VILNIUS UNIVERSITY

ŽIVILĖ STRAZDAITĖ-ŽIELIENĖ

INVESTIGATION OF THE IMPACT OF ENDORIBONUCLEASES ON BACTERIOPHAGE T4 EARLY TRANSCRIPTS

Summary of doctoral dissertation
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VILNIAUS UNIVERSITETAS

ŽIVILĖ STRAZDAITĖ-ŽIELIENĖ

ENDORIBONUKLEAZIŲ POVEIKIO ANKSTYVIESIEMS BAKTERIOFAGO T4 TRANSKRIPTAMS TYRIMAS

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INTRODUCTION

Virulent bacteriophages are phages which can only multiply on bacteria and destroy the cell by lysis after immediate replication of the virion. Bacteriophage T4 is a phage that infects *Escherichia coli.* Like most other virulent phages, T4 infects exponentially growing cells, which maintain active metabolic processes. T4 terminates these processes and takes over bacterial metabolism. This is achieved during the first few minutes of infection, when the phage-encoded factors modify proteins of *E. coli* to serve the development of the phage. The changes in gene expression occur at the levels of transcription, translation, and mRNA degradation. The T4 DNA sequences that act as the regulatory signals for gene expression also play an important role in the aforementioned processes.

The most important process that ensures a consistent expression of T4 genes is the temporal transcriptional regulation, when phage-encoded transcriptional regulators modify the RNA polymerase of the host cell and redirect it to recognize three different classes of T4 promoters: early, middle, and late (Miller et al., 2003). It takes less than 30 minutes for a switch from early to late T4 gene transcription to occur (O'Farrell and Gold 1973; Christensen and Young 1984; Kai et al., 1996). Such rapid transition cannot be achieved solely through changes in transcriptional activity; it also requires an effective and rapid mRNA degradation, which contributes to translation apparatus available for another gene expression and also increasing the pool of free nucleotides. These processes are closely related and interdependent and are regulated both by the structure of mRNA and protein modifications.

It is known that bacteriophage T4 also modifies a number of *E. coli* proteins involved in translation, thus redirecting these proteins to recognize the sequences that regulate the synthesis of phage peptides. Meanwhile the maturation and degradation of T4 mRNA are among the least investigated processes. The *E. coli* RNase E that is used throughout the phage development has been the first endonuclease shown to be involved in the processing of T4 mRNAs (Mudd et al., 1990), while the sequence-specific T4-encoded RegB endoribonuclease functionally inactivates most early transcripts and expedites their degradation. T4 RegB generates cuts in the middle of GGAG/U sequences located in the intergenic regions of early genes, mostly in translation initiation

regions (Uzan et al., 1988; Ruckman et al., 1989; Sanson and Uzan, 1993, 1995). By destroying the ribosome binding sites, RegB causes functional inactivation of early transcripts, thus facilitating the transition between early and subsequent phases of T4 gene expression (Sanson et al., 2000).

Although RegB targets most of the early transcripts, it is still unclear how this enzyme facilitates the mRNA degradation, and what is the role of RNase E as well as other *E. coli*-encoded RNases that have only a few targets in phage T4 mRNR identified so far. Moreover, there is no evidence regarding the modification of these endoribonucleases by T4. It has been found that the T4 infection changes the specificity of RNase E and G and, as a result, some of the phage-encoded transcripts become more resistant to these enzymes, while the resistance of *E. coli*-encoded transcripts drastically decreases (Yonesaki and Ueno, 2004). However, which phage T4-encoded factors modify ribonucleases and have the capacity to selectively stabilize or destabilize the mRNA remains unknown. It has been shown that during the T4 infection, Dmd is required for the regulation of mRNA stability in a stage-dependent manner (Ueno and Yonesaki, 2001). When a T4 *dmd* mutant infects *E. coli,* this RNase is activated after early and middle phage genes are expressed (Kai et al., 1998; Ueno and Yonesaki, 2001; Otsuka et al., 2003), and causes rapid degradation of most mRNAs at late stages, leading to a defect in T4 phage growth (Kai et al., 1996). The purified Dmd protein inhibits the activity of RNase LS but does not affect RNase E (Kanesaki et al., 2005; Otsuka and Yonesaki, 2005; Otsuka, 2007). The most recent research has shown that the T4 Dmd protein may inhibit the activity of other endonucleases as well (Otsuka and Yonesaki, 2012). These findings suggest that to control RNases phage T4 has developed a complex mechanism, which needs yet to be investigated. In particular, very little is known about the degradation of early mRNAs. Apart from the RNase RegB, no other *E. coli*- or phage-encoded proteins are known to be involved in this process.

To begin to address this knowledge gap, this study aimed to identify *E. coli* endoribonucleases that are involved in secondary processing of RegB-cleaved T4 mRNAs, to investigate whether RNases are being modified during the infection cycle and to determine what phage T4-encoded factors affect the activity of endoribonucleases.

GOAL OF THE DISSERTATION WORK

The goal of this study was to investigate the impact of *Echerichia coli* endoribonucleases on the early transcripts of phage T4 and their functional dependence on phage-encoded factors.

Towards this goal, the following specific objectives were formulated:

1. To find new RegB targets in bacteriophage T4-encoded transcripts.
2. To investigate the transcription of gene *segD*.
3. To identify *E. coli* endoribonucleases that are involved in secondary cutting of RegB-processed T4 mRNR.
4. To investigate whether RNases are being modified during the infection cycle and whether these modifications influence the origin of secondary cleavages.
5. To determine what phage T4-encoded factors affect the activity of endoribonucleases.
6. To investigate the degradation of RegB-processed T4 mRNAs.

SCIENTIFIC NOVELTY

The results of primer extension sequencing have revealed seven new RegB cleavage targets within SD or intergenic regions of the early T4 mRNAs, namely, *ndd.4*, *mobD*, *sp*, *segD*, *pin*, *dmd* and *arn*. The primer extension analysis of T4 *segD* mRNA has revealed a new early promoter that has been named $P_E$segD. Despite the deviation from the consensus sequence of T4 early promoters in -35 region at well-conserved first base position, this P_E is active during T4 infection. Three additional RegB-processed transcripts, namely, *nrdC.3*, *ndd* and *55.2*, carrying clear targets for secondary processing have been identified. Secondary processing of these genes occurs in the AU-rich sequences located 5-8 nt downstream of the primary cleavage. Using mutant strains deficient in RNase G, RNase E or both enzymes, the secondary endonucleolytic processing of several RegB-cleaved transcripts have been elucidated. The RNase G appears to be the main ribonuclease responsible for the secondary processing of T4

mRNA. Noteworthy, the RNase G targets in T4 mRNAs described in this study are the first that have been identified for this nuclease.

This study has revealed that the RNase G can be covalently modified during the T4 infection cycle. However, such modifications do not affect its activity towards the secondary sites in RegB-processed T4 mRNAs. In addition to these results, a new method has been proposed for a quick and inexpensive evaluation of protein modifications in the bacteria-phage system.

Another important finding is that the I/S phage T4K10, which is widely used for the construction of T4 mutants, encodes defective polynucleotidkinase (PNK). In this study it has been shown that the phage T4K10 carries two point mutations within the gene for polynucleotide kinase *pseT*, resulting in amino acid substitutions G14D and R229H. The G14D mutation impairs 5'-kinase activity *in vivo*, as well as *in vitro*, and leads to diminished processing at secondary sites of several RegB-cleaved transcripts. The *pseT* deletion mutants T4ΔPNK and T4K10ΔPNK, which have been constructed during this study, allowed to confirm the hypothesis that the degradation of transcripts carrying secondary cleavage sites is dependent on PNK. Noteworthy, this research was innovative in itself because of the application of a modern molecular technique qRT-PCR for the analysis of the degradation of bacteriophage-derived transcripts. It has been demonstrated that the PNK accelerates the degradation of RegB processed transcripts but the degradation can also proceed in a PNK-independent way.

THESIS STATEMENTS

1. The early promoter $P_E$segD, despite the deviation from the consensus sequence of T4 early promoters, is active during the T4 infection.
2. *E. coli* endoribonucleases E and G are involved in secondary processing of RegB-cleaved T4 mRNA.
3. RNase G is the main ribonuclease that cleaves all known secondary sites within RegB-processed transcripts.
4. T4K10-encoded PNK has a G14D mutation, which impairs the 5'-kinase activity.
5. The 5'-kinase activity of PNK determines the sensitivity of the secondary targets in RegB-processed mRNAs towards the RNase G and promotes their degradation.

DISSERTATION CONTENTS

The dissertation is written in Lithuanian and contains the following sections: Introduction, Review of the Literature, Materials and Methods, Results and Discussion, Conclusions, List of References (264 positions), Tables (7) and Figures (34). There are 148 pages in total.

MATERIALS AND METHODS

Bacterial Strains and Bacteriophages. *E. coli* strain B^E (*sup*^{0}) was a gift from dr. L. W. Black. *E. coli* K-12 strains GW10 (W3110 *zce-726::Tn10*), GW11 (GW10 *rng::cat*), GW20 (GW10 *rne-1*) and GW21 (GW10 *rne-1 rng::cat*) (Wachi et al., 1997) were kindly provided by dr. M. Wachi; *rng::cat* results in a truncated RNase G protein comprising only first 107 amino acids. N3433 (HfrH *lacZ43 λ−relA spoT1 thi-1*) and N3431 [N3433 *rne3071*(ts)] were kindly provided by dr. P. Régnier. *E. coli* DH5α was used for transformation and preparation of plasmid DNA. *E. coli* C41(DE3), a derivative of *E. coli* BL21(DE3) [*F− dcm ompT hsdS (rB − mB) gal λ(DE3)*] (Avidis) was used in plasmid-phage assays, as well as for superproduction of proteins. *E. coli* strains MH1, B^E-B^S (*sup*^{0}) and CR63 (*supD, ser*), the phage T4K10, as well as the standard supF-containing I/S cloning vector pBSPL0+ were kindly provided by dr. K. Kreuzer. T4K10 (*38amB262 51amS29 denAnd28 denB-rIIBΔrIIPT8*) permits genome substitutions using the T4 insertion/substitution (I/S) system (Selick et al., 1988; Kreuzer and Selick, 1994). *E. coli* strain MH1 (*araD139 ΔlacX74 galU galK hsdR rpsL*) was used as the plasmid-containing host for the initial infection with T4K10. *E. coli* B^E-BS (*sup*^{0}) is a selective host for phages carrying the integrated plasmid. *E. coli* CR63 (*supD ser*) was used for nonselective growth of T4K10 and T4 segregants that had lost the *supF*-containing plasmid. Wild-type bacteriophage $T4^+$ was kindly supplied by dr.W. B.Wood. T4regB^- (*regBL52*, Ruckman et al., 1989) was a gift from dr. M. Uzan. T4-related phages RB42 and RB49 were a gift from dr. K. Carlson. Phage T4_H88 was a gift from dr. L. W. Black. T4ΔregB was constructed earlier in our department. Phages T4ΔPNK, T4K10ΔPNK, T4K10PNK+, T4K10M14 and T4K10M229 were constructed in the course of this study.

RNA Preparations and Analysis of Phage mRNAs. Total RNA was used for RNA primer extension sequencing under conditions of primer excess using avian

myeloblastosis virus reverse transcriptase (AMV RT) as described by Uzan et al. (1988). Briefly, to highlight RNA cleavage positions, primers complementary to specific mRNAs were used. The oligonucleotide was 5'-end labelled by T4 PNK using [γ-^{32}P]ATP (Perkin Elmer) and separated from the labeled ATP by precipitation with ethanol in the presence of 2 M ammonium acetate. The dideoxy chain termination method of Sanger et al. (1977) was used to sequence RNA isolated from phage-infected cells by extension of a 5' end-labeled primer with an AMV RT (Promega). Total cellular RNA (50 µg) and P^{32}-labeled oligonucleotide (2 pmol) were annealed in a total volume of 10 µl containing AMV RT buffer (50 mM Tris-HCl (pH 8.3) 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM spermidine). The samples were incubated for 3 minutes at 60 °C and then rapidly frozen. After the thawing of samples on ice, 1 µl of AMV RT (5 units) was added. Four chain-termination reaction mixtures, each containing 2.5 µl of the annealing mixture and 2.5 µl of a solution containing all four dNTPs (2 mM each) and one of the four dideoxynucleotides (1 mM), in AMV RT buffer were incubated for 20 minutes at 48 °C. The reactions were stopped by adding 6 µl of stop solution (95 % formamide, 20 mM EDTA, 0.05 % bromphenol blue, 0.05 % xylene cyanol) and the samples were then analyzed on a denaturing polyacrylamide gel (6 % acrylamide, 8 M Urea, TBE). Reaction products were visualized with a Fujifilm FLA5100 phosphorimager.

Assays of the Activities of RNases in Phage-Infected Cells. To detect RegB cleavage sites in phage-induced transcripts, *E. coli* B^E or C41(DE3) was grown at 30 °C to A_{600} = 0.8 in LB medium and infected with T4$^+$, T4regB$^-$ (*regBL52*) or T4ΔregB at a multiplicity of infection (m.o.i.) of 10. The use of T4ΔregB allowed to avoid the expression of *regB* from the infecting genome. At the desired time, infected cells were collected, immediately lysed, and total cellular RNA was purified.

To determine the origin of the secondary cleavages, the *E. coli* GW10, GW11, GW20, GW21, N3433 and N3431 were grown in LB medium at 30 °C. With or without transferring of the cultures to 43 °C for 30 min, cells were infected with T4$^+$ (m.o.i=10). 43 °C is non-permissive for the mutant RNase E in GW20, GW21 and N3431. At 6 min after infection at 30 °C, or 4 min at 43 °C, cells were taken, immediately lysed and total cellular RNA was purified. Cleavages were analyzed by primer extension sequencing of phage-specific mRNAs.

Assays of the Activities of RNases in Plasmid-Phage Systems. To detect secondary cleavage sites in the transcripts induced from the recombinant plasmids, *E. coli* C41(DE3), harboring pT4regB-T4nrdC.3′, pT4regB-T4g55.2′ or pT4regB-T4motB′, were grown in LB medium with ampicillin (50 μg/ml) at 30 °C to A_{600} = 0.5. The transcripts to be tested, as well as RegB protein, were induced by addition of IPTG to 1 mM. After 30 min, an aliquot of the culture was infected with T4ΔregB (m.o.i.=10) and total RNA was extracted at 6 min post-infection. Samples from the uninfected culture were taken at 30, 40 and 50 min after the addition of IPTG. Harvested cells were immediately lysed and total cellular RNA was purified. RNAs induced from plasmids were analyzed by primer extension sequencing.

Identification of Protein Modifications. Recombinant plasmids encoding proteins RNase G or EF-Tu fused to an N-terminal leader peptide containing 10 tandem histidines were introduced into *E. coli* C41(DE3) cells. Single ampicilin-resistant colonies were inoculated into LB medium containing 0.05 mg/ml ampicilin, and cultures were grown at 37 ºC until the A_{600} reached 0.3. Then cultures were induced by addition of IPTG to 1 mM, and subsequently incubated for 1 h with continuous shaking. One sample was then infected with $T4^+$, while the other one was left uninfected (control). Samples were taken at 10 min after infection. Cells were harvested by centrifugation, sonicated and the induced proteins were purified using His-Spin Protein Miniprep (Zymo Research) columns following all subsequent procedures of Zymo Research recommendations.

Construction of T4 Phage Mutants. Gene *pseT* mutants of phage $T4^+$ or T4K10 were constructed using I/S system (Selick et al. 1988). For this, the *pseT* deletion was first constructed *in vitro* on the basis of I/S vector pBSPL0+ by fusing two DNA fragments flanking *pseT* gene. A plasmid carrying wild-type T4 *pseT* gene was also constructed using the I/S cloning vector pBSPL0+. Standard procedures for isolation and manipulation of plasmid DNA, and for construction and verification of recombinant plasmids were used throughout (Sambrook et al. 1989).

The resulting plasmids were introduced into *E. coli* MH1 cells. The cells were grown at 37 °C to an A_{600} = 0.6 in LB medium and then were infected with the I/S phage T4K10 (m.o.i = 3). Infected cells were grown at 37 ºC 120 min and the cross between phage and recombinant plasmid was terminated by the addition of 1/10 of chloroform. Progeny phages carrying integrated *supF*-containing plasmids within their genomes and

subsequent segregant phages that had lost the *supF*-containing plasmid were selected by plating on the *E. coli* strains B^E-B^S (*sup*^{0}) and CR63 (*supD, ser*) respectively.

Protein Production and Purification. Recombinant plasmids for overexpression of the native or mutated T4 *pseT* genes were constructed on the basis of expression vector pET16b (Novagen). Recombinant plasmids carrying the native or mutated T4 *pseT* genes were introduced into *E. coli* C41(DE3) cells. Single ampicilin-resistant colonies were inoculated into 40 ml of LB medium containing 0.05 mg/ml ampicilin, and cultures were grown at 37 ºC until the A_{600} = 0.3. Then the temperature was gradually lowered to 17 ºC, cultures were induced by addition of IPTG to 1 mM, and subsequently incubated for 16 h with continuous shaking. Cells were harvested by centrifugation and the induced proteins were purified using His-Spin Protein Miniprep (Zymo Research) columns following manufacturers recommendations. Finally, purified recombinant proteins were dialyzed against T4 PNK storage buffer (20 mM Tris-HCl (pH 7.5), 25 mM KCl, 0.1 mM EDTA, 2 mM DTT and 50 % (v/v) glycerol). Concentrations of the purified proteins were determined using Bradford reagent with bovine serum albumin as the standard (Thermo Fisher Scientific, Vilnius).

***In Vitro* 5'-Polynucleotide Kinase Assay.** Reaction mixtures (10 µl) containing 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 25 µM [γ-P^{32}]ATP, 10 pmol of a synthetic 5'-OH DNA oligonucleotide d(GTGCTCGATCCAATGCCAGT CAGGACAAGATTGCGTAGCTTCTG) and 100, 75, 50, 25, 10 or 5 ng of an appropriate recombinant PNK were incubated for 5 min at 37 ºC. The reactions were stopped by adding 6 µl of stop solution. The mixtures were analyzed by electrophoresis through a denaturing polyacrylamide gel (6 % acrylamide, 8 M Urea, TBE). The radiolabelled oligonucleotide products were visualized and quantitated using Fujifilm FLA5100 phosphorimager.

qRT-PCR

RNA Extraction and Purification. *E.coli* C41(DE3) cells were grown at 30 °C to A_{600} = 0.8 in LB medium and then were infected with phages T4K10PNK or T4ΔPNK (m.o.i= 10). The first sample of the cells was collected 5 min after infection (sample at 0 min point) and rifampicin was added (12 µg/ml). Other samples were collected 3, 5, 7, 10 and 20 min after rifampicin had been added, lysed immediately and total cellular

RNA was purified using ZR RNA MiniPrep™ RNA isolation kit following the manufacturer's protocol. Total RNA was eluted from the matrix with 25 μl of RNase-free water. Residual genomic DNA was removed by incubating the RNA solution with 0,1 u/μl of RNase-free DNase I (Thermo Scientific, Vilnius) in 10× reaction buffer with $MgCl_2$ for 30 min at 37 °C followed by addition of 50 mM EDTA and incubation 10 min at 75 °C to inactivate the DNase I.

Quantitative Real-Time PCR. Manufacturers' instructions were followed for setting one-step qRT-PCR reactions (SensiMix Probe One-Step Kit, Bioline Reagents). Briefly, for one-step, the liquid handling system created a mastermix and primer/probe. Mastermix and sample were then added to each tube (15 μl total volume) and contents mixed. The real-time PCR reactions were performed using Rotor-Gene 6000 5-plex HRM model (Corbett Research, Australia). The RT step involved incubation at 42 °C for 30 min. The PCR cycling conditions included an initial denaturation of 95 °C for 10 min followed by 30 cycles of 95 °C for 5 sec and 60 °C for 50 sec. qRT-PCR assays were performed fourfold in triplicate. All samples were tested for the presence of residual DNA during quantitative real-time PCR with RT-minus control.

Data Analysis. Quantitative PCR assays were analyzed and cycle threshold (Ct) values determined using the Rotor-Gene 1.7.87 software version.

RESULTS AND DISCUSSION

Identification of the New RegB Processing Sites within Bacteriophage T4 mRNAs

In order to find more T4 phage transcripts implicated in RegB mediated secondary cleavage, a systematic study of DNA sequences in the regions carrying prereplicative genes was performed. Potential RegB sites were tested for whether they could be substrates for RegB. Primer extension sequencing was performed on mRNAs extracted from *E. coli* infected by either $T4^+$ or $T4regB^-$ (T4regBL52). Putative RegB sites located within the Shine–Dalgarno or intergenic regions of T4 mRNAs were tested first. Analysis revealed 7 new RegB targets in the SD or intergenic regions of T4 mRNAs (Fig. 1).

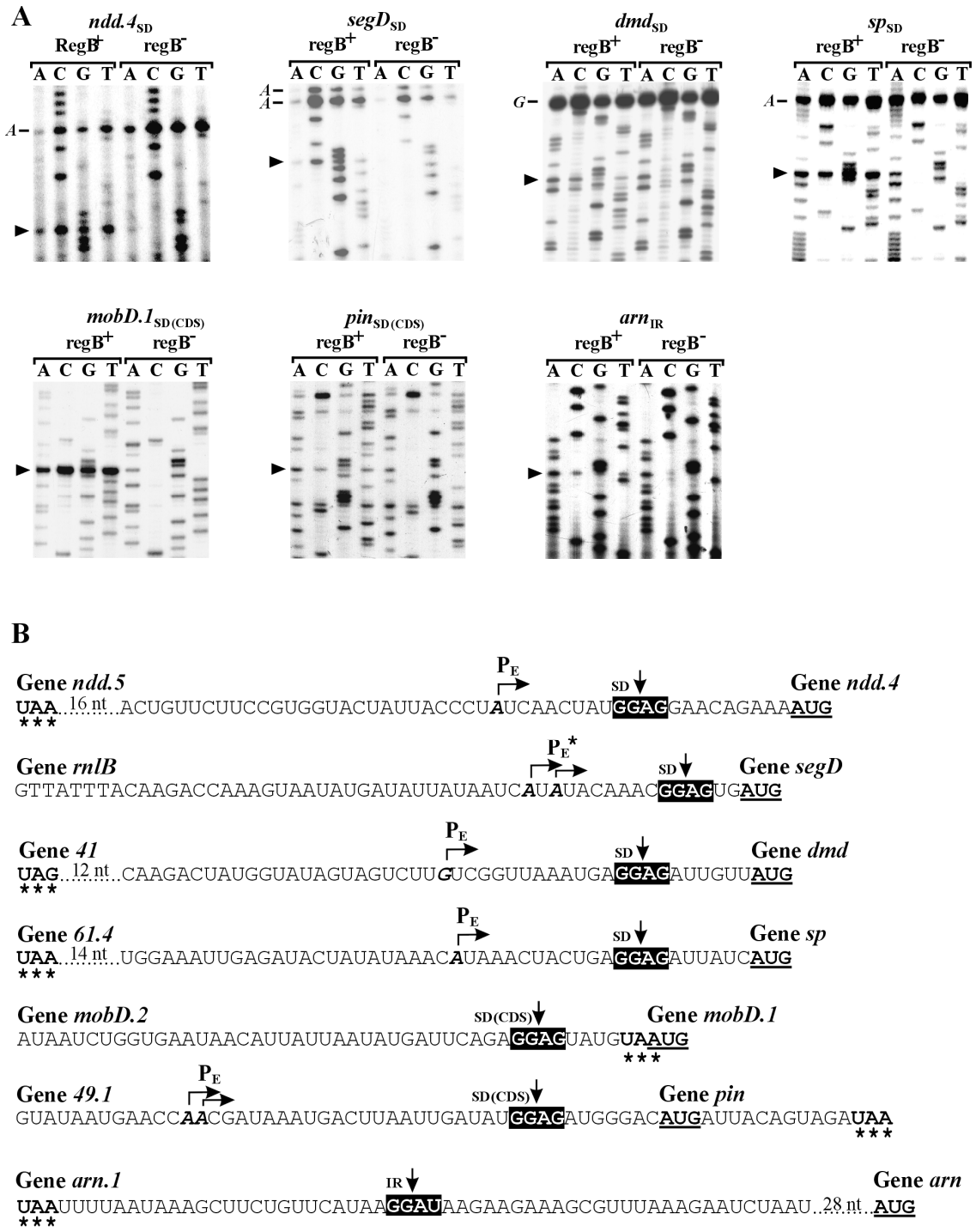

Fig. 1 Mapping of RegB targets within SD sequences for genes *ndd.4*, *segD*, *dmd*, *sp*, *mobD.1* and *pin* and within intergenic region upstream gene *arn* (A). Primer extension sequencing reactions were done on RNA isolated from *E. coli* B^E cells at 4 min post-infection at 30 °C with $T4^+$ or $T4regB^-$. The sequencing lanes are labeled with the dideoxynucleotides used in the sequencing reactions. Triangles indicate the 5' ends generated by RegB processing. The initiating nucleotides of the transcripts for genes *ndd.4*, *segD*, *dmd* and *sp* are noted. (B) Nucleotide sequences of the 5' flanking regions of genes *ndd.4*, *segD*, *dmd*, *sp*, *mobD.1*, *pin* and *arn*. The GGAG and GGAU motifs are shown with black backgrounds. Initiation codons are shown in boldface and underlined; termination codons for the upstream genes are given in bold and marked with asterisks. Vertical black arrows designate the positions of RegB cleavages. The initiating nucleotides for the early transcripts are shown in italic and boldface. P_E^* - shows initiating nucleotides of *segD* transcrips induced from newly detected early promoter.

The GGAG/U motifs of the targets showed different susceptibilities to RegB. Three strong RegB targets were located within the SD sequences of *ndd.4*, *sp* and *mobD.1* transcripts. Three poorly-cut GGAG/U motifs were found in the SD sequences of *segD*, *dmd* and *pin* transcripts, and one poorly processed RegB target was found upstream of the early gene *arn*.

Primer extension reaction on transcript from the region of the gene *segD* revealed 5' ends coming from the putative promoter. So, the putative promoter sequences that could potentially serve as the early promoters have been analyzed and their activity has been tested *in vivo.*

Analysis of *In Vivo* Activity of Bacteriophage T4 Putative Early Promoter

First, the nucleotide sequence of the genomic region that harbors a putative promoter has been analyzed, and a good match to the -10 consensus sequence as well as a plausible spacer region have been identified. However, the -35 region of the putative promoter deviated from the consensus sequence of T4 early promoters (ATTTACA) at the first position (GTTTACA). Since it is known that the first and the third positions are usually well-conserved among early T4 promoters (Wilkens and Rüger, 1994), the activity of the aforementioned putative promoter sequence has been tested *in vivo.*

For this, total RNA was isolated from the T4-infected *E. coli* cells in the absence or presence of chloramphenicol (Cm) and the transcripts were analyzed by primer extension analysis. Chloramphenicol inhibits synthesis of new proteins. Early transcripts can be seen in the presence of chloramphenicol, since only early transcription is independent of *de novo* T4 protein synthesis. Analysis of the transcripts that were isolated from the T4-infected *E. coli* cells in the absence of chloramphenicol revealed initiating nucleotides of transcripts as well as the 5' ends generated by RegB. In contrast, in the case of the RNA isolated from the T4-infected *E. coli* cells in the presence of chloramphenicol only initiating nucleotides of transcripts were observed (Fig. 2). These results confirmed that the putative promoter sequence is an active early promoter *in vivo*.

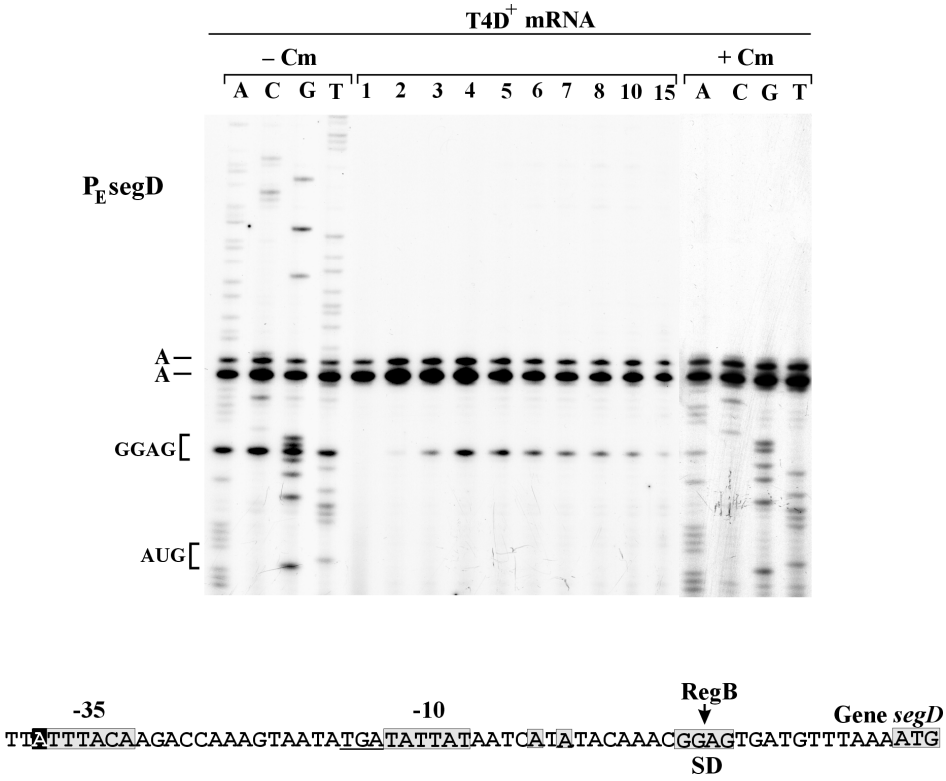

Fig. 2. Primer extension analysis of transcripts for gene *segD* of bacteriophage T4. Primer extension sequencing was done on RNA isolated from *E. coli* B^E cells at 3 min post-infection with bacteriophage $T4^+$ in the absence or presence of chloramphenicol at 30 ºC. Primer extension reactions of RNA isolated at 1 to 15 min post-infection from the cells that were infected with phage $T4^+$ at 30 ºC are shown. The sequencing lanes are labeled with the dideoxynucleotides used in the sequencing reactions. The post-infection time (minutes) at which each RNA was isolated is noted on the top of the autoradiograph. The initiating nucleotides of transcripts and the 5'-end nucleotide of the endoribonuclease RegB-truncated transcript are noted. Nucleotide sequence of the 5' flanking region of gene *segD* of phage T4 are shown at the bottom of the autoradiograph. The -35 and -10 regions of early promoters and the initiating nucleotides for transcripts are shown on grey background. The base that differs from the consensus is on black background. Vertical arrow denotes the position of RegB cleavage

Primer extension analysis revealed that the transcripts initiated from this promoter appear immediately after infection and their amount increases up to at least 4 min after infection (Fig. 2). This feature is also characteristic to the transcription initiated from early T4 promoters. Thus, the aforementioned new early promoter has been designated as $P_E$segD.

Identification of *E. coli* Endoribonucleases Involved in Secondary Processing of RegB-cleaved T4 mRNAs

Primer extension reactions on transcripts from regions of the genes *ndd, nrdC.3* and *55.2* revealed strong RT stops corresponding to RegB cuts at the GGAG and GGAU motifs, as well as additional stops corresponding to the secondary cuts within RegB-

processed transcripts (Fig. 3 A–C). The observed secondary cleavages occur in AU-rich sequences downstream of the processed (primary) sites (Fig. 3 D), indicating that the cuts were generated by another nuclease.

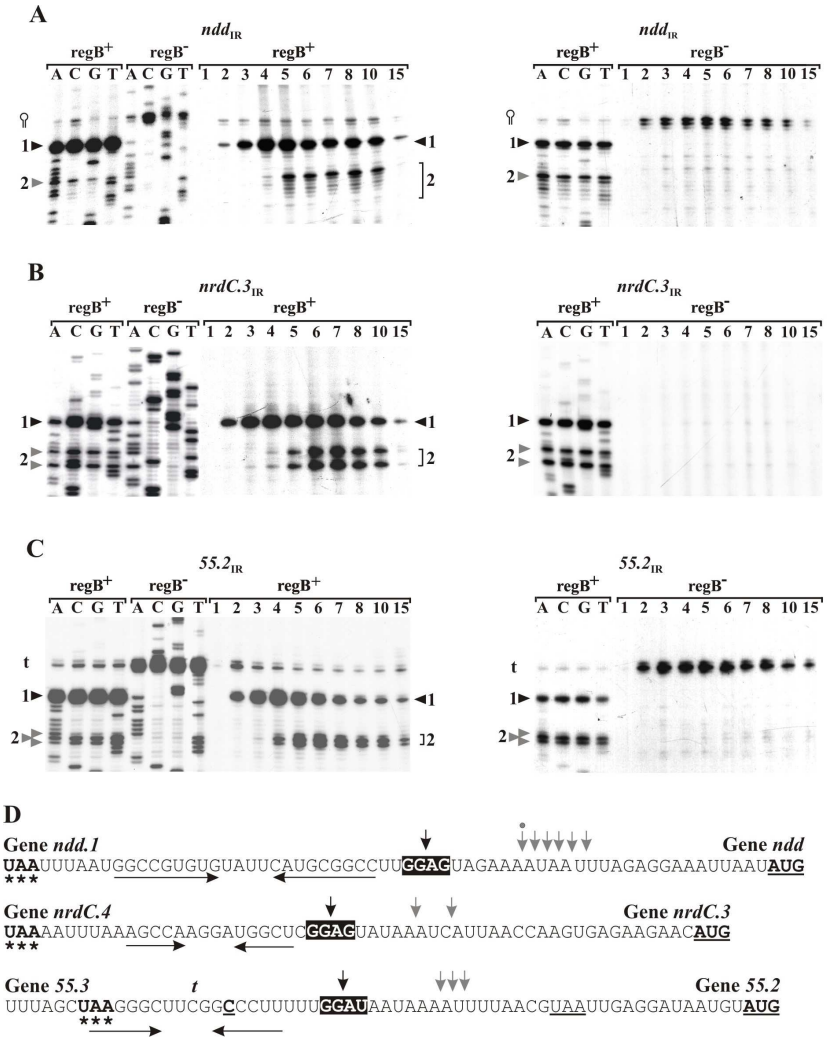

Fig. 3. Primer extension analyses of mRNAs from the intercistronic regions upstream of genes *ndd* (A), *nrdC.3* (B) and *55.2* (C). Primer extension sequencing used RNA isolated from *E. coli* B^E cells at 4 min post-infection at 30 °C with $T4^+$ or T4 $regB^-$. The sequencing lanes are labeled with the dideoxynucleotides used in the sequencing reactions. Primer extension reactions of RNA isolated 1–15 min post-infection from the cells that were infected with $T4^+$ or T4$regB^-$ at 30 °C are next to the sequencing lanes. The time (min) of post-infection that RNA was isolated is at the top. Black triangles marked with (1) indicate the 5' ends generated by RegB processing, grey triangles marked with (2), as well as brackets, show the 5' ends generated by secondary cleavages. The stem-loop symbol in the case of the *ndd* transcript indicates the RT stops at the hairpin structure, while for the *55.2* transcript t marks the RT stops at the probable rho-independent terminator (Miller et al., 2003). Nucleotide sequences of the 5' flanking regions of T4 genes *ndd*, *nrdC.3* and *55.2* **(D)** are presented at the bottom. The GGAG and GGAU motifs are shown with black backgrounds. Initiation codons are in boldface and underlined, termination codons for upstream genes are in bold and marked with asterisks. Convergent arrows denote the inverted repeats of palindromic sequences. Vertical black arrows designate the positions of RegB cleavages, grey arrows denote the positions of secondary cuts, the most efficient being marked by a point.

The group of RT stops (labeled 2), with the major stop mapping to an A, 6 nucleotides downstream of the cleaved GGAG motif, was observed in the polycistronic

transcript just upstream of gene *ndd* (Fig. 3 A). The 5' ends assigned to the additional cleavage events first appear at 4 min after infection reach a maximum at 5 min and continue to be present thereafter. The secondary cleavages appear with a 2 min delay, with respect to RegB processing event in the middle of the GGAG. Primer extension analysis of the kinetics of 5' ends accumulation for the T4regB$^-$ infection indicates that the secondary processing events are dependent on the primary cleavage by RegB (Fig. 3 A). Two bands indicating the 5' ends generated by secondary processing of RegB-processed transcripts were detected in the early polycistronic transcript synthesized from the *nrdC.3* region (Fig. 3 B). A doublet band, mapping 5 and 8 nucleotides downstream of the processed GGAG sequence can be detected at 3 min and culminates 6–7 min after infection. These bands appear with a one-minute delay, with respect to the bands indicating the 5' ends generated by RegB at the primary site, and are absent after T4regB$^-$ infection.

In the case of primer extension experiments carried out with the gene *55.2* specific primer, a triplet of bands indicating the 5' ends of transcripts assigned to secondary cleavages maps 6 to 8 nucleotides downstream of the cleaved GGAU motif (Fig. 3 C). Analysis of the kinetics of 5' end accumulation shows that secondary processing becomes efficient 5 to 7 min after infection with phage T4$^+$ and is dependent on RegB. Taken together, these results show that the RegB-processed transcripts from the intercistronic regions just upstream of *ndd*, *nrdC.3* and *55.2* become susceptible to further processing by an unknown nuclease after initial cleavage by RegB.

Secondary processing of RegB-cleaved transcripts from intercistronic regions upstream of the genes *ndd*, *nrdC.3* and *55.2* occurs in the AU-rich sequences located downstream of the primary cleavage. This fact encouraged to test the possibility of RNase E involvement, since this enzyme prefers to cut within single-stranded regions that are AU-rich. Therefore, the RNA isolated from wild-type [N3433] and *rne3071* (ts) [N3431] isogenic *E. coli* strains after infection at 30 °C and 43 °C has been analyzed. The results (not shown) suggest that RNase E alone is either not involved in the secondary cleavage of *ndd*, *nrdC.3* and *55.2* mRNAs or its activity is masked by another nuclease with overlapping specificity, e.g. RNase G, which is a structural homologue of RNase E and has similar cleavage site specificity.

To test whether the mRNAs truncated at secondary sites were products of *E. coli* RNase G cleavage, total RNA was extracted from isogenic wild-type [GW10] and RNase G-deficient [GW11] strains after T4 infection (Figs. 4 and 5).

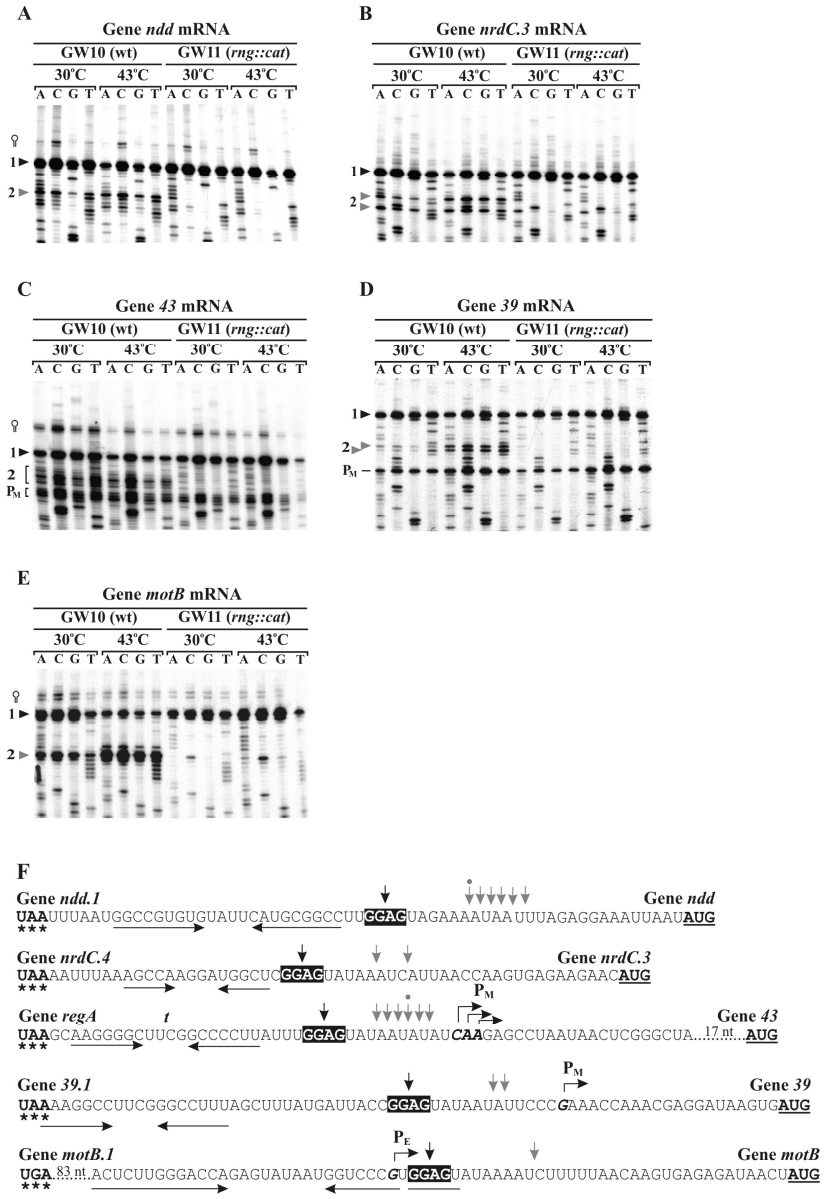

Fig. 4. The role of RNase G in the secondary processing of mRNAs from the intercistronic regions upstream of genes *ndd* (A), *nrdC.3* (B), *43* (C), *39* (D) and *motB* (E). Primer extension sequencing of RNA isolated from *E. coli* GW10 (wild-type) and GW11 (*rng::cat*) cells at 6 min (30 °C) and 4 min (43 °C) post-infection with T4$^+$. Sequencing lanes are labeled with the dideoxynucleotides used. Black triangles marked with (1) indicate the 5' ends generated by RegB, grey triangles marked with (2) show the 5' ends generated by secondary cleavage. The symbol associated with *ndd*, *43* and *motB* transcripts indicates the RT stops at the hairpin structure. Initiating nucleotides for middle-mode transcripts are noted. Nucleotide sequences of the 5' flanking regions of T4 genes *ndd*, *nrdC.3*, *g43*, *g39* and *motB* **(F)** are presented at the bottom. The GGAG motifs are shown with black backgrounds. Initiation codons are in boldface and underlined, termination codons for upstream genes are in bold and marked with asterisks. Convergent arrows denote the inverted repeats of palindromic sequences. Vertical black arrows designate the positions of RegB cleavages, grey arrows denote the positions of secondary cuts, the most efficient being marked by a point. Initiating nucleotides for the early and middle transcripts are shown in italic boldface.

mRNA was analyzed by primer extension sequencing using primers specific to *ndd*, *nrdC.3*, *55.2* and for genes *43*, *motB*, *cef* and *39*, whose mRNAs had been found previously to undergo RegB-dependent primary and secondary processing (Hsu and Karam, 1990; Sanson and Uzan, 1993). In wild-type cells, RT stops corresponding to the cleavages at primary and secondary sites were detected (Figs. 4 and 5), whereas in the absence of RNase G the 5' ends of mRNAs truncated at the secondary sites were not detected in the intergenic regions upstream of genes *ndd*, *nrdC.3*, *43*, *39* and *motB* (Fig. 4), indicating that the host-encoded RNase G generates those 5' ends.

However, the 5' ends reflecting the RT stops at the secondary sites of mRNAs upstream of genes *55.2* and *cef* (Fig. 5) were still observed in the RNase G-deficient strain, although the intensity of the bands (especially that of the gene *55.2* mRNA) was lower than in the wild-type strain (Figs. 5 A and B). These results suggest that RNase G is not sufficient to account for the secondary cleavage events in the *55.2* and *cef* mRNAs and another enzyme most likely also participates.

Using a different set of isogenic strains, the involvement of both RNase E and RNase G has been tested. In agreement with experiments using *rne3071* there was no significant difference in accumulation of secondary cleavage products of gene *55.2* transcripts isolated from wild-type and *rne-1* (ts) strains at either 30 °C or 43 °C (non-permissive for *rne-1*) (Fig. 5 A). Very weak RT stops were observed in the *rne-1 rng::cat* double mutant strain at 30 °C, indicating that in the absence of RNase G another ribonuclease attacks the same targets of the *55.2* transcript. At 43 °C, however, no 5' ends resulting from cleavage at the secondary sites could be detected. These results indicate that both RNase G and RNase E cleave the same target just upstream of the gene *55.2*.

Analysis of the transcription pattern of the region just upstream of *cef* (Fig. 5 B) also leads to the same conclusion. It should be noted that RegB is active in these experiments, regardless of any inactivation of RNases G and E. Thus, RNase G is solely responsible for cutting within the AU-rich sequences downstream of the RegB-processed primary sites near T4 *ndd*, *nrdC.3*, *motB*, *43* and *39*, while both RNases G and E recognize the same sites within mRNAs upstream of the T4 genes *55.2* and *cef*. However, judging by the intensity of bands corresponding to the RT stops (Fig. 5), the

contribution of RNase E to the secondary cleavage is small and RNase G appears to be the main ribonuclease that cleaves all known secondary targets.

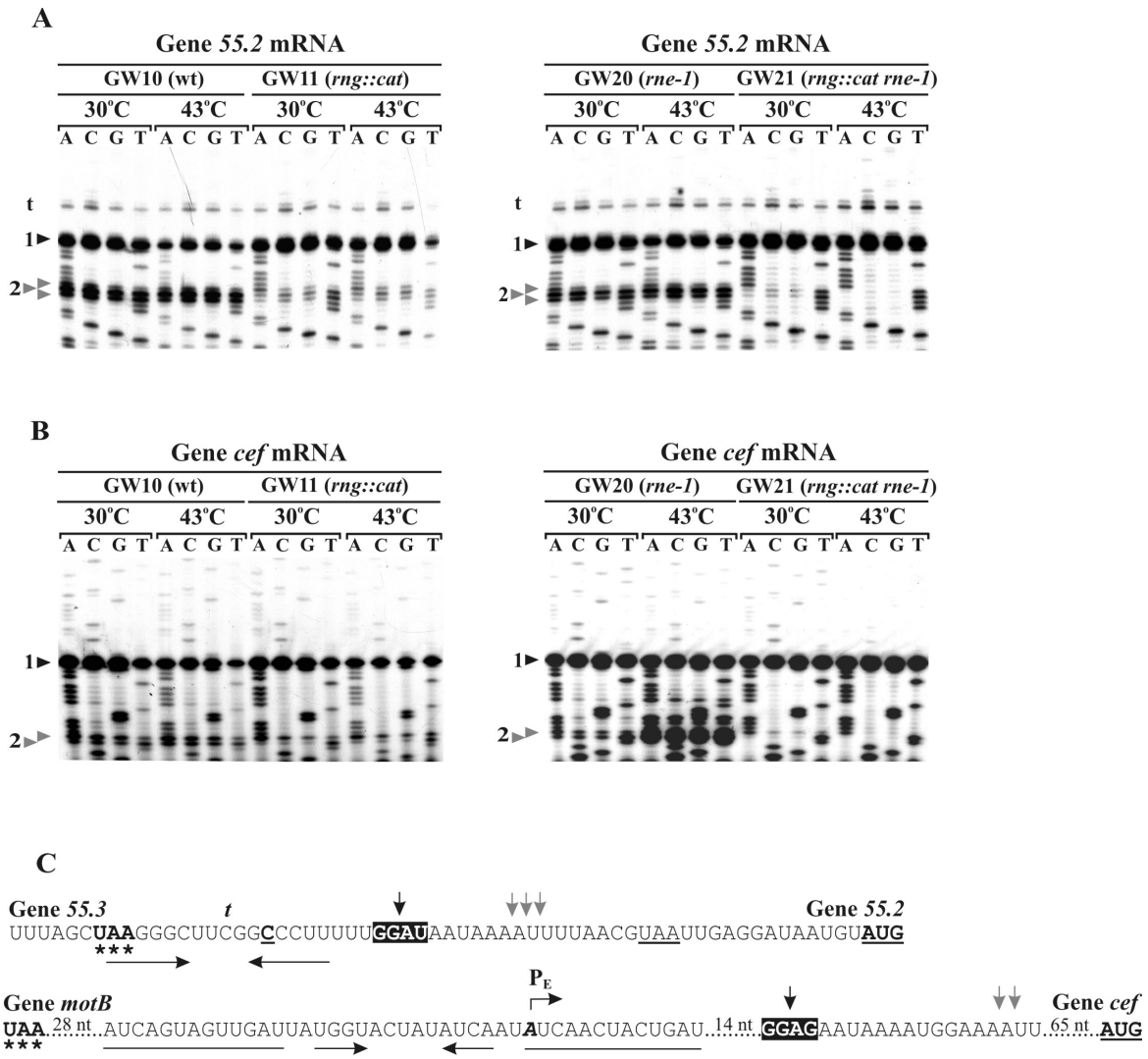

Fig. 5. The roles of RNases G and E in the secondary processing of mRNAs from the intercistronic regions upstream of genes *55.2* (A) and *cef* (B). Primer extension sequencing of gene *55.2* mRNA used RNA isolated from *E. coli* GW10 (wild-type), GW11 (*rng::cat*), GW20 (*rne-1*) and GW21 (*rng::cat rne-1*) cells at 6 min (30 °C) and 4 min (43 °C) post-infection with $T4^{+}$. Sequencing lanes are labeled with the dideoxynucleotides used. Black triangles marked with (1) indicate the 5' ends generated by RegB, grey triangles marked with (2) show the 5' ends generated by secondary cleavage. Nucleotide sequences of the 5' flanking regions of T4 genes *55.2* and *cef* **(C)** are presented at the bottom. The GGAU and GGAG motifs are shown with black backgrounds. Initiation codons are in boldface and underlined, termination codons for upstream genes are in bold and marked with asterisks. Convergent arrows denote the inverted repeats of palindromic sequences. Vertical black arrows designate the positions of RegB cleavages, grey arrows denote the positions of secondary cuts, the most efficient being marked by a point. Initiating nucleotide for the early transcript are shown in italic boldface. For the *55.2* transcript t marks the RT stops at the probable rho-independent terminator (Miller et al., 2003).

The RNase E/G family prefers AU-rich single-stranded RNA substrates carrying a 5' monophosphate (Mackie, 1998, 2000; Jiang et al., 2000; Tock et al., 2000). However,

T4 RegB produces 5'-OH carrying RNA. It is thus surprising that RNases G and E appear responsible for secondary cleavages within RegB-processed transcripts, and it is possible that T4 infection influences the activities of RNases E and G.

The plasmids pT4regB-T4g55.2', pT4regBT4nrdC.3' and pT4regB-T4motB' each containing the T4 *regB* gene and the proximal parts of genes *55.2*, *nrdC.3*, and *motB*, respectively, together with their 5' upstream regions carrying primary and secondary sites for the endoribonucleases have been constructed. Plasmid-derived mRNAs were isolated from uninfected and T4ΔregB-infected *E. coli* C41(DE3) and analyzed by primer extension sequencing using plasmid specific primers. A control experiment using plasmid-free cells infected by $T4^+$ or T4ΔregB revealed that the 5' end patterns of the *nrdC.3*, *55.2* and *motB* mRNAs (Figs. 6 A–C) were the same as observed in *E. coli* B^E or GW10 after infection with $T4^+$ or $T4regB^-$ (Fig. 3 B–C and 4 E). Plasmid-derived *nrdC.3* and *55.2* transcripts in uninfected cells are efficiently processed at their primary site by RegB, but are resistant to secondary processing (Fig. 6 A and B).

After infection by T4ΔregB transcripts derived from the plasmids pT4regB-T4g55.2' and pT4regBT4nrdC.3' are truncated at both primary and secondary sites, and at the same positions as *nrdC.3* and *55.2* transcripts in $T4^+$-infected cells (Fig. 3 B and C). Plasmid-derived pT4regB-T4motB' transcripts (Fig. 6 C) were processed at primary and secondary sites in both uninfected and T4ΔregB-infected cells, although in the latter case the cuts at secondary sites were stronger than in uninfected cells. Thus, the data suggest that cleavage of RegB-processed transcripts by RNases E and G is stimulated by T4 infection.

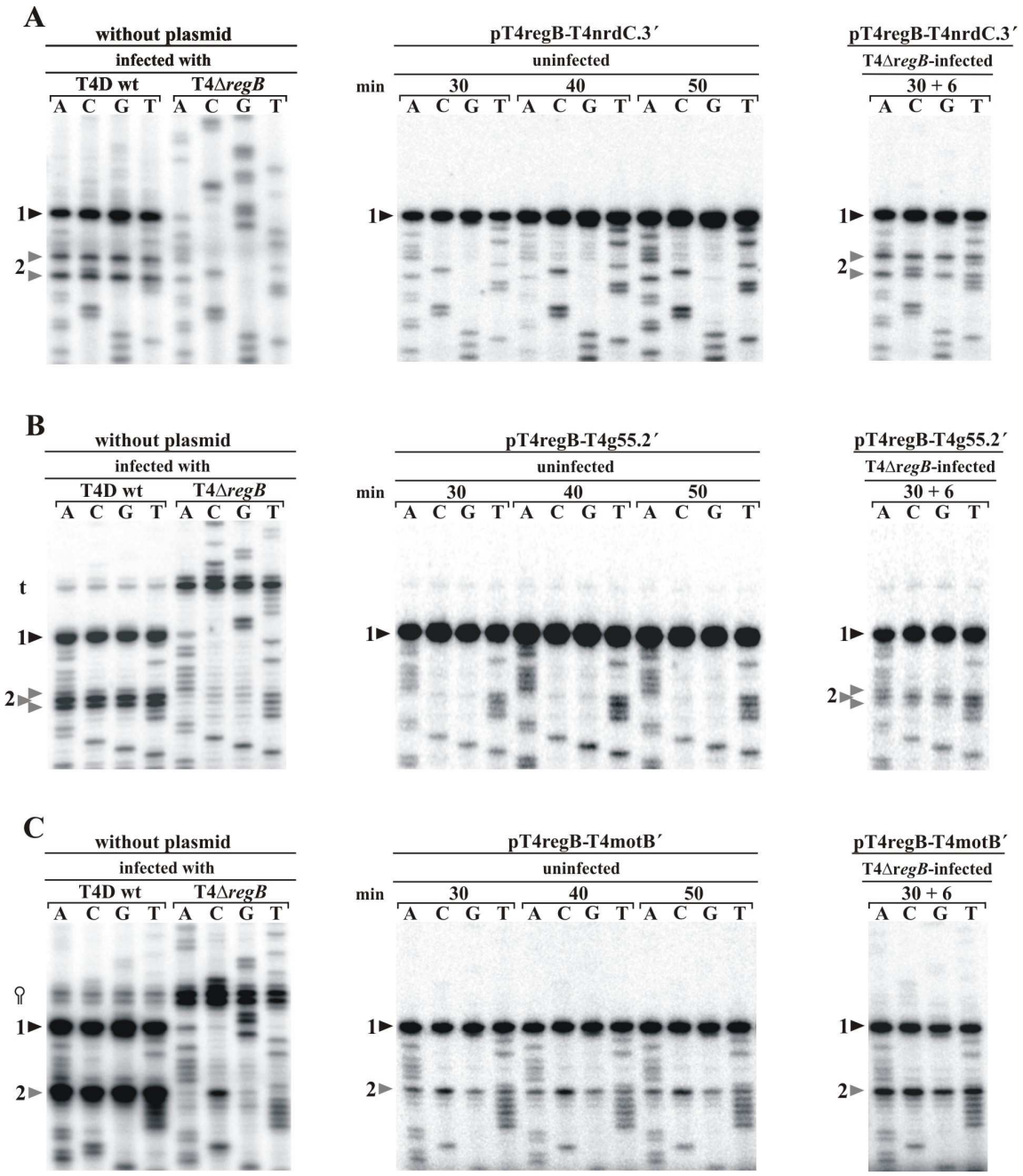

Fig. 6. Susceptibility of RegB-processed T4 mRNAs from the intercistronic regions upstream of genes *nrdC.3* (A), *55.2* (B) and *motB* (C) to the host RNases in plasmid-phage systems. Primer extension sequencing of RNA isolated from *E. coli* C41(DE3) cells harboring recombinant plasmids pT4regB-T4nrdC.3', pT4regB-T455.2' or pT4regB-T4motB', without phage infection or 6 min post-infection with phage T4ΔregB at 30 °C. Transcripts were induced from the plasmids by the addition of IPTG to 1 mM 30 min before infection. Samples from uninfected cells were taken 30, 40 and 50 min after induction with IPTG. The control experiments (first panels) show primer extension sequencing of the T4-induced transcripts isolated from *E. coli* C41(DE3) at 6 min after infection with $T4^+$ or T4ΔregB at 30 °C. The sequencing lanes are labelled with the dideoxynucleotides used, the time (min) of induction with IPTG (and infection) is noted. The black triangles marked with (1) indicate the 5' ends generated by RegB, grey triangles marked with (2) show the 5' ends generated by secondary cleavage. For the *55.2* transcript t marks the RT stops at the probable rho-independent terminator (Miller et al., 2003). The symbol for the *motB* RNA indicates the RT stops at the hairpin structure.

It should be noted that the regions containing RegB-dependent secondary sites share some common features. Secondary cleavages take place in the AU-rich regions downstream of the processed primary sites. Palindromic sequences predicted to fold into stem-loops lie upstream of the primary and secondary sites in all cases. To test whether these stem-loops affect cleavages at the primary and secondary sites, the proximal parts of genes *55.2, nrdC.3* together with their 5' upstream regions with and without palindromic sequences for the stem-loop were cloned. No differences were observed in the RNA processing patterns (data not shown). It was concluded that, after cleavage by RegB the terminal hairpins at the 3' extremities may be required to stabilize the upstream mRNAs.

Determination of Bacteriophage T4 Encoded Factors, which May Affect the Activities of Endoribonucleases E and G

In order to find out, which of bacteriophage T4 encoded factors may affect the activities of endoribonucleases, the investigation in the following two stages was pursued. First, it was tested whether RNases can be modified during the infection cycle and whether this plays a role in the origin of secondary cleavages. Second, the attempt to find such a bacteriophage T4 mutant, which would not contain secondary cleavage sites in appropriate early transcripts, was made.

Study of Possible Modification in RNase G

The plasmid pET16b_rnag containing *E. coli rnag* gene fused to an N-terminal tag containing 10 tandems of histidines has been constructed. pET16b_rnag plasmid was introduced into *E. coli* C41(DE3) cells, grown until the A_{600} reached 0.3, then cultures were induced by addition of IPTG to 1 mM. One hour later a part of the culture was infected with bacteriophage $T4^+$ and the remaining part of the culture was left uninfected. After 10 min cells were harvested by centrifugation, disrupted by sonification and the induced proteins were purified using His-Spin Protein Miniprep (Zymo Research) columns following all subsequent procedures of Zymo Research recommendations. Purified recombinant proteins were analyzed by SDS PAGE (Fig. 7).

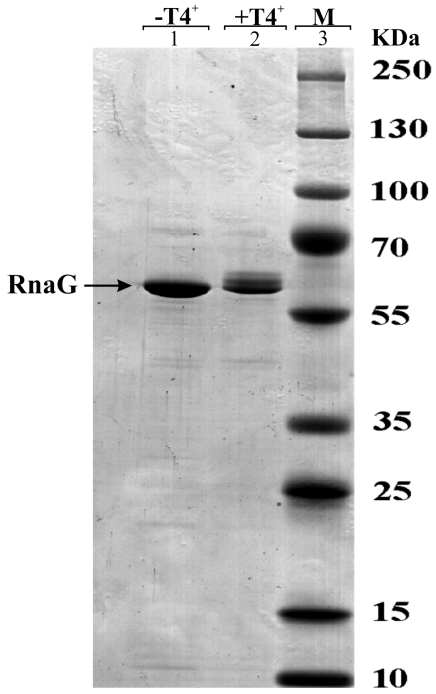

Fig. 7. Endoribonuclease G electrophoretic mobility assay in denaturing protein electrophoresis. Samples were loaded on SDS–PAGE gel and protein bands were visualized by coomassie brilliant-blue staining. The first line indicates RnaG protein, which is isolated after induction with IPTG, 2 - RnaG protein isolated after induction and T4 infection, 3 – molecular mass standards – PageRuler^{TM} Plus Prestained Protein Ladder.

Electrophoresis analysis revealed that infection with phage T4 changed RnaG electrophoretic mobility. After infection, RnaG protein becomes heterogeneos and moves in electrophoretic field as three separate proteins of higher molecular weight. The molecular weight of RnaG protein can change as a result of some additional functional groups that covalently bound to protein amino acids. Post-translational modifications usually can alter the intracellular location of the protein, its stability and functions.

To test the hypothesis that RNase G modification(s) takes place in the early phase of bacteriophage T4 infection, recombinant plasmids pET16b_rnag were introduced into *E. coli* C41(DE3) cells. Then the culture was induced by addition of IPTG to 1 mM and infected with bacteriophage T4. The samples were taken at 1, 3, 5 and 10 min after the start of infection. Proteins were purified using histidine-binding columns and analysed as described previously. It was found that one minute after the start of infection RNase G electrophoretic mobility changes – protein moves in electrophoretic field as two proteins of higher molecular weight (Fig. 8). Five minutes after infection, protein moves in

electrophoretic field as three separate bands. So it can be supposed that ribonuclease G could be modified in the early period of infection.

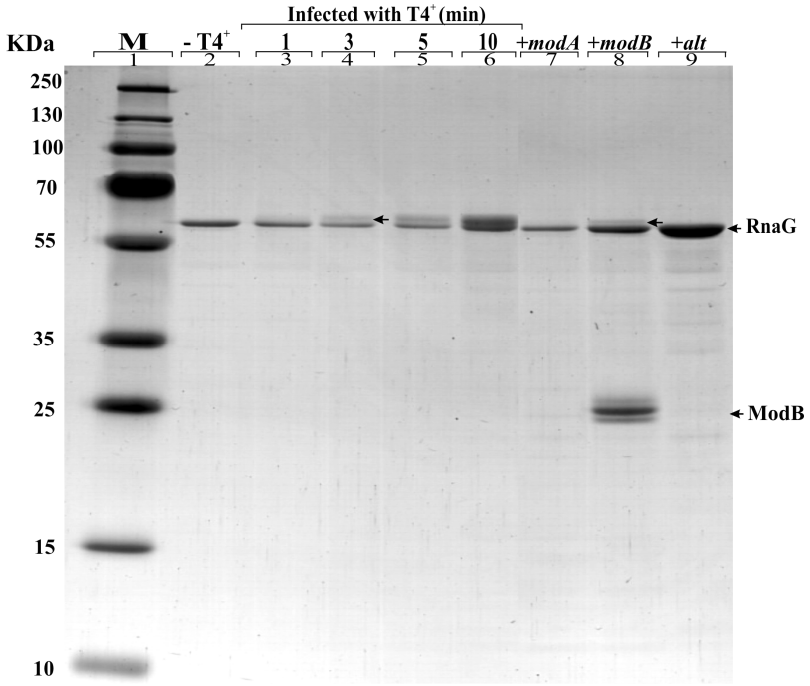

Fig. 8. Analysis of recombinant RNase G electrophoretic mobility assay after co-expression with genes *modA*, *modB* and *alt* encoded ribosiltransferases. Samples were loaded on standard SDS–PAGE gel and protein bands were visualized by coomassie brilliant-blue staining. The first line indicated molecular mass standards – PageRuler^{TM} Prestained Protein Ladder Plus, 2 - RnaG protein, which is isolated after induction with IPTG, 3 – 6 RnaG protein isolated after induction and infection of phage T4 after 1, 3, 5 and 10 min, 7 – 9 – *rnag* co-expression with phage T4 ADP-ribosyltransferases genes *modA*, *modB* and *alt*. The black arrow denotes the resulting equivalent protein mass changes after 3 min of infection and after the co-expression with *modB*.

To test whether RNase G can be ADP-ribosylated, a system for the co-expression of cloned genes in *Escherichia coli* was constructed. Co-expression typically involves the transformation of *E. coli* with several plasmids that have compatible origins of replication and independent antibiotic selection for maintenance. So first, the vectors p128_21_1 and p128_16b that were compatible with recombinant plasmid pET16b_rnag were constructed. Also, the plasmids p128_21_1_modA, p128_16b_modB and p128_16b_alt each containing one of phage T4 encoded ADP-ribosyltransferase genes were constructed independently. Recombinant plasmids were co-transformed into *E. coli* C41(DE3) cells, grown until the A_{600} reached 0.6, then cultures were induced by addition of IPTG to 1 mM and after two hours cells were harvested by centrifugation, disrupted by sonification and the induced proteins were purified using His-Spin Protein Miniprep

(Zymo Research) as recommended by manufacturer. Purified recombinant proteins were analyzed by SDS PAGE (Fig. 8). It was found that proteins ModA and Alt do not change RNase G electrophoretic mobility, while ribosyltransferase ModB does. After two hours of coexpresion of RnaG and ModB, RnaG protein is seen in electrophoresis field as two bands (Fig. 8). Taken together, such data suggest that RNase G is modified during bacteriophage T4 infection and it may be ADP-ribosylation. On the other hand it seems most likely that RnaG undergoes additional yet unidentified modification or modifications.

Analysis of Transcripts of Bacteriophage T4 Deletion Mutants

Previously, it was shown that endoribonuclease RegB acts in concert with the *E. coli* RNases G and E to process some T4 early transcripts during infection. Moreover, it was suggested that T4 infection could alter the preference of RNases G and E to cleave 5'-OH-terminated substrates with enhanced efficiency.

In order to detect which T4-encoded factor is responsible for stimulation of RNase G activity, the attempt to find such bacteriophage T4 deletion mutant, which would not contain secondary cleavage sites in appropriate early transcripts, has been made. Analysis has revealed that phage mRNAs are resistant to cleavage by host RNases, when cells are infected with the insertion/substitution (I/S) system phage T4K10 (Fig. 9).

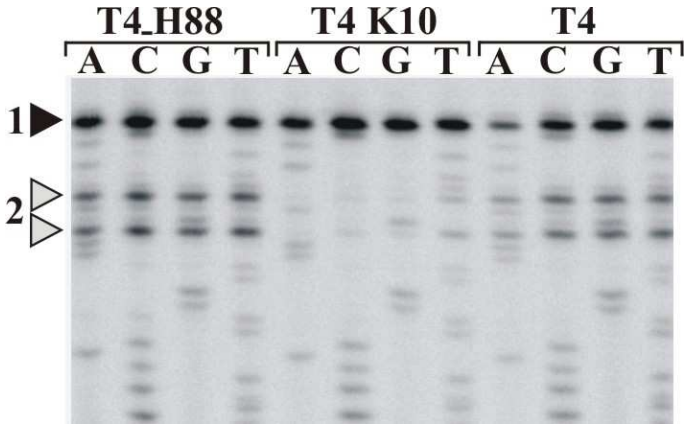

Fig.9. Analysis of transcripts for gene *nrdC.3* of bacteriophage T4 and T4 deletion mutants. Primer extension sequencing of gene *nrdC.3* mRNA used RNA isolated from *E. coli* C41(DE3) cells at 5 min (30 °C) post-infection with T4_H88, T4K10 or $T4^{+}$. Sequencing lanes are labeled with the dideoxynucleotides used. Black triangles marked with (1) indicate the 5' ends generated by RegB, grey triangles marked with (2) show the 5' ends generated by secondary cleavage.

Phage T4K10 was obtained from dr. K. Kreuzer laboratory. Multiply mutant T4 I/S phage T4K10 was generated by genetic crosses between T4 mutants obtained earlier by UV or chemical mutagenesis (Selick et al, 1988). Genotype of the I/S phage is defined as *amB262* (*gene 38*) *amS29* (gene *51*) *nd28* (*denA*) *rIIPT8* (*denB-rII* deletion). It is known that ΔrIIPT8 deletion encompases twelve genes with largely unknown functions. First, it was assumed that one of those genes can affect RNases activity related to the origin of secondary cuts in RegB-processed T4 mRNA. Hence, phage T4 mutant T4_H88, which has defective *rIIB* gene, was tested. The analysis revealed that this gene does not affect RNases activity (Fig. 9). Other genes from ΔrIIPT8 region by complementation studies were also tested, but no one was responsible for the origin of secondary cuts in RegB-processed T4 mRNA (data not shown). So it was assumed that T4K10 might have additional mutations in the other genes.

It was hypothesized that phage T4-encoded PNK may influence secondary cuts. Bacteriophage T4 *pseT* gene encodes PNK enzyme, which catalyzes both the phosphorylation of 5'-hydroxyl polynucleotide termini and the hydrolysis of 3'-phosphomonoesters and 2',3'-cyclic phosphodiesters (Novogrodsky et al., 1966; Cameron and Uhlenbeck 1977). *In vivo,* T4 PNK plays a role of a healing enzyme involved in $tRNA^{Lys}$ repair together with the RNA ligase in response to cleavage catalyzed by host enzymes (Amitsur et al 1987). Hence, the potential role of bacteriophage T4 PNK in the phosphorilation of mRNA has been investigated. In this case T4K10 PNK should be defective, so the *pseT* gene of this phage was sequenced. The results indicated the existence of two point mutations that alter amino acids: glycine changes to aspartic acid (G14D) and arginine to histidine (R229H). In addition, the first substitution is in the conserved position of 5' kinase domain, so there could be a strong probability that the PNK 5' kinase function is abolished. Second substitution is in non conserved position, so it might be insignificant. To determine whether or not phage T4K10 PNK is functional, phage T4 and T4K10 p*seT* genes were cloned into the expression vectors p128_21_1.

In order to determine whether the phage T4K10 PNK point mutation affects the activity of PNK and herewith mRNA phosphorylation, the study was carried out in two different plasmids systems. Previously it was shown that transcripts induced from recombinant plasmids pT4regB-T4nrdC.3 are efficiently processed at the primary site by

RegB but are resistant to secondary processing (Fig. 6 A, p. 23). In order to find out if PNK alone, without phage T4 infection, has a role in the origin of secondary cleavages, the assay in co-expression systems was performed. *E. coli* C41(DE3) cells were transformed with recombinant plasmid pT4regB-T4nrdC.3' or co-transformed with pT4PNK or pT4K10PNK. It was discovered that *nrdC.3* transcripts were processed at primary and secondary sites in both co-expression systems, although in the case of co-expression with pT4PNK the cuts at secondary sites were stronger than in co-expression with pT4K10PNK (Fig. 10).

This finding suggests that T4K10 PNK retains its partial activity. More generally, it may be concluded that PNK alone is a sufficient factor for stimulating the activity of endoribonucleases E and G, whereas the modification of RNase G during infection has no influence on the origin of secondary cleavages.

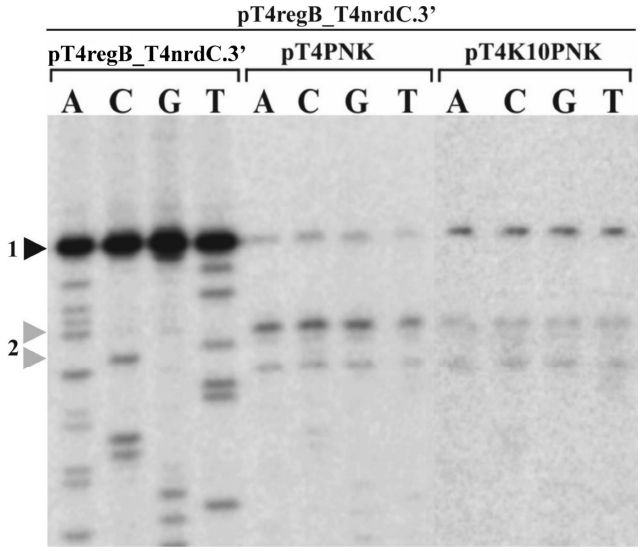

Fig. 10. Analysis of plasmid-derived *nrdC.3* transcripts coexpressed with cloned T4 or T4K10 *pseT* genes. Primer extension sequencing of RNA isolated from *E. coli* C41(DE3) cells harboring recombinant plasmids pT4regB-T4nrdC.3', pT4regB-T4nrdC.3' and pT4PNK or pT4regB-T4nrdC.3' and pT4regB-T4nrdC.3' and pT4K10PNK at 30 °C. Transcripts and PNK from the plasmids were induced by the addition of IPTG to 1 mM 30 min before infection. Samples were taken 30 min after induction with IPTG. The sequencing lanes are labeled with the dideoxynucleotides used. The black triangles marked with (1) indicate the 5' ends generated by RegB and the grey triangles marked with (2) show the 5' ends generated by secondary cleavage.

In order to determine which amino acid replacement leads to a partial loss of PNK activity, T4K10 mutants carrying single mutation within the *pseT* gene were constructed, and the functional gene was recovered as well. For this, phage T4 gene *pseT* was inserted into cloning vector pBSPL0+. Recovery of the *pseT* mutations of the T4K10 phage was

accomplished by the genetic crosses between phage T4K10 and a recombinant plasmid p0-T4*pseT* carrying T4 wild-type *pseT* gene. As a result of homologous recombination between the sequences of cloned and genomic *pseT* genes, we obtained three mutants on the T4K10 genetic background: T4K10PNK+ carrying wild-type *pseT*; T4K10M14 carrying *pseT* gene encoding PNK with the G14D substitution; and T4K10M229 carrying *pseT* gene encoding PNK with the R229H substitution.

To detect if secondary cleavages appear in the phage-induced transcripts, the *E. coli* C41(DE3) cells were infected with phages T4, T4K10, T4K10M14, T4K10M229 and T4K10PNK+. Isolated mRNAs were analyzed by primer extension sequencing using specific primer nrdC.3(R) (Fig. 11).

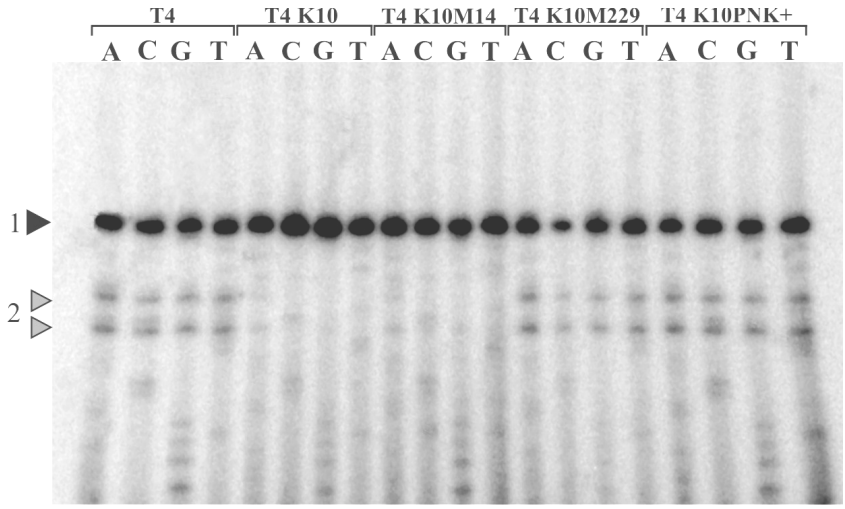

Fig. 11. Analysis of transcripts for gene *nrdC.3* of bacteriophage T4 and T4 *pseT* mutants. Primer extension sequencing of *nrdC.3* mRNA isolated from *E. coli* C41(DE3) cells at 6 min post-infection at 30 ºC with T4, T4K10, T4K10M14, T4K10M229 and T4K10PNK+ phages. The sequencing lanes are labeled with the dideoxynucleotides used in the sequencing reactions. Black arrows marked with (1) indicate the 5' ends of primary cleavage generated by RegB and grey arrows marked with (2) show the 5' ends of secondary cleavages generated by RNase G, stimulated by T4 PNK.

As shown in figure 11, gene *nrdC.3* transcripts after infection with phages T4, T4K10M229 and T4K10PNK+ are truncated at both primary and secondary sites, whereas in case of infection with T4K10 and T4K10M14 mRNA are efficiently processed only at their primary site by RegB. Absence of the secondary cuts within *nrdC.3* transcripts from T4K10 and T4K10M14 infections indicated that PNK protein encoded by these phages might be inactive. To ensure this, recombinant PNK proteins from T4 and T4K10 were produced and their *in vitro* 5'-kinase activities were tested.

Recombinant plasmids carrying wild-type *pseT* gene or mutated T4 *pseT* gene from phage T4K10 genome were constructed on the basis of expression vector pET16b. After transformation of the *E. coli* C41(DE3) cells and induction of expression of the cloned genes, recombinant proteins were purified using His-affinity columns. Purified recombinant proteins contained short N-terminal polyhistidine tags, which do not affect kinase activity or quaternary structure of T4 PNK (Wang and Shuman 2001).

T4 PNK catalyses transfer of the γ-phosphate from ATP or other nucleotides to a 5'-hydroxyl terminus of either DNA or RNA, which can equally serve as the substrates for this enzyme (Richardson 1965; Novogrodsky et al 1966). The 5'-polynucleotide kinase activities of the recombinant proteins were tested by *in vitro* phosphorylation reactions of DNA oligonucleotide having 5'-hydroxyl. γP^{32}-labeled ATP was used as a phosphate donor. The radiolabeled oligonucleotide products were resolved by electrophoresis in polyacrylamide gels and visualized and quantitated using a Fujifilm FLA5100 phosphorimager (Fig. 12).

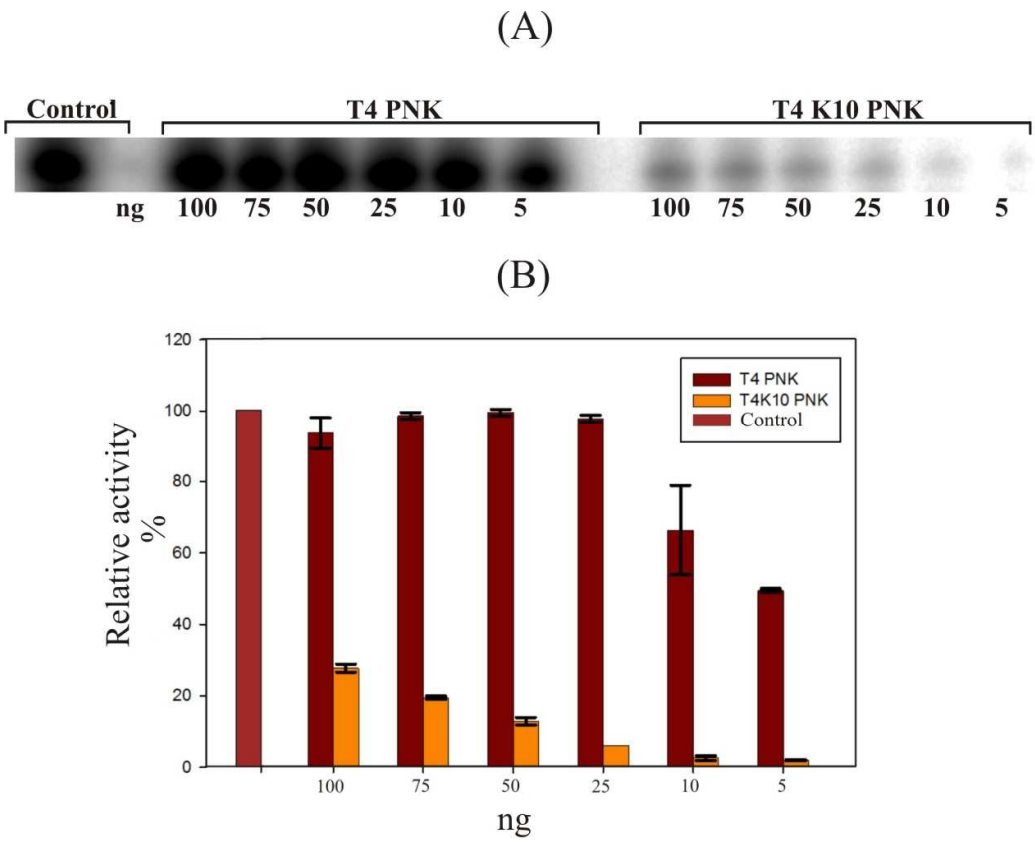

Fig. 12. Comparison of phage T4 and T4K10 5'-kinase activity of recombinant PNK proteins. The activity of the wild-type PNK was assigned 100%. **A)** T4 PNK and T4K10 PNK activity assay of the P^{32} primer labeling reaction, **B)** T4 and T4K10 PNK activity assessments in graphical expression.

The results of *in vitro* reactions presented in Figure 12 indicate that T4K10 mutant PNK was defective in catalysis and retained only less than 5 % of the wild-type PNK activity.

Interestingly, both G14 and R229 aa have been previously changed by A (alanine), but these substitutions had no effect on either 5'-kinase or 3'-phosphatase activities of recombinant proteins (Wang and Shuman 2001). This is not surprising for R229, which occurs in the non-conservative position and there are no catalytically important residues located nearby (Galburt et al. 2002). Meanwhile, G14 is conserved in PNK close homologues and is located in the vicinity of K15 and S16, which are essential for 5'-kinase activity. Presumably, substitution of G14 by a small alanine preserved the structural integrity of the PNK active site and did not affect phosphorylation process, while displacement of G14 by the aspartic acid distorted active site and ablated 5'-kinase activity.

Investigation of PNK Influence on Degradation of Phage T4 Early Transcripts

In order to determine whether PNK has an influence on degradation process of phage T4 transcripts *nrdC.3*, *motB* and *g39* quantitative real-time PCR analysis was performed. qRT-PCR can be used to demonstrate quantitative changes in mRNA levels (Halford, 1999), so an assay based on the detection of changes in mRNA levels under the influence of PNK was performed. First, the *pseT* deletion mutant T4K10ΔPNK of phage T4K10 using I/S system was constructed (Selick et al. 1988). Then *E. coli* C41(DE3) cells were infected by phages T4K10PNK+ or T4K10ΔPNK. Rifampicin, which prevents initiation of new transcripts by binding to the β subunit of RNA polymerase (Campbell, 2001), was added to bacterial cultures. Samples were harvested before rifampicin addition, and 3, 5, 7, 10 and 20 minutes after rifampicin addition. Isolated mRNAs were analyzed by qRT-PCR using sequence-specific DNA probes consisting of oligonucleotides that were labeled with a florescent reporter and specific primers. Real-time PCR and HRM assays were performed in line with Manufacturers' instructions. qRT-PCR was performed four times (using RNA isolated in four different experiments) as triplicates. Each transcript had negative control without input of any template. The Rotor-Gene 1.7.87 software version was used for processing the results.

First, the influence of PNK on gene *nrdC.3* transcripts was examined. As shown in figure 13, there are significant differences between mRNA degradation levels, when functional *pseT* gene is presented in bacteriophage genome and when it is deleted.

As already mentioned, 5 min after infection with bacteriophage T4K10PNK+ or T4K10ΔPNK rifampicin was added to bacterium culture. RNAP molecules in elongation complexes with RNA and DNA are resistant to rifampicin binding. Once elongating RNAPs terminate transcription and release RNA and DNA, they become susceptible to rifampicin binding, which traps them in newly formed open complexes (Herring et al., 2005). For this reason, increasing levels of transcripts is still observed from 0 to 3 min (Fig. 13 B). During this period, new transcripts are still synthesized, but the degradation of mRNA also commences.

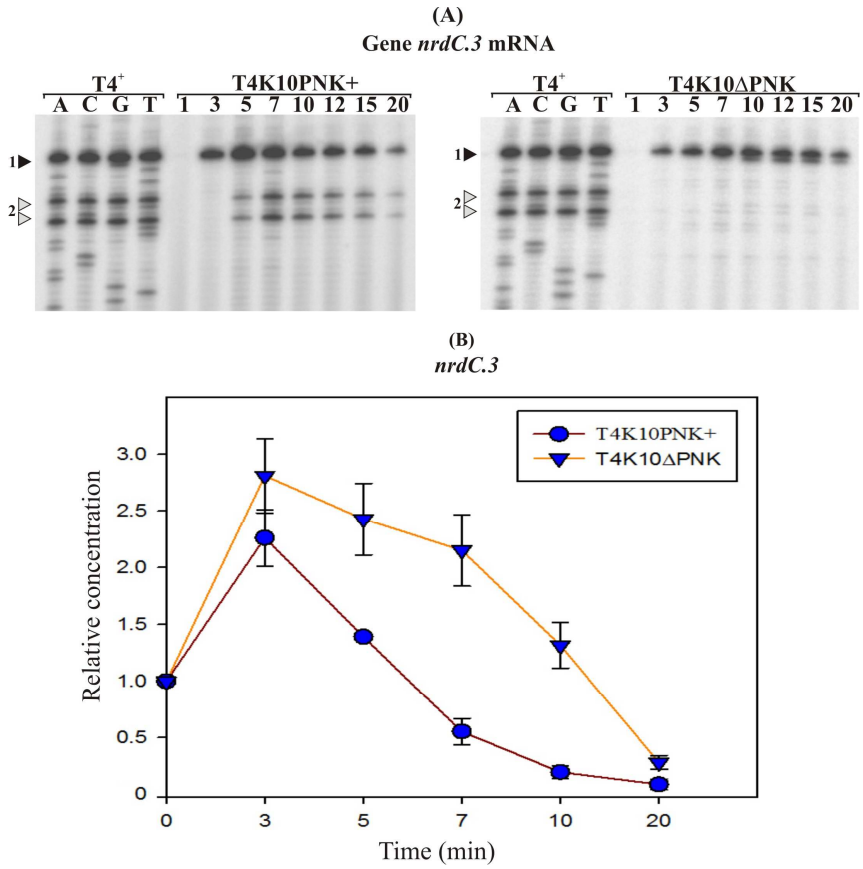

Fig. 13. Analysis of PNK influence on gene *nrdC.3* transcript degradation. A) Primer extension sequencing using RNA isolated from *E. coli* C41(DE3) cells at 6 min post-infection at 30 °C with T4^{+}. The sequencing lanes are labeled with the dideoxynucleotides used in the sequencing reactions. Primer extension reactions of RNA isolated 1, 3, 5, 7, 10, 12, 15 and 20 min post-infection from the cells that were infected with T4K10PNK+ or T4K10ΔPNK phages at 30 °C are next to the sequencing lanes. The time (min) of post-infection that RNA was isolated is at the top. Black triangles marked with (1) indicate the 5' ends generated by RegB processing and grey triangles marked with (2) show the 5' ends generated by secondary cleavages. **B)** Grafical representation of mRNA degradation revealed by qRT-PCR assay. RNA was isolated from *E. coli* C41(DE3) cells at 0, 3, 5, 7, 10, and 20 min from the cells that were infected with T4K10PNK+ or T4K10ΔPNK phages at 30 °C. Data analysis was performed using Rotor-Gene 7.1.87 software.

The analysis of *nrdC.3* transcripts derived from cells infected with T4K10PNK+ has revealed that they degraded much faster than those derived from T4K10ΔPNK infected cells. Thus in the absence of PNK the transcripts become more stable (Fig. 13 B). These results suggest that *nrdC.3* transcripts degradation is dependent on PNK, but later on the degradation can also proceed in a PNK-independent way.

The gene *motB* was chosen for degradation study because hydrolysis of its transcripts differs from other analyzed transcripts, and it seems that *motB* mRNR secondary processing can take place both in the presence of PNK, or without it (Fig. 14 A).

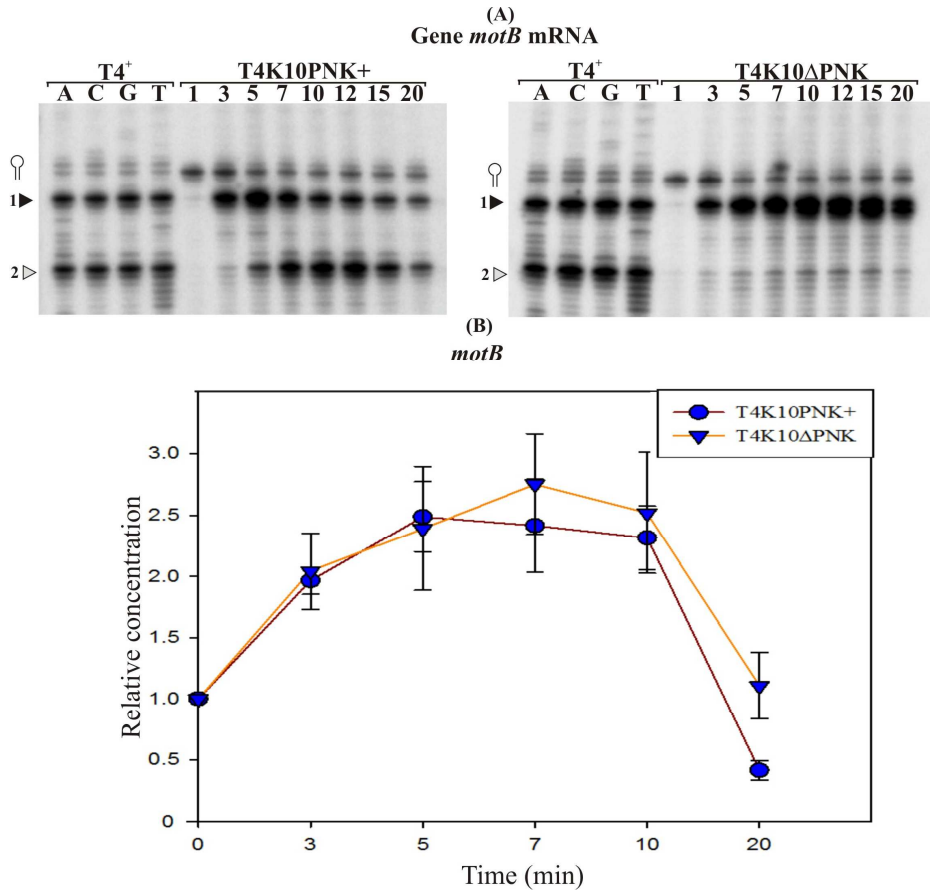

Fig. 14. Analysis of PNK influence on gene *motB* transcript degradation. A) Primer extension sequencing using RNA isolated from *E. coli* C41(DE3) cells at 6 min post-infection at 30 °C with T4^{+}. The sequencing lanes are labeled with the dideoxynucleotides used in the sequencing reactions. Primer extension reactions of RNA isolated 1, 3, 5, 7, 10, 12, 15 and 20 min post-infection from the cells that were infected with T4K10PNK+ or T4K10ΔPNK phages at 30 °C are next to the sequencing lanes. The time (min) of post-infection that RNA was isolated is at the top. Black triangles marked with (1) indicate the 5' ends generated by RegB processing and grey triangles marked with (2) show the 5' ends generated by secondary cleavages. **B)** Grafical representation of mRNA degradation revealed by qRT-PCR assay. RNA was isolated from *E. coli* C41(DE3) cells at 0, 3, 5, 7, 10, and 20 min from the cells that were infected with T4K10PNK+ or T4K10ΔPNK phages at 30 °C. Data analysis was performed using Rotor-Gene 7.1.87 software.

The results of this study indicated that there were no significant differences between the degradation rate of the transcripts derived from cells infected with T4K10ΔPNK or T4K10PNK+ (Fig. 14 B), but mRNA derived from T4K10PNK+ infected cells degraded a little bit faster. This finding suggests that PNK has minor influence on *motB* mRNA degradation. Taken together, all results presented in this work suggest that *motB* transcripts can be equally degraded in both the PNK-dependent and independent way.

It was known that gene *39* is transcribed from the early promoter into the long polycistronic early transcripts and from a proximal middle promoter into monocistronic middle transcripts (Stitt and Hinton, 1994). What distinguishes the two overlapping transcripts in each case is that the polycistronic species can be cleaved by RegB in the upstream intergenic regions, whereas the monocistronic middle transcripts cannot. It was therefore appropriate to examine PNK influence in this case. The results indicate that *g39* transcripts isolated from cells infected with T4K10PNK+ are degraded more rapidly than those isolated from the T4K10ΔPNK-infected cells, however, the difference between the amounts over the time is similar (Fig. 15). This can be explained by the fact that polycistronic early transcripts are degraded rapidly (by RegB and PNK-dependent manner), while monocistronic middle transcripts are more stable and degraded by RegB and PNK independent manner.

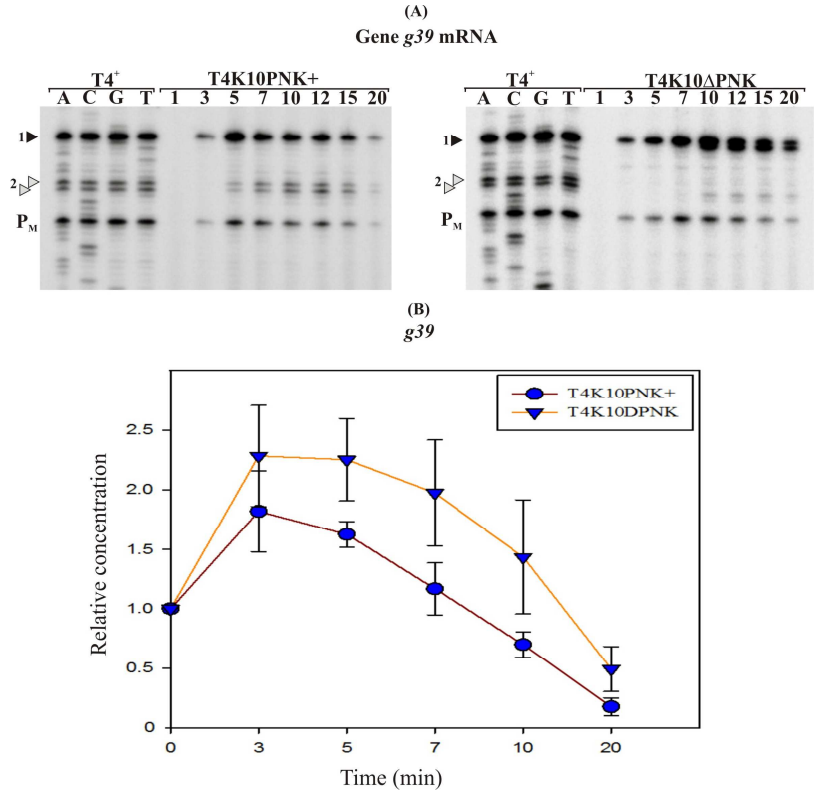

Fig. 15. Analysis of PNK influence on gene *39* transcript degradation. A) Primer extension sequencing using RNA isolated from *E. coli* C41(DE3) cells at 6 min post-infection at 30 °C with T4^{+}. The sequencing lanes are labeled with the dideoxynucleotides used in the sequencing reactions. Primer extension reactions of RNA isolated 1, 3, 5, 7, 10, 12, 15 and 20 min post-infection from the cells that were infected with T4K10PNK+ or T4K10ΔPNK phages at 30 °C are next to the sequencing lanes. The time (min) of post-infection that RNA was isolated is at the top. Black triangles marked with (1) indicate the 5' ends generated by RegB processing and grey triangles marked with (2) show the 5' ends generated by secondary cleavages. **B)** Grafical representation of mRNA degradation revealed by qRT-PCR assay. RNA was isolated from *E. coli* C41(DE3) cells at 0, 3, 5, 7, 10, and 20 min from the cells that were infected with T4K10PNK+ or T4K10ΔPNK phages at 30 °C. Data analysis was performed using Rotor-Gene 7.1.87 software.

Based on the known *in vivo* functions of the PNK, its deficiency may have several consequences during phage development, major of which concern gene expression processes. As mentioned above, T4 PNK is involved in tRNA repair and accelerates mRNA degradation through 5'-phosphorylation of their hydroxyl termini left by ribonuclease RegB. In this study, we show that processing at the secondary sites of seven T4 transcripts (*ndd*, *nrdC.3*, *55.2*, *cef*, *motB*, *g43* and *g39*) is impaired in the absence of an active kinase. However, even in the absence of clearly visible secondary cuts, 5'-terminal phosphorylation can stimulate distant cleavage events within other transcripts by RNases E and G and modulate their stability (Jiang et al. 2000; Feng et al. 2002; Jiang and Belasco 2004). It can be concluded that both the T4 RNase RegB, as well as PNK stimulate activities of *E. coli* RNases E and G and are directed to accelerate degradation of phage T4 early transcripts.

CONCLUSIONS

1. The SD sequences of genes *ndd.4*, *mobD*, *sp*, *segD*, *pin*, *dmd* and the intergenic region upstream gene *arn* have RNase RegB cleavage sites.
2. Early *segD* mRNA is transcribed from the newly detected early promoter $P_E$segD that contains the untypical -35 sequence.
3. RegB-processed *nrdC.3*, *ndd* and *55.2* mRNAs carry AU rich clear secondary cleavage targets located just downstream of RegB cleavage sites.
4. *E. coli* endoribonucleases E and G are involved in secondary processing of the RegB-cleaved T4 mRNAs, but RNase G is the main ribonuclease that cleaves all known secondary sites of RegB-processed transcripts.
5. RNase G can be modified during the T4 infection cycle. However, such modifications do not affect the activity of this nuclease towards secondary cuts in the RegB-processed T4 mRNAs.
6. The T4K10 gene *pseT* contains two point mutations leading to the amino acid substitutions G14D and R229H. The G14D mutation impairs 5'-kinase activity of T4K10 PNK.
7. Phage encoded RNase RegB, as well as PNK stimulate activities of *E. coli* RNases E and G and are directed to accelerate degradation of phage T4 early transcripts.

LIST OF PUBLICATIONS

1. Truncaitė L., Stoškienė G., Kalinienė L., **Strazdaitė Ž.**, Nivinskas R. (2006). *In vivo* and *in vitro* activities of the bacteriophage T4 early promoters. *Biologija.* 3:8-11.
2. Zajančkauskaite A., Truncaite L., **Strazdaite-Žieliene Ž.**, Nivinskas R. (2008). Involvement of the *Escherichia coli* endoribonucleases G and E in the secondary processing of RegB-cleaved transcripts of bacteriophage T4. *Virology*. 375:342-353.

3. **Strazdaitė-Žielienė Ž.,** Zajančkauskaitė A., Kalinienė L., Meškys R., Truncaitė L. (2013). A mutation in the gene for polynucleotide kinase of bacteriophage T4 K10 affects mRNA processing. *Archives of Virology.* Doi: 10.1007/s00705-013-1800-x

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SANTRAUKA

Šio darbo tikslas buvo ištirti bakteriofago T4 ankstyvųjų transkriptų degradacijos mechanizmus, nustatyti šiame procese dalyvaujančius fermentus ir jų tarpusavio veikimo priklausomybę.

Pirmas šio darbo uždavinys buvo nustatyti naujus endoribonukleazės RegB skėlimo taikinius ankstyvuosiuose fago transkriptuose bei patikrinti kai kuriuos potencialius taikinius, išsidėsčiusius viduriniųjų ar vėlyvųjų genų srityse. Šiame darbo etape pavyko patvirtinti 6 naujus RegB hidrolizuojamus taikinius, esančius genų *ndd.4*, *segD*, *dmd*, *sp*, *mobD.1* ir *pin* SD srityse ir 1 taikinį esantį prieš geną *arn*. Analizuojant *segD* transkriptus, kurie buvo laikomi vėlyvaisiais (Luke ir kt., 2002), buvo aptiktas iki šiol nenustatytas *in vivo* aktyvus ankstyvasis promotorius $P_E$segD, kurio –10 srities seka ir tarpiklio sritis atitiko tipines fago T4 P_E promotoriaus sekas, o –35 srities seka (ATTTACA) buvo netipinė. Darbo metu buvo patvirtinta, kad šis promotorius yra aktyvus ir pakankamai stiprus *in vivo*, taigi RegB hidrolizuojami *segD* transkriptai yra ankstyvieji.

Ieškant naujų endoribonukleazės RegB taikinių, buvo aptikti 3 transkriptai (*ndd, nrdC.3* ir *55.2*), kurie turėjo antrinius skėlimo taikinius AU turtingose sekose, esančiose už RegB taikinių. Šiame darbe patvirtinome, kad visi 7 antrinius taikinius turintys transkriptai tampa tinkamais substratais kitoms nukleazėms tik po pradinio kirpimo, kurį vykdo endoribonukleazė RegB. Šie transkriptai buvo pasirinkti, kaip modelis tolimesnės RegB kirptų transkriptų degradacijos tyrimams.

Analizuojant mRNR, išskirtą iš RNazės E ts jautrių *E. coli* kamienų nustatėme, kad RNazė E arba visiškai nedalyvauja genų *ndd*, *nrdC.3* ir *55.2* transkriptų degradavime, arba nukleazės aktyvumas maskuojamas kitos nukleazės, turinčios panašų specifiškumą. Todėl buvo patikrintas panašius taikinius atpažįstančios RNazės G galimas poveikis fago T4 RegB kirptiems transkriptams. Šio darbo metu, naudojant RNazės G mutantinius kamienus, nustatyta, kad RNazė G yra atsakinga už antrinių skėlimų atsiradimą AU turtingose sekose prieš bakteriofago T4 genus *ndd, nrdC.3*, *43*, *motB* ir *39*. Tuo tarpu RNazės E ir G abi kartu gali atpažinti tuos pačius antrinius taikinius, esančius mRNR prieš genus *55.2* ir *cef*. Apibendrinus tyrimų rezultatus galima teigti, kad RNazės E vaidmuo antriniame tirtų transkriptų skaldyme yra nedidelis, ir RNazė G yra pagrindinė

nuklеazė, skelianti visus iki šiol žinomus RegB kirptų transkriptų antrinius taikinius. Šie taikiniai yra pirmieji fago T4 transkriptuose nustatyti RNazės G taikiniai.

RNazės E ir G teikia pirmenybę tiems RNR substratams, kurie 5' gale turi monofosfatinę grupę (Mackie, 1998, 2000; Baker ir Mackie, 2003; Jiang ir kt., 2000). Tačiau buvo žinoma, kad po endoribonukleazės RegB skėlimo, transkripto gale lieka 5' OH grupė (Saida ir kt., 2003), o tokie transkriptai nėra geri substratai endoribonukleazėms E bei G. Įklonavus T4 *regB* geną bei RegB ir antrinius taikinius turinčius genus ir indukavus jų raišką nuo plazmidžių, buvo pastebėta, kad antriniai mRNR kirpimai plazmidinėse sistemose neatsiranda. Jie būdavo stebimi tik plazmidines sistemas dar papildomai infekavus ir bakteriofagu $T4^+$. Tai rodė, kad fago koduojami veiksniai turi įtakos *E. coli* RNazių E ir G aktyvumui. Todėl tolimesni tyrimai buvo skirti nustatyti, kokie fago baltymai veikia RNazių aktyvumą RegB kirptų ankstyvųjų fago transkriptų atžvilgiu. Tyrimų modeliu buvo pasirinkta pagrindinė šio proceso RNazė G ir jos hidrolizuojami transkriptai. Buvo tikrinama RNazės G modifikavimo galimybė, o taip pat panaudojant delecinius T4 mutantus buvo bandoma nustatyti fago baltymus, darančius įtaką antrinių skėlimų atsiradimui.

Tikrinant RNazės G modifikavimo galimybę, buvo klonuotas jos genas bei indukuotas ir išgrynintas rekombinantinis baltymas. Dalis indukuotų ląstelių prieš baltymo išgryninimą buvo infekuojama fagu $T4^+$. Analizuojant baltymus SDS PAGE nustatyta, kad keičiasi po faginės infekcijos išgryninto RnaG baltymo elektroforetinis judrumas. Po infekcijos rekombinantinis baltymas RnaG tampa nevienalytis, juda elektroforetiniame lauke tarsi trys atskiri, panašios molekulinės masės baltymai. Atlikus *rnag* koekspresijos su T4 riboziltransferazių genais (*alt*, *modA* ir *modB*) tyrimus, nustatėme, kad RNazė G faginės infekcijos metu gali būti ribozilinama baltymo ModB. Tačiau taip pat panašu, kad infekcijos metu RNazė G gali būti ne tik ribozilinama, bet ir patiria ne vieno tipo modifikaciją.

Lygiagrečiai su RNazės G modifikavimo tyrimais buvo tiriami laboratorijos kolekcijoje esančių T4 delecinių mutantų transkriptai, siekiant rasti tokį mutantą, kurio infekcijos metu RegB kirptų transkriptų antriniai skėlimai nevyktų. Tyrimų modeliu naudojant *nrdC.3* transkriptus nustatyta, kad I/S sistemos (Selick ir kt., 1988) fago T4K10 infekcijos metu ties antriniais taikiniais jie nėra karpomi. Patikrinus šio fago delecinės srities ΔrIIPT genų įtaką antriniams skėlimams, buvo parodyta, kad nė vienas

iš tirtų delecinės srities genų nebuvo atsakingas už RNazių E/G aktyvumo RegB kirptų transkriptų atžvilgiu susilpnėjimą. Fagas T4K10 buvo sukonstruotas kryžminant atskirų genų mutantus, gautus cheminės ar UV mutagenezės būdu (Selick ir kiti, 1988), todėl buvo didelė tikimybė, kad gali būti pažeisti ir kiti jo genai. Vienas iš kandidatų, galinčių įtakoti antrinių kirpimų atsiradimą buvo fago T4 geno *pseT* koduojamas fermentas polinukleotidkinazė (PNK). PNK fosforilina $tRNR^{Lys}$ antikodono 5'-OH liekaną, paliktą po nukleazės PrrC skėlimo (Amitsur ir kt., 1987). Tai buvo vienintelė iki šiol žinoma biologinė T4 PNK funkcija. Buvo nuspręsta patikrinti fago T4K10 *pseT* geno nukleotidinę seką. Nustačius ją, buvo pastebėta, jog šiame gene yra dvi taškinės mutacijos, keičiančios aminorūgštis – G14D ir R229H. Pirmoji buvo konservatyvioje padėtyje ir arti PNK 5' kinazės aktyviojo centro, o kita – nekonservatyvioje padėtyje ir toli nuo aktyviųjų centrų.

Įterpus T4 ir T4K10 *pseT* genus į ekspresijos vektorius, indukavus jų raišką ir išgryninus rekombinantinius baltymus buvo atlikti jų *in vitro* 5'-kinazinio aktyvumo tyrimai. Nustatyta, kad T4K10 PNK aktyvumas siekia tik apie 5 % natyvios PNK aktyvumo. Taip pat buvo atliktas koekspresijos eksperimentas, po kurio buvo nustatyta, kad plazmidinėje sistemoje pateiktos PNK užtenka, kad nuo plazmidės indukuoti *nrdC.3* transkriptai būtų fosforilinami ir dėl to atpažįstami RNazės G bei efektyviai hidrolizuojami antrinių taikinių vietose. Šis tyrimas paneigė hipotezę, kad RNazės G modifikavimas T4 infekcijos metu yra reikalingas antrinių skėlimų atsiradimui, nes esant rekombinantinei PNK ir be infekcijos fagu jie buvo stebimi.

Sukonstravus T4K10 viengubus mutantus ir ištyrus jų RegB kirptus transkriptus, buvo nustatyta, kad G14D mutaciją turinti PNK nestimuliuoja RNazės G aktyvumo. Ši mutacija yra PNK 5'-kinazės domene, todėl greičiausiai yra pažeista 5' kinazės funkcija. Tuo tarpu, R229H pakaita yra įvykusi PNK 3'-fosfatazės nekonservatyvioje padėtyje, todėl greičiausiai nesutrikdė šios funkcijos arba ji yra nesvarbi RNazės G stimuliavimui. Vėliau buvo sukonstruoti T4Δ*pseT* ir T4K10Δ*pseT* deleciniai mutantai ir ištirti jų visi 7, antrinius taikinius turintys, RegB hidrolizuojami transkriptai. Rezultatai patvirtino visų septynių transkriptų antrinės hidrolizės priklausomybę nuo fago T4 koduojamos PNK. Buvo padaryta išvada, kad fago koduojama PNK fosforilina visų tirtų fago ankstyvųjų RegB kirptų transkriptų 5'OH galus ir taip sukuria palankius substratus *E. coli* RNazėms E/G, kurios toliau hidrolizuoja minėtuosius transkriptus.

Darbo pabaigoje kiekybinio realaus laiko PGR metodu buvo įvertinta fago koduojamos PNK įtaka ankstyvųjų genų *nrdC.3*, *motB* ir *g39* transkriptų degradacijai. Tyrimams naudoti fagų T4K10ΔPNK ir T4K10PNK+ infekcijos eigoje išskirti transkriptai. Visų tirtų transkriptų degradacija yra greitesnė esant PNK, tačiau ilgainiui jie degraduojami ir nuo PNK nepriklausomu keliu. Buvo padaryta išvada, kad tirtų transkriptų stabilumas sumažėja esant veikliai PNK, tačiau sumažėjimas priklauso nuo konkretaus transkripto endoribonukleolitinės hidrolizės ypatumų.

Apibendrinant gautus rezultatus galima teigti, kad tiek T4 RNazės RegB, tiek ir PNK sąlygotas *E. coli* RNazių E ir G veikimas yra skirtas ankstyvųjų transkriptų degradacijai pagreitinti.

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