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Post-transcriptional control of gene *regB* expression in T4-related bacteriophage VR7

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The bacteriophage VR7 with a T4-type morphology is classified as a T4-related phage. VR7 appeared to contain the gene *regB* encoding endoribonuclease RegB that introduces cuts within mRNA in the middle of tetranucleotide GGAG as observed for RegB of most of T4-relatives. The characteristic feature of RegB of T4-related phages is the ability to autoregulate its synthesis level by attacking GGAG motifs located within the RBS region and the coding sequence of its own mRNA. RegB of T4-related phages efficiently process GGAG within SD sequence and, less efficiently, the GGAG motifs located in coding sequences. Here we show that the expression of the VR7 gene *regB* is also autoregulated by introducing cuts within GGAG motifs located in the SD region and coding sequence within the transcript of the gene *regB*. Unexpectedly, the cleavage efficiency differs from that observed for other T4-related phages. VR7 RegB degrades its own mRNA by introducing a strong cut within the first GGAG motif located in the coding sequence and, less efficiently, in GGAG located in the SD sequence. Thus, it is the first example of such an unusual *regB* gene expression regulation pattern at the post-transcriptional level in T4-related phages.

Key words: T4-related phages, bacteriophage VR7, gene *regB*, endoribonuclease RegB

INTRODUCTION

RNA processing and degradation are an integral part of the metabolic machinery of the cell, and these processes are important for determining the levels of gene expression. *Escherichia coli* employs a large number of distinct RNases that regulate mRNA stability and provide a potent gene expression governing mechanism.

RNases also play a central role in all aspects of bacteriophage T4 RNA metabolism, including mRNA decay, conversion of RNA precursors to their mature forms as well as the end-turnover of certain RNAs. Additionally, T4 encodes its own endoribonuclease RegB, which participates in the turnover of early mRNAs of T4 by introducing cuts within GGAG motifs located in the intergenic regions, and in many cases these are Shine–Dalgarno (SD) sequences of early genes [1–5]. Thus, the RegB endoribonuclease provides a mechanism whereby a subset of viral genes is rapidly and irreversibly turned off shortly after infection [6, 7]. Moreover, T4 RegB cleaves its own mRNA and thus controls the expression level of the gene *regB* [7].

More than 150 bacteriophages with a morphology similar to T4 have been identified [8, 9]. On the basis of the sequence comparison of the major structural genes, T4-related phages were classified into four subgroups: the T-evens, pseudo T-evens, schizo T-evens and exo T-evens [10–12]. The phages RB69 and TuIa were also considered as T-evens [10, 12] but other studies indicated that these phages could occupy an 'intermediate' position [10, 13] and were entitled as 'mezzo T-evens' (H. Krisch, personal communication).

A number of T4-related phages that have been previously classified as T-evens [14] showed the presence of gene *regB* homologues in their genomes [15]. The deduced primary structures of RegB endoribonuclease of 32 T4-related phages were well conserved and almost identical to the T4 RegB (98.6–100%). As expected, the RegB protein of the 'mezzo T-even' phages RB69 and TuIa differed from the T4 RegB at a higher level (identity to T4 RegB was 77.5%). The RegB of the pseudo T-even phage RB49 diverged mostly from the rest of phages examined (43%) [15]. The pseudo T-even phage RB42 [15] and T-even phage KVP40 [16] appeared to lack RegB at all. The targets participating in *regB* gene expression control were found within transcripts of the gene *regB* in most of these phages [15]. This indicates that autogeneous regulation of gene *regB* expression is common to T4-related phages.

Recently a bacteriophage VR7 has been isolated from environmental water [17]. The purified particle of

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bacteriophage VR7 resembled the virion morphology of the family *Myoviridae*, A2 morphotype, which is indistinguishable from the bacteriophage T4. A comparison of the structural protein profile of the isolated phage VR7 showed that it displayed a similar protein electrophoretic migration pattern with the major capsid protein gp23 of bacteriophage T4 [17]. The results indicate that VR7 has a T4-type morphology and thus could be classified as a T4-related phage. This observation has raised a question of presence of the gene *regB* in the genome of the T4-related phage VR7. The aim of our present work was to test if the phage VR7 encodes endoribonuclease RegB. Furthermore, we wished to determine the expression pattern of the gene *regB* in case RegB nuclease is present within the VR7 genome.

MATERIALS AND METHODS

Bacteriophage and bacteria strain. Bacteriophage VR7 [17]. *E. coli* strain B^E (*sup*^{0}), a gift from Dr L. W. Black, was used for preparation of total mRNA.

PCR and sequencing analysis. The DNA fragments of bacteriophage VR7 were amplified by PCR using T4-specific primers. Later, primers based on the obtained VR7 DNA sequences were synthesized and used to fill the gaps of DNA sequences in the region tested. PCR was carried out as described [18]. The sequencing reactions [19, 20] were carried out using a CycleReader™ DNA sequencing kit (Fermentas AB). Phage-specific primers for PCR and sequencing procedures were used as follows:

T4-specific primers: Pr. 1
5'-CAGTTAAGAGGAGAATAACATGAC
(19 nt upstream of gene *regB*) and Pr. 2
5'-GTGCTTTTCTCGAGCTTACCTCATTG
(complementary to 13–49 nt of gene *vs.1*).

VR7-specific primers: Pr. 3
5'-CTGAAAGTAGGCTCTACGCTATGGGC
(complementary to 45–71 nt of gene *vs.1*), Pr.
VR7regB-CDS-2 5'-GTTCTTGTCTGTGATTTCCAGACG
(complementary to 309–333 nt of gene *regB*), Pr.
VR7regB-RBS 5'-GCCTGCTTCTTTACAAGCCTTG
(complementary to 92–114 nt of gene *regB*).

RNA preparation and primer extension analysis of phage mRNA. *E. coli* B^E (*sup*^{0}) were grown at 30 °C to a density of 3×10^8 cell/ml in LB medium and the culture then was aliquoted. One minute before infection a single aliquot was treated with chloramphenicol, whereas the remaining culture was left untreated. Afterwards both aliquots were infected with bacteriophage VR7 at a m.o.i. of 10. Four minutes post infection, total RNA from VR7 infected *E. coli* cells was phenol-extracted as described [6, 21]. The oligonucleotides Pr. VR7regB-RBS, Pr. VR7regB-CDS-2 (see above) and Pr. VR7regB-CDS-1 5'-GTCAATTTCACGTTGGATTGCACGG were 5'-end labeled by T4 polynucleotide kinase (Fermentas AB) with [γ-^{32}P]ATP (Amersham Biosciences) and separated by precipitation with ethanol in the presence of 2 M ammonium acetate. Primer extension and RNA sequencing were carried out under conditions of primer excess, using avian myeloblastosis virus reverse transcriptase (Promega), essentially as described [6].

RESULTS AND DISCUSSION

A characteristic property of ribonucleases is the ability to autoregulate their synthesis in the cell by controlling the degradation rate of their own mRNA [22-25]. Previous studies have shown that T4 endoribonuclease RegB efficiently cleaves its mRNA within the GGAG motif located in the SD region and less efficiently the three additional GGAG motifs located in the coding sequence of the transcript for the gene *regB* [7] (Fig. 1). Recently Piešiniene et al. [15] have shown that 32 T-even phages harbor the similar signals for transcriptional and post-transcriptional autogeneous regulation of *regB* expression: most of them carry the GGAG motif in the SD region and three or at least two GGAG motifs within the coding sequence of the gene *regB* [15], whereas mezzo T-even phages RB69 and TuIa contained the GGAG motif in the SD region and only one GGAG downstream from the initiation codon AUG (Fig. 1) [15].

We wondered if phage VR7 [17], preliminarily classified as T4-related, contains gene *regB* as common to a large number of T4-related phages. T4-specific primers were used to detect T4 homologous DNA in the phage VR7. The primers for *regB* region were employed (see Material and Methods) and DNA amplification products of expected size were detected (data not shown). PCR and sequencing analysis allowed us to map the VR7 gene *regB* within the VR7 genome which appeared to reside upstream of gene *vs.1* as found in other T4-related phages (Fig. 1). The transcript for the VR7 gene *regB* is initiated from an early promoter immediately upstream the gene (Fig. 1). The deduced primary structure of VR7 RegB showed a 59.7% homology with that of T4 RegB. The VR7 RegB homology to the RegB of mezzo T-even phage RB69 (TuIa) as well as the pseudo T-even phage RB49 was 62.9% and 44.2%, respectively.

Analysis of the nucleotide sequence of the VR7 gene *regB* revealed three GGAG motifs: the first motif located within the Shine–Dalgarno sequence and two additional GGAG motifs located within the coding sequence of the transcript of the VR7 gene *regB* (Fig. 1). The number of GGAG motifs contained within the transcript of the VR7 gene *regB* and the RegB primary structure homology rate show the phage VR7 to be related to mezzo T-even phages. With the aim to support this idea, we tested whether the protein encoded by the VR7 gene *regB* is an active endoribonuclease capable to autoregulate its synthesis in a manner common to mezzo T-even phages.

We examined the activity of VR7 RegB endoribonuclease towards GGAG motifs located within the SD region and the coding sequence of the transcript for the gene *regB*. To this end, *E. coli* B^E cells were infected with the phage VR7. Total RNA was extracted four

minutes post infection and subjected to primer extension sequencing.

As expected, VR7 RegB appears to be an active endoribonuclease that introduces cuts in the middle of tetranucleotide GGAG (Fig. 2) as do RegB of T4-related phages [15].

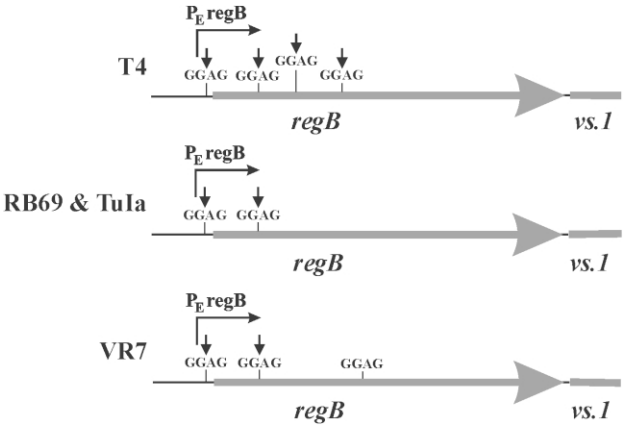

Fig. 1. Schematic representation of genomic region with gene *regB* of bacteriophages T4, RB69 (TuIa) and VR7. The early promoters are shown as well as positions of potential RegB cleavage sites within SD sequences and in the coding sequences of *regB*. Vertical arrows show the sites susceptible to the RegB cleavage

Analysis of the VR7 RegB cleavage pattern within the RBS region shows the 5' end of mRNA assigned to the transcription initiation and 5' end of mRNA cleaved at the middle of the GGAG motif of the SD region upstream the translation initiation codon AUG. The second cut occurred in the proximal part of the coding sequence, whereas the third GGAG motif located within coding sequence remained stayed resistant to the VR7 RegB attack (Fig. 2).

To ensure that the observed cuts in the GGAG motifs were caused by VR7 endoribonuclease RegB but not by *E. coli* endonucleolytic activity, primer extension sequencing experiments were also carried on RNAs that had been extracted from the cells after infection with VR7 in the presence of the protein synthesis inhibitor chloramphenicol.

The primer extension sequencing of the VR7 gene *regB* mRNA extracted after the treatment of cells with chloramphenicol has shown that 5' ends indicating RegB cleavage within the SD and coding sequences are absent in the transcript of the VR7 gene *regB* in the presence of a protein synthesis inhibitor (Fig. 2). Only the transcriptional start site is detected as the 5' end (Fig. 2). Thus, the VR7 gene *regB* transcript is not processed when the synthesis of RegB protein is inhibited, indicating that both cleavages within the transcript of the VR7 gene *regB* are phage-induced (Fig. 2).

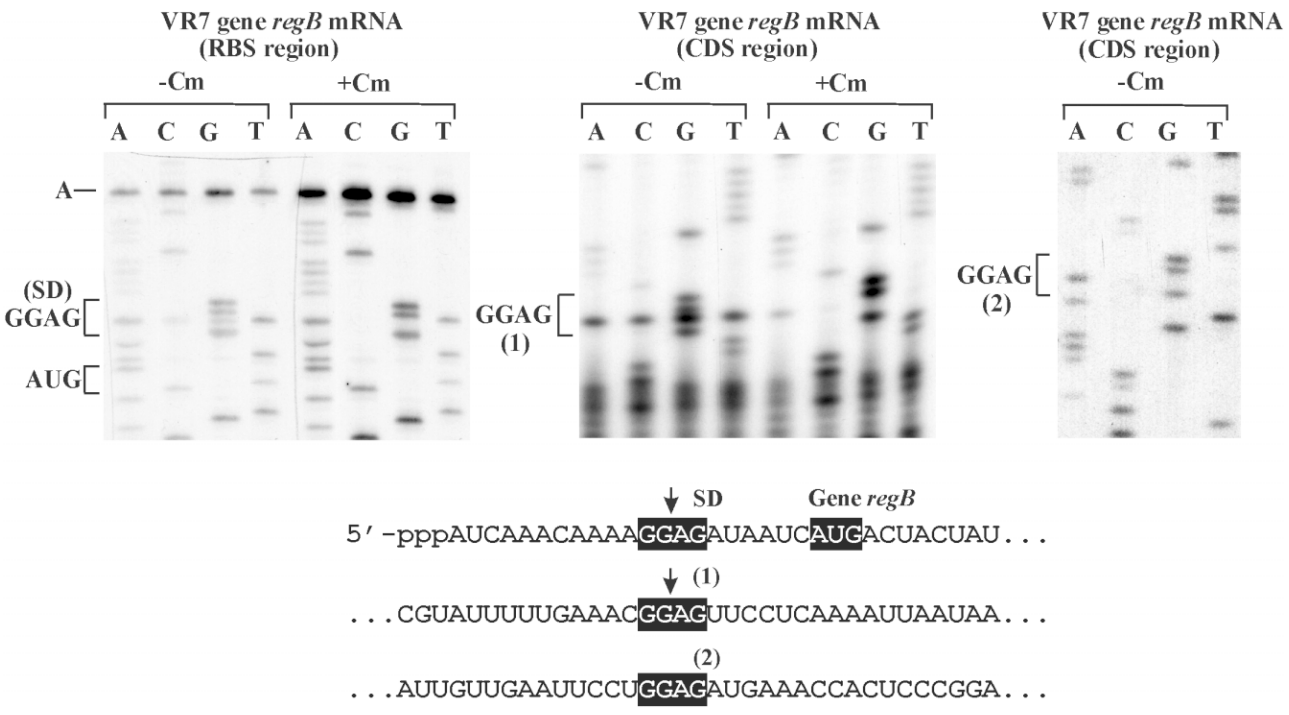

Fig. 2. Susceptibility of VR7 transcript for gene *regB* to the RegB endoribonuclease of phage VR7. The VR7 mRNA was isolated from *E. coli* B^E cells at 4 min post infection with phage VR7 in the presence or absence of chloramphenicol at 30°C. Primer extension reactions were performed as described in Materials and Methods. The sequencing lanes are labeled with the dideoxynucleotides used in the sequencing reactions. The initiating nucleotide, the 5' ends of RegB processed transcripts as well as the initiation codon for gene *regB* are noted. The primers used in primer extention reactions Pr. VR7regB-RBS, Pr. VR7regB-CDS-1 and Pr. VR7regB-CDS-2 are complementary to the sequence of gene *regB* of phage VR7. The nucleotide sequences of 5' flanking region and the coding sequence of gene *regB* of phage VR7 are shown underneath

The results clearly indicate that VR7 RegB processes its own mRNA within two GGAG motifs. This information confirms the previous observation suggesting that the phage VR7 is related to mezzo T-evens. The obtained results indicate that RegB of phage VR7 regulates its synthesis by introducing two cuts within its own mRNA as observed for the mezzo T-even phages RB69 and TuIa [15].

Hence the autoregulation of the expression at the post-transcriptional level is also natural to *regB* of phage VR7. However, the results revealed certain discrepancies in the cleavage pattern obtained for VR7 RegB. The region of the transcript for the gene *regB* encompassing GGAG motifs within the SD and proximal coding sequence was tested twice with two different primers, Pr. VR7regB-RBS and Pr. VR7regB-CDS-1, allowing to visualize both cleavage sites simultaneously. In both cases the processing of the GGAG motif within the SD sequence appeared less efficient than the processing of the second GGAG motif located within the proximal part of the coding sequence (Fig. 2). Usually, endoribonucleases RegB of T4-related phages regulate their synthesis level as well as the turnover of early mRNAs by introducing a strong cut within the SD sequence, whereas GGAG motifs within the coding sequence are processed with a less efficiency [2, 3, 7, 15]. It is known that a more efficient cleavage of the GGAG motif within the RBS region is caused by a positive effect of the ribosomal protein S1 that targets the same sequence as does RegB [26, 27]. The less efficient processing of GGAG motifs within the coding sequence is explained by various context-dependent local secondary structures that may impair the processing of GGAG motifs [26 –28]. Hence, the cleavage pattern of VR7 RegB differs from that of T4: the primary motif participating in VR7 gene *regB* expression control is the first GGAG motif located within the proximal part of the coding sequence. The GGAG motif within the RBS region has a less influence on gene *regB* expression control, whereas the second GGAG motif within the coding sequence has no influence at all.

It should be noted that the degradation pattern of the VR7 transcript of the gene *regB* represents a first example where inactivation of the gene *regB* mRNA is performed mainly by processing the GGAG within the coding sequence instead of that within the RBS region.

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T4 GIMININGO BAKTERIOFAGO VR7 GENO *regB* EKSPRESIJOS POTRANSKRIPCINĖ REGULIACIJA

Santrauka

Bakteriofagui VR7 yra būdinga T4 tipo morfologija, todėl jis yra priskiriamas T4 gimininių fagų grupei. Fagas VR7 turi *regB* geną, koduojantį endoribonukleazę RegB, kuri specifiškai kerpa iRNR per tetranukelotidų GGAG vidurį. T4 gimininių fagų *regB* geno ekspresijai yra būdinga autogeninė reguliacija, kai šio geno koduojama RegB nuklеazė kerpa savo iRNR GGAG motyvus, esančius Šaino-Dalgarno ir koduojančioje sekose. Mes nustatėme, kad fago VR7 *regB* geno ekspresija taip pat reguliuojama autogeniškai, kadangi VR7 RegB kerpa savo iRNR per du GGAG motyvus, esančius SD ir koduojančioje sekose. Tačiau, priešingai T4 gimininių fagų RegB, VR7 RegB efektyviau kerpa savo iRNR per pirmąjį GGAG motyvą, esantį koduojančioje sekoje, tuo tarpu SD sekos GGAG motyvas yra kerpamas silpniau. Taigi toks T4 gimininго fago VR7 *regB* geno ekspresijos reguliacijos potranskripciniame lygyje pobūdis nustatytas pirmą kartą.