Analysis of sequence-specific endoribonuclease RegB gene in T4-related bacteriophages

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We have determined the existence of sequence-specific endoribonuclease gene *regB* in the genomes of 15 T4-related phages. The nucleotide sequences of gene *regB* of 14 T4-related phages share a high homology (97.8–99.1%) with T4. In phage RB69, the nucleotide sequence of gene *regB* differs considerably from that of T4 (73.2%). The amino acid sequences of endoribonuclease RegB are highly conserved in 14 T4-related phages (98–100%). The RegB of phage RB69 shows a substantial amino acid sequence divergence from T4 (23.2%). Nevertheless, we have demonstrated that RegB endoribonuclease of phage RB69 is active during the early period of infection and introduces cuts in the Shine–Dalgarno sequence of gene *30.7*.

Key words: T4-related bacteriophages, gene *regB*, endoribonuclease RegB

INTRODUCTION

Bacteriophages utilise host ribonucleases to process and degrade their mRNAs. RNase E plays a major role in bacteriophage T4 mRNA degradation [1, 2]. Besides, T4 transcripts are processed by an endoribonuclease encoded by the phage itself [3]. The T4 RegB endoribonuclease, made shortly after infection, cleaves mRNAs in the middle of the sequence GGAG or, in a few cases, in the sequence GGAU [4–6]. The most efficient cleavages occur in GGAG motifs located in intergenic regions, and in many cases these are Shine–Dalgarno sequences of early genes [3]. Thus, the RegB endoribonuclease provides a mechanism whereby a subset of viral genes is rapidly and irreversibly turned off shortly after infection [4, 5]. In contrast, it has no effect on most of those GGAG motifs located in the late or middle messengers [3, 4, 6].

Gene *regB* is transcribed from a typical early promoter, immediately upstream of the gene [6]. It seems to be autoregulated, since *regB* mRNA is cleaved by the RegB nuclease within its Shine–Dalgarno sequence, and, to a lesser extent, at three places within its coding sequence [5]. PCR amplification revealed the presence of gene *regB* in the genomes of T4-related phages K3, RB70, T2 and T6 [7], but no genes have been sequenced. In this study we have demonstrated that posttranscriptional control of gene expression with the participation of RegB endoribonuclease is typical of the T4-related bacteriophages tested.

MATERIALS AND METHODS

Phages and bacteria. Phages T2, T6, M1, K3, Ox2, and TuIb were obtained from Prof. U. Henning (Tübingen, Germany). Phages RB2, RB3, RB14, RB15, RB23 and RB69 were provided by Prof. K. Carlson (Uppsala, Sweden). Bacteriophages RB6, RB10 and RB26 were obtained from Prof. W. B. Wood (Boulder, USA).

All phages were grown in *Escherichia coli* B^E (sup^o) (provided by Dr. L. W. Black), except for TuIb grown in *E.coli* CR63 (supD) (supplied by Dr. K. N. Kreuzer).

Enzymes and isotope. T4 polynucleotide kinase and *Taq* DNA polymerase were obtained from Fermentas AB. Avian myeloblastosis virus reverse transcriptase was obtained from Promega. [γ-^{33}P]ATP and [γ-^{32}P]ATP were supplied by Amersham Biosciences.

PCR analysis of gene *regB* in the T4-related phage genomes. A series of gene *regB* of T4-related bacteriophages was amplified by PCR [8] using the gene *regB* specific primers. Phage plaques were used as a source of DNA templates for PCR amplification [9]. The PCR protocol involved 30 cycles with a program of denaturation at 92 °C for 1 min, primer annealing at 50 °C for 1 min, and extension at 72 °C for 1 min. In some cases the annealing temperatures had to be adapted for some of these templates. For example, the RB69 PCR product was obtained at an annealing temperature of 48 °C.

Direct sequencing of PCR DNA fragments. Sequencing was performed with a CycleReader DNA

sequencing kit (Fermentas AB) using the same primers. The nucleotide sequences were submitted to the EMBL/GenBank database. Their accession numbers are given in the appropriate figures.

RNA preparation and primer extension analysis of phage mRNAs. Total RNA from phage T4 infected *E. coli* cells was phenol-extracted as described [4]. Primer extension analysis and RNA sequencing were carried out under conditions of primer excess, using avian myeloblastosis virus reverse transcriptase, as described [4, 10].

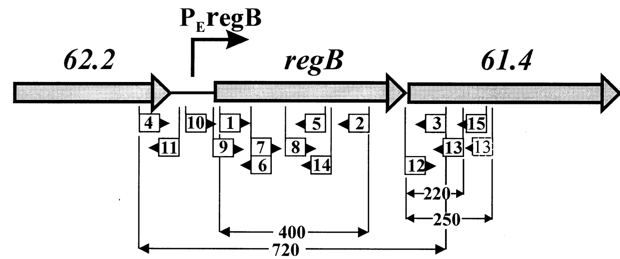

Fig. 1. The genomic region with genes *62.2*, *regB* and *61.4* of bacteriophage T4. The position of early promoter is shown. Boxes with arrows indicate oligonucleotide primers used in PCR analysis and sequencing reactions

RESULTS AND DISCUSSION

We have amplified and sequenced the gene *regB* in the genomes of 15 T4-related phages using PCR primers (Table 1) based on the T4 gene *regB* sequence [11]. Initially, using two PCR primers, Pr. 1 and Pr. 2, within the coding sequence of gene *regB* we obtained a PCR product of 400 bp of 14 T4-related phages (Fig. 1). The phage RB69 did not yield the expected PCR fragment with these primers. Another pair of primers, Pr. 4 and Pr. 3, flanked the gene *regB* sequence and was used to amplify the analogue of this gene in the genomes of 15 T4-related phages. Fourteen T4-related bacteriophages yielded PCR fragments of 720 bp, while phage RB69 did not produce the expected PCR fragment again. The sequences of gene *regB* of 14 T4-related phages were determined from the obtained PCR products. Some other primers (Fig. 1) were also used to define the nucleotide sequence of gene *regB* in T4-related phages.

A pair of primers Pr. 12–Pr. 13 based on the sequence of gene *61.4* of phage T4 was used to amplify a fragment from phage RB69 under specific PCR conditions. We obtained two PCR products of 220 bp and 250 bp using this pair of primers (Fig. 1). The fragments were sequenced and shown to contain a gene *61.4* analogue. Using some special primers Pr. 15 and Pr. 14 for phage RB69 we determined a complete nucleotide sequence of gene *regB* of RB69.

The nucleotide sequences of gene *regB* of 14 T4-related phages share a high homology with nucleic acid of phage T4 (97.8–99.1%). All of them have an identically positioned *regB* early promoter sequence, –35 and –10 boxes separated by 15 bp. The promoter sequence, the ribosome binding site and the start codon of gene *regB* are essentially identical with T4. Meanwhile the nucleotide sequence of gene *regB* of phage RB69 differs considerably from that of T4 (73.2%). In the case of this phage, the –10 and –35 boxes of the *regB* early promoter have some nucleotide

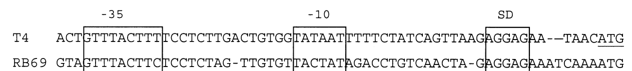

Fig. 2. Alignment and comparison of early promoter sequences, Shine–Dalgarno sequences and start codons of the *regB* gene of bacteriophages T4 and RB69. The positions of the promoters –35 and –10 boxes and the Shine-Dalgarno (SD) sequences are indicated in the first line of the panel. The initiation codons are underlined

Table. **Oligonucleotide primers used in PCR and sequencing reactions**

Primer	Primer sequence	Position
Pr. 1	5′-GCTTCGTCGTCACTTTGAGTCGG	gene *regB*, 39–61 nt
Pr. 2	5′-GAAGCCTTTCCTGGTAAACGACG	gene *regB,* 432–415 nt
Pr. 3	5′-GGACATATCTGAAAGTATGCTCGGAGC	gene *61.4,* 80–53 nt
Pr. 4	5′-GAGGTTCGTGCCGAAGTTTCAAAGTC	gene *62.2,* 223–249 nt
Pr. 5	5′-GAATTCATTAACTTCTAAAACATG	gene *regB*, 241–217 nt
Pr. 6	5′-GACTCCTGCTGCTTTTGATGCCTC	gene *regB*, 118–91 nt
Pr. 7	5′-GAGGCATCAAAAGCAGCAGG	gene *regB*, 91–111 nt
Pr. 8	5′-GACCATGTTTTAGAAGTTAATGA	gene *regB*, 212–236 nt
Pr. 9	5′-GAGGAGAATAACATGACTATCAA	12 nt before gene *regB*
Pr. 10	5′-GTTTACTTTTCCTCTTGACTGTGGTA	58 nt before gene *regB*
Pr. 11	5′-ACAGTTATTCTTTAAATCTAATC	gene *62.2*, 284–261 nt
Pr. 12	5′-CAATGAGGTAAGCATGAGAAAAGCAC	gene *regB*, 457–483 nt
Pr. 13	5′-ACTCCGCCAAAGCTTTCTTGCC	gene *61.4*, 232–201 nt
Pr. 14	5′-GCCTGGTAGTCCAAGAGACTTTGCAGC	gene *regB*, 120–94 nt
Pr. 15	5′-ATACGCATGGTGACCTTTCTTATC	gene *61.4*, 256–232 nt

changes (Fig. 2) and the spacing between them is only 14 bp. The Shine–Dalgarno sequence and the start codon are exactly the same as in T4 and other 14 T4-related phages, but separated by 8 bp instead of 6 bp in T4. The coding sequence of gene *regB* in RB69 was shortened by one codon and the termination codon was changed from UGA (in T4) to UAG.

A comparison of amino acid sequences of the RegB endoribonuclease shows a substantial amino acid se-

quence homology among the 14 T4-related phages. The primary structures of sequence-specific endoribonuclease RegB of the phages tested indicate the following levels of amino acid identity with T4: 100% for T6, RB14 and RB26, 99.3% for M1, K3, O × 2 TuIb, RB2 and RB15 and 98.6% for T2, RB3, RB6 RB10, and RB23. The RB69 RegB endoribonuclease amino acid sequence is 76.8% identical to that of T4 (Fig. 3).

The early gene *30.7* of T4 carries a GGAG motif as a Shine–Dalgarno sequence [12]. This tetranucleotide could be a potential RegB cleavage site. Moreover, this region is well conserved in the bacteriophage RB69 genome. Figure 4 shows that transcripts for gene *30.7* of phage T4, as well as RB69, are efficiently processed in the middle of GGAG Shine–Dalgarno sequence by endoribonuclease RegB during phage infection.

Thus, our results indicate that posttranscriptional control of gene expression with participation of RegB endoribonuclease is typical of all the T4-related bacteriophages tested. The activity of sequence-specific endoribonuclease RegB was demonstrated by primer extension analysis of mRNA for gene *30.7* even in the case of phage RB69.

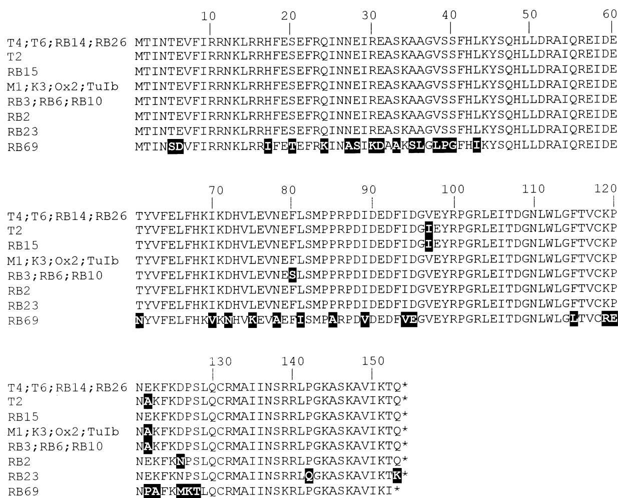

Fig. 3. Comparison of amino acid sequences of the RegB endoribonuclease of T4-related phages tested. Protein sequences of 15 T4-related phages were aligned to the T4 sequence using the ClustalW program. Amino acid motifs common to all of T4-related phages tested are indicated by a white background. Sequences shown with a black background were not well conserved. An asterisk indicates a termination codon. The nucleic acid sequences have been deposited in the GeneBank Database under accession numbers: AJ488513 (for phage T2), AJ488518 (T6), AJ488514 (M1), AJ488515 (K3), AJ488516 (Ox2), AJ488517 (TuIb), AJ488519 (RB2), AJ488520 (RB3), AJ488521 (RB6), AJ488522 (RB10), AJ488523 (RB14), AJ488524 (RB15), AJ488525 (RB23), AJ488526 (RB26) and AJ439451 (RB69)

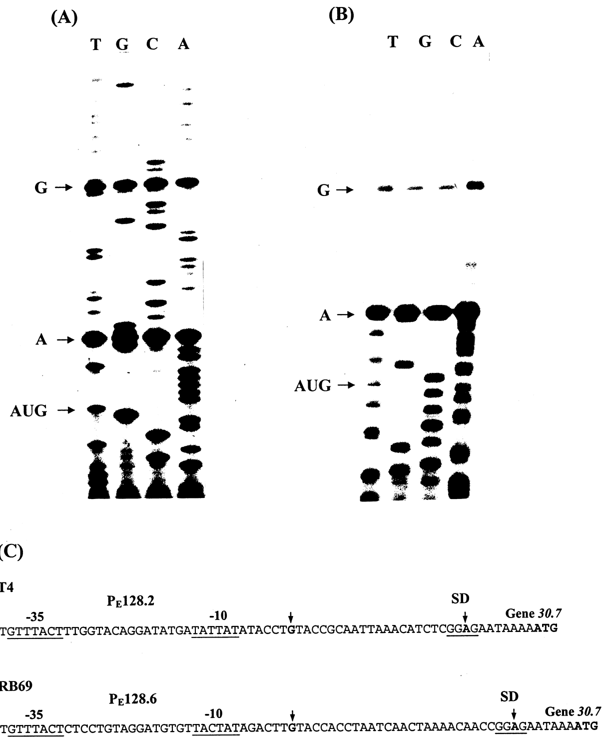

Fig. 4. Primer extension sequencing of transcripts for gene *30.7* of bacteriophages T4 (A) and RB69 (B). The nucleotide sequence of the 5' flanking region of gene *30.7* of phages T4 and RB69 are shown in (C). Primer extension reactions were performed as described in Materials and Methods, using total RNA isolated from *E. coli* B^E cells 7 min post infection with a phage. The sequencing lanes are labeled with dideoxynucleotides used in the sequencing reactions. The initiating nucleotides for early transcripts, the 5′ ends of RegB processed transcripts, as well as initiation codon for gene *30.7* are noted. The following primers were used in primer extension reactions: Pr. 16, 5′-GTATCAATAGAACCTG CAATACC, complementary for 62–39 nt of the gene *30.7* of phage T4 and Pr. 17, 5′-GGAGTCATGCACT GTTCGATTTCAC, complementary for 123–97 nt of gene *30.7* of RB69.

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ENDORIBONUKLEAZĖS REGB, ATPAŽĮSTANČIOS SPECIFINĘ SEKĄ, GENO ANALIZĖ T4 GIMININGUOSE BAKTERIOFAGUOSE

S a n t r a u k a

Polimerazinės grandininės reakcijos (PGR) metodu buvo suamplifikuoti 15 T4 giminingų bakteriofagų DNR fragmentai su genu *regB*: 14 T4 giminingų bakteriofagų amplifikacijos produktai buvo gauti naudojant pradmenis, susintetintus pagal jau žinomą bakteriofago T4 geno *regB* seką, o fago RB69 atveju DNR fragmentas PGR metodu buvo gautas tik pritaikius specialius pradmenis ir polimerazinės grandininės reakcijos sąlygas.

Atlikus 15 T4 giminingų bakteriofagų *regB* genų nukleotidų sekų palyginamąją analizę nustatyta, kad 14 T4 giminingų fagų *regB* genų homologija T4 *regB* genui yra labai aukšta (97,8–99,1%), o fago RB69 atveju ši homologija daug žemesnė – 73,2%. Be to, 14 fagų atveju *regB* reguliacinės sekos (ankstyvasis promotorius, Shine-Dalgarno seka ir transliacijos iniciacijos kodonas) išlieka absoliučiai konservatyvios. Fago RB69 geno *regB* ankstyvojo promotoriaus –35 ir –10 sritys yra pakitusios, o atstumas tarp jų sumažėjęs.

14 tirtų T4 giminingų fagų RegB endoribonukleazės yra labai homologiškos T4 koduojamai RegB endoribonukleazei (98–100%). Fago RB69 RegB endoribonukleazės homologija T4 RegB nukleazei – 76,8%. Netgi fago RB69 atveju ankstyvojo geno *30.7* informacinė RNR yra skaidoma per Shine-Dalgarno sekos GGAG motyvą.

Taigi nustatyta, kad potranskripcinė genų ekspresijos reguliacija, kai RegB endoribonukleazė inaktyvuoja ankstyvųjų genų informacines RNR, perkirpdama jas per Shine-Dalgarno sekų GGAG motyvus, yra būdinga visiems tirtiems T4 giminingiems fagams.