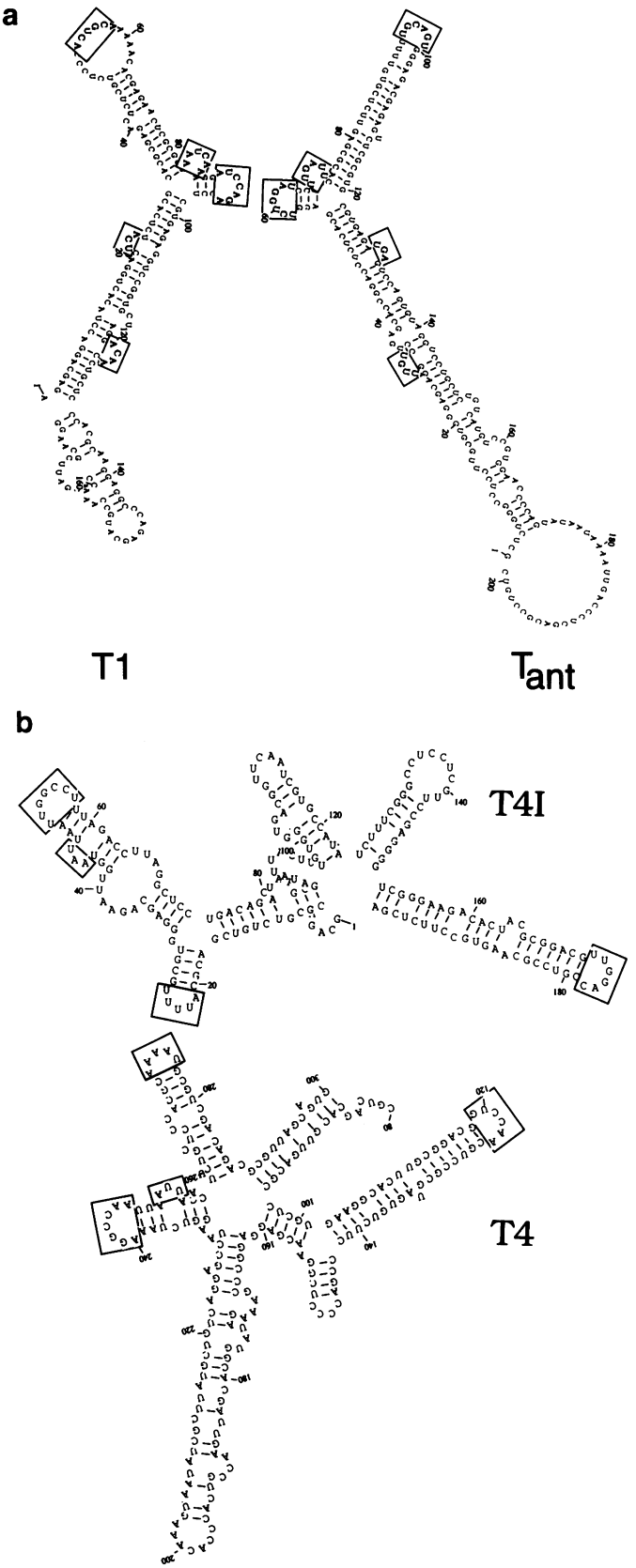


**Figure 5.** Secondary structure predictions for a) the natural sense-antisense RNA pair T1/$T_{ant}$ and b) the genetically engineered pair T4/T4I. Matching unpaired nucleotides which could participate in the initial duplex-forming 'kissing reaction' are boxed. In b) the prediction for T4 encompasses the nucleotides 80-300, corresponding to the region complementary to the first 190 nt of T4I.

## DISCUSSION

The ds/ss-RNA processing mediated by the halophage $\phi$H transcript $T_{ant}$ is the only reported instance of antisense-mediated gene processing in the archaebacteria. Since no activity has been shown *in vitro*, the possibility remained that the processing enzyme were able to act only on the T1/$T_{ant}$ RNA duplex (4). In this paper, we show, by generating a novel antisense RNA species *in vivo*, that the activity is indeed structure specific, cutting at the ends of a duplex RNA structure. The T4/T4I RNA duplex generated when P03-pT4I transformants are infected with $\phi$H is never present in phage-infected wild-type cells, but the transcript T4 is nevertheless processed by the transformants at the end of the T4/T4I RNA duplex in the same way as the naturally occurring T1/$T_{ant}$ ss/ds-RNA.

We did not observe any processing signals in the T4 RNA at the 5′ overhang, which may indicate that the enzymatic activity is limited to 3′ overhangs. Such are present at both ends of the T1/$T_{ant}$ RNA duplex (4).

The antisense-regulated processing shows similarities to that of OOP-cII in coliphage λ (21−24), where OOP forms an RNA duplex with the 3′ end of the cII mRNA and the duplex is subsequently processed by RNaseIII. The RNaseIII activity in *E.coli* cuts 13 bp into the duplex region and the RNA species 5′ to the cut is preferentially degraded. In contrast, the RNA processing acting in *H.salinarium* recognises the change from dsRNA to ssRNA and cuts at the edge of the duplex. Since the processing activity studied in the present work is host encoded and would presumably act on any ds/ss-RNA structure, we expect antisense-RNA mediated gene regulation in *H.salinarium* to be far more common than the single instance (4) described so far.

The additional cut at a point 17 bp into the T4/T4I duplex (but not in the T1/$T_{ant}$ duplex) may indicate that an RNaseIII-like activity is active as well in *H.salinarium*. Several RNA processing activities have been described in this organism (16, 25, 26). The products of the ds/ss-RNA processing event are not rapidly degraded (4) and no processing was observed further than 17 bp into the T4/T4I duplex.

The processing of T4 is not complete in the transformants and the cells are not immune from phage infection. The immunity mediated by $T_{ant}$ is also low (10 times reduction in plating efficiency relative to wild type), but the processing does seem to be more efficient in the T1/$T_{ant}$ case. One reason may be a limited degree of sequence requirements for the processing enzyme; indeed an enzyme acting on every 3′ RNA overhang would cut many naturally occurring small RNA hairpins and may be harmful to the organism. Only the activity and not the specificity of the enzyme seems to be reduced though; the processing of T4/T4I is on the same base as in the case of T1/$T_{ant}$. More detailed studies probably need an *in vitro* system, though such systems are notoriously difficult to establish for halobacterial proteins, the RNA polymerase being a case in point (27).

Another reason for the incomplete processing in the T4/T4I system may be that the secondary structures of the sense-antisense RNAs are less conductive to duplex formation than the T1/$T_{ant}$ structures. A secondary structure prediction for the naturally occurring pair T1/$T_{ant}$ (Fig. 5a) shows that nearly all unpaired regions of the sense transcript have matching loops in the antisense transcript. In the case of the pair T4/T4I (Fig. 5b) several of the loops in the sense transcript are parts of stems in the antisense transcript and *vice versa*. Hence, the initial 'kissing reaction' (28) in the process of duplex formation is probably less

efficient in the T4/T4I case. Since nature has had a long time evolutionary to perfect the T1/$T_{ant}$ system, these differences are not surprising.

An unexpected observation that has no direct connection with the RNA processing studies was that the changing of the promoter for T4, at a point downstream of the start point for transcription, resulted in a change of the initiation nucleotide. The only change is that the nucleotides AC downstream of the G where wild-type transcription starts have been shifted to CA (Fig. 3). In the transformants, transcription starts two nt downstream of the wild-type start point (Fig. 2, lanes 2 and 3) and transcription also seemed weaker in this case. This indicates that in *H.salinarium*, sequences downstream of the initiation point of transcription may influence promoter strength and the choice of initiation nucleotide. Regrettably, studies in depth of promoter functions in the halobacteria must also await a still-lacking *in vitro* system.

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