Primary structure and activity of endoribonuclease RegB encoded by bacteriophage TuIa

L. Piešinienė,
L. Truncaitė,
A. Zajančkauskaitė,
R. Nivinskas

Institute of Biochemistry, Mokslininkų 12, Vilnius, LT-2600, Lithuania, rimasn@bchi.lt

We have established the primary structure and activity of sequence-specific endoribonuclease RegB encoded in the genome of T4-related bacteriophage TuIa. The nucleotide sequence of gene *regB* of phage TuIa is 73.2% identical to that of T4. The amino acid sequence of the RegB endoribonuclease of phage TuIa diverges more than 23% from that of T4. Nevertheless, we have demonstrated that RegB endoribonuclease of phage TuIa retains the same specificity as T4 RegB and introduces cuts in the GGAG motif of the Shine–Dalgarno sequence of TuIa early mRNA.

Key words: bacteriophage TuIa, gene *regB*, endoribonuclease RegB

INTRODUCTION

The T4 endoribonuclease RegB is involved in the inactivation of the phage early messenger RNAs [1–5]. This nuclease recognizes the GGAG motif in the Shine–Dalgarno sequence of these messengers and specifically cleaves between the second G and the A in the tetranucleotide sequence [6]. Surprisingly, GGAG motifs carried by middle and late mRNA escape RegB processing [1, 2, 4, 5]. The tetranucleotide GGAG located in a coding sequence is poorly recognized, if at all [2]. Thus, the sequence-specific endoribonuclease RegB is specifically required for normal turnover of early mRNAs and regulation of translation of several early genes [4]. This endonuclease activity is found to be encoded by the *regB* gene. The *regB* gene is transcribed from a typical early promoter located immediately upstream of the gene. The expression of the *regB* gene seems to be regulated autogenously by attacking its own mRNA within the GGAG motif in the Shine–Dalgarno sequence and at three additional GGAG motifs within its coding sequence [7]. The *regB* gene is found in several other T4-related phages [8], but only in the case of phage T4 the primary structure, activity and specificity of the sequence-specific endoribonuclease RegB have been determined.

Recently, it has been shown that T4-related phage TuIa is evolutionarily distant from the T4 [9]. We wished to determine if a sequence-specific endoribonuclease RegB analogue is also encoded in the genome of phage TuIa and cuts specifically in the GGAG motif of the Shine–Dalgarno sequence of early mRNA.

MATERIALS AND METHODS

Bacteriophage and bacteria strains. Bacteriophage TuIa was obtained from Dr. U. Henning. T4D wild-type was kindly supplied by Dr. W. B. Wood, T4 *regB*$^-$ (*regB*L52) was a gift of Dr. M. Uzan. The following *Escherichia coli* strains were used: CR63 (supD) (supplied by Dr. K. N. Kreuzer) was used as the host to prepare phage stock of TuIa, B^E (sup^0) obtained from Dr. L. W. Black was used for propagation of phages T4D wild-type and T4 *regB*$^-$.

PCR and sequencing procedures. The PCR reactions were carried out as described [11]. The conditions used for the amplification reaction involved 30 cycles consisting of a 60 s denaturation at 92 °C, a 60 s annealing at 55 °C and a 60 s extension at 72 °C. In some cases the annealing temperature had to be adapted for some of templates. For example, TuIa PCR products with primers Pr. 1–Pr. 2 were obtained at the annealing temperature of 48 °C. The DNA template for the reaction was either in the form of purified phage DNA [12] or denatured phage particles [13]. PCR was performed using *Taq* DNA polymerase (Fermentas AB) with Pr. 1, 5'-CAATGAGGTAAGCATGAGAAAAGCAC, corresponding to 457–483 nt of T4 gene *regB*, Pr. 2, 5′-ACTCCGCCAAAGCTTTCTTGCC, complementary to 223–201 nt of T4 gene *61.4* and Pr. 6, 5′-GAGGAGAATAACATGACTATCAA, identical to the sequence starting at 1 nt upstream the Shine–Dalgarno sequence of the T4 gene *regB*.

The sequencing reactions were carried out using a CycleReader DNA sequencing kit (Fermentas AB). The DNA template for the sequencing reactions was in the form of phage DNA purified and digested with restriction enzyme SspI (Fermentas AB) [14] or a PCR fragment. The primers Pr. 3, 5′-ATA CGCATGGTGACCTTTCTTATC, complementary to 256–232 nt of TuIa of gene *61.4*, Pr. 4, 5′-GCCT GGTAGTCCAAGAGACTTTGCAGC, complementary to 120–94 nt of TuIa of gene *regB*, and Pr. 5, 5′-GACTCCTGCTGCTTTTGATGCCTC, complementary to 118–91 nt of TuIa of gene *regB* were used in these sequencing reactions. The oligonucleotides were 5'-end-labeled by T4 polynucleotide kinase (Fermentas AB) with [γ-^{33}P]ATP (Amersham Bioscience). The nucleotide sequence of gene *regB* of TuIa has been deposited in the EMBL/GenBank database. The accession number is given in the appropriate figure.

RNA preparation and primer extension analysis of phage mRNAs. Total RNA from phage-infected *E. coli* cells was phenol extracted as described [6]. RNA was analyzed by primer extension under conditions of primer excess, using avian myeloblastosis virus reverse transcriptase as described [6]. Two oligonucleotides were used to prime reverse transcriptase Pr. 7, 5′-GTATCAATAGAACCTGCAATACC, complementary to 62–39 nt of the gene *30.7* coding sequence of phage T4, and Pr. 8, 5′-GGAGTCATG CACTGTTCGATTTCAC, complementary to 123–97 nt of the gene *30.7* coding sequence of phage TuIa. Oligonucleotides were 5'-end-labeled with [γ-^{32}P]ATP (Amersham Bioscience).

RESULTS AND DISCUSSION

We have amplified and sequenced the gene *regB* in the genome of T4-related phage TuIa. Initially, we tried to obtain PCR products of gene *regB* of TuIa with a variety of primers based on the T4 *regB* coding sequence or promoter sequence upstream the gene, but the phage did not yield the expected PCR fragment.

Using two PCR primers based on the T4 sequence, one at the 3' end of gene *regB,* Pr. 1, and the other at the beginning of gene *61.4,* located just downstream the gene *regB,* Pr. 2, [15] we obtained two PCR products, 220 bp and 250 bp, from the TuIa genome under specific PCR conditions (see Materials and Methods) (Fig. 1). The fragments were sequenced and shown to contain a gene *61.4* analogue. Primer Pr.3 based on the obtained sequence of gene *61.4* of TuIa was used to sequence the genomic DNA of TuIa (Fig. 1). The complete nucleotide sequence of gene *regB* of TuIa was determined using the same method with specific primers for phage TuIa Pr. 4 and Pr. 5. To confirm the sequence of the gene *regB* of TuIa, a PCR fragment of 570 bp with a pair of primers Pr. 6–Pr. 3 was obtained and sequenced.

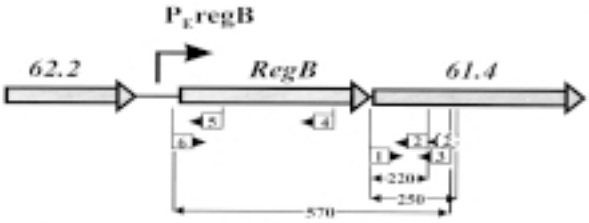

Fig. 1. The genomic region with genes *62.2*, *regB* and *61.4* of bacteriophage T4. The position of early promoter is shown. Boxes with arrows indicate oligonucleotide primers used in PCR analysis and sequencing reactions

The deduced nucleotide sequence of gene *regB* of phage TuIa indicates that it is 73.2% identical with the *regB* gene of T4. In the case of phage TuIa, the *regB* early promoter –35 and –10 boxes exhibit some changes (Fig. 2). The spacing between –35 and –10 boxes is reduced by 1 bp. The Shine–Dalgarno sequence and a start codon are exactly the same as in T4, but separated by 8 bp instead of 6 bp in T4. The coding sequence of gene *regB* in TuIa is shortened by one codon and the termination codon is changed from UGA (in T4) to UAG.

Comparison of the amino acid sequence of the RegB endoribonuclease of phage TuIa revealed a substantial amino acid sequence divergence from T4. The TuIa RegB endoribonuclease amino acid sequence was 76.8% identical to that of T4 (Fig. 3).

The early gene *30.7* of T4 carries the GGAG motif as a Shine–Dalgarno sequence [16]. This tetranucleotide could be a potential RegB cleavage site. Our primer extension experiments show that new 5′

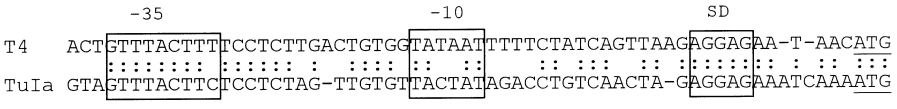

Fig. 2. Alignment and comparison of the early promoter sequences, Shine–Dalgarno sequences and the start codons for the *regB* gene of T4 and TuIa. The positions of the promoter's –35 and –10 boxes and the Shine–Dalgarno (SD) sequences are indicated in the first line of the panel. The initiation codons are underlined. A dash (-) indicates that the nucleotide at this position is deleted and a double dot (:) that the nucleotides are identical

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              10         20         30         40         50         60
               |          |          |          |          |          |
T4     MTINTEVFIRRNKLRRHFESEFRQINNEIREASKAAGVSSFHLKYSQHLLDRAIQREIDE
TuIa   MTINSDVFIRRNKLRRIFETEFRKINASIKDAAKSLGLPGFHIKYSQHLLDRAIQREIDE
       ****::********** **:***:** .*::*:*: *:..**:*****************

              70         80         90         100        110        120
               |          |          |          |          |          |
T4     TYVFELFHKIKDHVLEVNEFLSMPPRPDIDEDFIDGVEYRPGRLEITDGNLWLGFTVCKP
TuIa   NYVFELFHKVKNHVKEVAEFISMPARPDVDEDFVEGVEYRPGRLEITDGNLWLGLTVCRE
       .********:*:** ** **:***.***:****::******************:***:

              130        140        150
               |          |          |
T4     NEKFKDPSLQCRMAIINSRRLPGKASKAVIKTQ
TuIa   NPAFKMKTLQCRMAIINSKRLPGKASKAVIKI-
       *  **  :**********:********** .
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Fig. 3. Comparison of the amino acid sequences of the RegB endoribonuclease of T4 and TuIa phages. The protein sequence of phage TuIa was aligned to the T4 sequence using the ClustalW program. An asterisk (*) means that the residues in the column are identical; a double dot (:) means that conserved substitutions have been observed; a dot (.) means that semi-conserved substitutions are observed; a dash (–) stands for a deleted amino acid. The nucleic acid sequence has been deposited in the GeneBank Database under accession number AJ490327

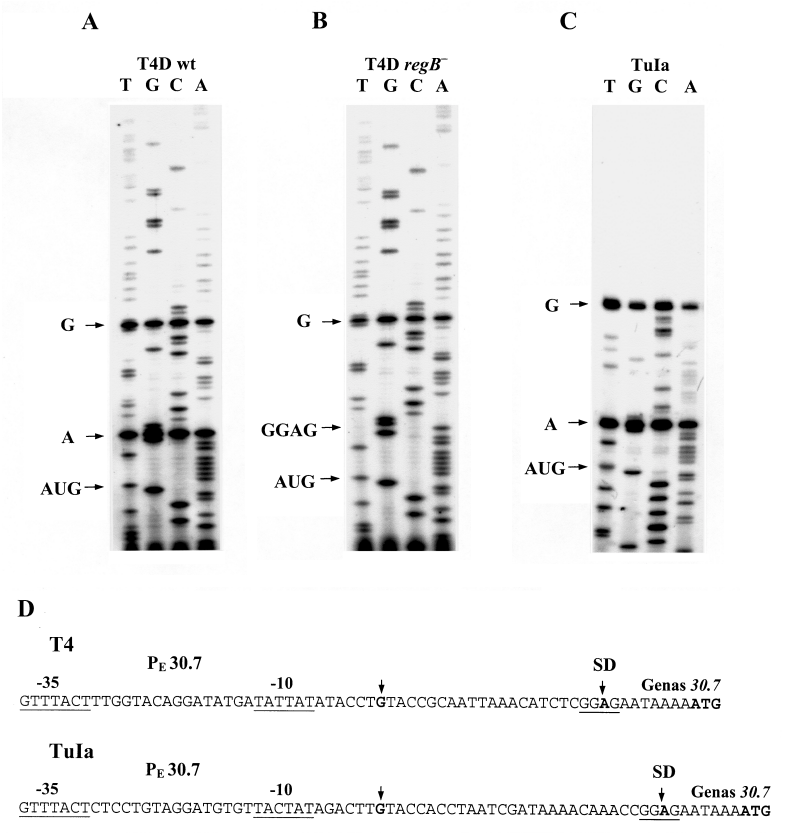

Fig. 4. Primer extension reaction of transcripts for the gene *30.7* of bacteriophages T4D wilde-type (A), T4 *regB*⁻ (B) and TuIa (C). The nucleotide sequences of the 5′ flanking region of gene *30.7* of phages T4 and TuIa are shown in (D). Primer extension reactions were performed as described in Materials and Methods, using total RNA isolated from *E. coli* B^E cells 7 min post infection with phage. The sequencing lanes are labeled with the dideoxynucleotides used in the sequencing reactions. The initiating nucleotide for early transcripts, the 5' end nucleotide of RegB processed transcripts, as well as the initiation codon for gene *30.7* are noted.

ends resulting from post-transcriptional RegB cleavage event appear at the nucleotide A positioned 9 nt upstream gene *30.7* initiation codon in a T4D wild-type phage infection (Fig. 4A). The latter 5′ ends are absent in the case of infection with the T4 mutant lacking RegB processing activity (Fig. 4B). Figure 4C shows that transcripts for the gene *30.7* of phage TuIa are efficiently processed in the middle of the GGAG Shine–Dalgarno sequence by endoribonuclease RegB during phage infection.

Thus, in spite of the endoribonuclease RegB of TuIa sequence divergence from that of T4, our results indicate that post-transcriptional control of gene expression with the participation of RegB endoribonuclease is typical of both bacteriophages, T4 and TuIa. The activity of sequence-specific endoribonuclease RegB was demonstrated by primer extension analysis of mRNA for gene *30.7* in the case of phages T4 and TuIa.

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L. Piešinienė, L. Truncaitė, A. Zajančkauskaitė, R. Nivinskas

BAKTERIOFAGO TuIa ENDORIBONUKLEAZĖS ReGB PIRMINĖ STRUKTŪRA IR AKTYVUMAS

S a n t r a u k a

T4 giminingo bakteriofago TuIa DNR fragmentai *regB* geno srityje buvo gauti pritaikius specialius pradmenis ir polimerazinės grandininės reakcijos sąlygas. Nustačius bakteriofago TuIa *regB* geno nukleotidų seką matyti, kad ji 73,2% homologiška fago T4 *regB* sekai. Be to, fago TuIa geno *regB* ankstyvojo promotoriaus –35 ir –10 sritys yra pakitusios ir atstumas tarp jų sumažėjęs. Tačiau TuIa *regB* Shine-Dalgarno seka ir transliacijos iniciacijos kodonas išlieka absoliučiai konservatyvūs. Fago TuIa RegB endoribonukleazės homologija T4 RegB nukleazei – 76,8%. Paaiškėjo, kad abiejų fagų atveju ankstyvojo geno *30.7* informacinė RNR yra skaldoma 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 T4 gimininga fagui TuIa.