Volume 296, number 3, 267–270 FEBS 10611 January 1992

Translation enhancing properties of the 5′-leader of potato virus X genomic RNA

D.A. Zelenina[b], O.I. Kulaeva[a], E.V. Smirnyagina[a], A.G. Solovyev[a], N.A. Miroshnichenko[b], O.N. Fedorkin[a], N.P. Rodionova[a], S.Yu. Morozov[a] and J.G. Atabekov[a]

[a]*Department of Virology, Moscow State University, Moscow 119899, USSR* and [b]*Institute of Microbiology, Academy of Sciences of the USSR, Moscow 117811, USSR*

Received 20 November 1991

The double-stranded DNA copy corresponding to the 5′-nontranslated αβ-leader of potato virus X (PVX) genomic RNA (positions –3 to –85 according to AUG initiator) was chemically synthesized and fused to the transcription plasmids containing three different reporter genes: neomycinphosphotransferase type II (NPT II) gene, *Bacillus thuringiensis* coleopteran-specific toxic protein gene and β-glucuronidase (GUS) gene. Expression of the reporter genes in vitro and in plant protoplasts (in the case of GUS gene) reveals that the αβ-leader of PVX RNA acts as a translation enhancer despite the presence of the upstream vector-derived sequence and irrespective of the length of the spacer sequence preceding the reporter genes.

Recombinant DNA; Translational enhancement; Plant virus

1. INTRODUCTION

The 5′-untranslated leaders of various mRNAs have been shown to enhance translation of foreign eukaryotic and prokaryotic genes in vivo and in vitro [1–5]. The 5′-leader of potato virus X (PVX) RNA (83 nt apart from cap structure) consists of two sequences referred to as α-sequence (41 nt 3′ to cap) and β-sequence (42 nt upstream from the first AUG) [6]. We have tested αβ-sequence for its translation-enhancing ability using the gene construct in which neomycin phosphotransferase I gene (NPT I) with deleted 5′-terminal region (including the AUG initiator) was under the control of the initiation codon of the PVX 5′-proximal 165K protein gene in T7-transcription plasmid. The αβ-leader which contained no vector-derived sequences has been shown to enhance the translation of NPT I gene in different cell-free protein-synthesizing systems by 6–40-fold [6]. In comparative experiments [6] it has been found that the PVX αβ-leader was almost equally effective as tobacco mosaic virus RNA leader, which was known to be the most efficient translational enhancer [1,7].

In the most versatile transcription vectors containing T7 or SP6 promoters for in vitro usage, or cauliflower mosaic virus (CaMV) 35S promoter for in vivo expression, the polylinker region is positioned downstream from the transcription initiation site. Routine genetic engineering procedure for fusing of any regulatory element (like the αβ-leader) and foreign gene usually results in insertion of the nucleotide spacers between the engineered elements. Therefore, the αβ-carrying chimeric mRNAs constructed by routine procedure will typically contain the vector-derived 5′-nucleotides and/or spacer sequences. To determine the influence of the vector-derived 5′-terminal sequences and the spacer sequences located between the αβ-leader and the AUG initiation codon, we have fused the αβ-leader (namely, the sequence positioned –3 to –85 according to AUG-initiator of the 165K protein gene) to the T7-transcription plasmids containing different reporter genes.

Our results show that the αβ-sequence significantly stimulates translation of chimeric mRNAs despite the presence of the vector-derived sequences upstream from αβ-leader and the spacer sequences preceding the reporter genes.

Abbreviations: NPT, neomycin phosphotransferase; PVX, potato virus X; GUS, β-glucuronidase.

Correspondence address: D.A. Zelenina, Institute of Microbiology, Academy of Sciences of the USSR, Moscow 117811, USSR.

2. MATERIALS AND METHODS

2.1. *Construction of plasmids for the expression of the reporter genes*

The following expression vectors were used: pTZ18R (Pharmacia-LKB) containing T7 promoter for in vitro transcription and pRT101 and pRT103 plasmids which are plant transient-expression vectors containing CaMV 35S promoter and multiple cloning site [8].

The plasmid pTZ-NEO was assembled by insertion of a *Bgl*II-*Bam*HI DNA fragment containing NRT II gene from pNEO (Pharmacia-LKB) into the *Bam*HI site of pTZ18R and selected further by sequence analysis of the recombinant plasmids isolated from the transformed *E. coli* strain XL-1B (Stratagene) cells.

The plasmid pTZ-TOX was assembled by insertion of a *Bam*HI-*Hin*dIII PCR-generated DNA fragment containing *Bacillus thuringiensis* coleopteran-specific toxic protein gene (Dobrzhanskaya et al., unpublished data) into the *Bam*HI-*Hin*dIII-cut pTZ18R.

The GUS gene from pJI110-00 (kindly provided by Dr. T.M.A. Wilson) was excised as a *Nco*I (filled in with Klenow domain of DNA polymerase I)-*Sal*I fragment and ligated into *Bam*HI(filled)-*Sal*I-cut pTZ18R to give pTZ-Gus. The *Nco*I-*Sal*I fragment of pJI110-00 was additionally ligated into *Eco*RI-*Sal*I-cut pTZ18R using *Eco*RI to *Nco*I adaptor to give pTZ-G12.

To create control plasmid pRT-GUS containing GUS gene under the control of 35S promoter, *Nco*I-*Sal*I (filled) fragment of pJI110-00 was ligated into *Nco*I-*Sma*I-cut pRT103.

To fuse the sequence of $\alpha\beta$-leader and those of reporter genes, the plasmids pTZ-NEO, pTZ-TOX and pTZ-GUS were digested with *Eco*RI and *Sma*I and then separated electrophoretically from the excised short DNA fragment. The linearized plasmid DNAs were recovered from agarose gel and ligated to a double-stranded 83 bp synthetic oligodeoxyribonucleotide (Fig. 1A) containing the $\alpha\beta$-sequence flanked by an *Eco*RI cohesive end and a blunt end (oliogonucleotides were synthesized and kindly provided by V.A. Efimov). Preceding the ligase reaction, the individual oligonucleotide DNA strands were phosphorylated at the 5′ terminus and annealed. The resulting plasmids pTZ-NEO$\alpha\beta$, pTZ-TOX$\alpha\beta$ and pTZ-GUS$\alpha\beta$ were screened by DNA sequence analysis.

The plasmid pRTGUS$\alpha\beta$ was constructed by insertion of the fused $\alpha\beta$-GUS sequence, contained in an *Eco*RI-*Hin*dIII (filled) fragment from pTZ-GUS$\alpha\beta$, between the *Eco*RI and *Sma*I sites of pRT101.

2.2. *In vitro transcription and translation*

Plasmids pTZ-NEO$\alpha\beta$, pTZ-TOX$\alpha\beta$ and pTZ-GUS$\alpha\beta$, as well as control constructs pTZ-NEO, pTZ-TOX, pTZ-G12 and pGEM3Z-GUS (kindly provided by Dr. E. Kuzmin) were digested with *Hin*dIII, which recognized a unique site downstream of each reporter gene. Transcription with T7 RNA polymerase (Promega Biotec.) was as described previously [9]. The primary structures of the leader regions of $\alpha\beta$-containing and control RNA transcripts of the reporter genes are shown (Fig. 1).

Synthetic RNA transcripts were translated in rabbit reticulocyte lysates essentially as described previously [9]. [^{35}S]Methionine (Amersham) was used as a radioactive label.

2.3. *Protoplast preparation and electroporation*

Protoplasts were obtained from *Hordeum vulgare* leaves grown in climatic chamber (25°C). The undersurfaces of 7-day-old plant leaves (10–12 cm in length) were abraded and then floated (usually 2 h at

Fig. 1. The primary structures of the double-stranded synthetic oligodeoxyribonucleotide containing $\alpha\beta$-sequence (A); different 5′-terminal nontranslated leaders of the in vitro transcripts containing: NPT II gene (B), *B. thuringiensis* toxic protein gene (C) and GUS gene (D). Initiation AUG codon is included.

28°C) in enzyme solution (2% Cellulysin (Calbiochem); 0.1% Macerase (Calbiochem); 10% mannitol, pH 5.5). Protoplasts were isolated by filtration through Miracloth, pelleted by centrifugation at 100 × *g* for 65 s and then washed by two sedimentation cycles (100 × *g* for 65 s). The protoplasts were resuspended in 10% D-mannitol, pH 5.5.

A total of 0.4 × 10^6 protoplasts was suspended in approximately 5 × 10^5/ml of 10% mannitol and incubated at 0°C for 30 min. The protoplasts were mixed with pRTGUS or pRTGUS$\alpha\beta$ DNA (20–40 μg/ml) and exposed to electric pulse of 1 ms at 750 V/cm. The electroporated protoplasts were incubated for 30 min at 0°C. After incubation protoplasts were suspended in 10% mannitol at room temperature, pelleted by centrifugation at 100 × *g* for 70 s and resuspended in Aoki-Takebe media. After incubation for 20 h at 22°C the protoplasts were collected by centrifugation.

2.4. *Determination of β-glucuronidase activity*

Fluorometric assays of GUS activity were performed on protoplasts and in vitro-synthesized enzyme. Protoplasts harvested by centrifugation were used in GUS extraction buffer (50 mM sodium phosphate, pH 7.0, 10 mM β-mercaptoethanol, 10 mM Na_2EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100). The samples were taken from the clarified extracts, mixed with 4-methyl umbelliferyl β-D-glucuronide (MUG) (final concentration 0.5 mM) and incubated at 37°C for 60 min. GUS activity was tested by using 100 μl-reactions containing 1:10 dilutions of protoplast lysate. Activity assays for in vitro-synthesized GUS were conducted by using 1:100 dilutions of the cell-free system. Reaction samples after incubation with MUG were diluted with 0.4 M Na_2CO_3 up to 10 ml. Fluorescence (excitation 365 nm, emission 455 nm) of each sample was determined in a Hitachi Fluorescence Spectrophotometer 850.

3. RESULTS AND DISCUSSION

Uncapped synthetic RNAs derived from pTZ-NEO$\alpha\beta$, pTZ-NEO, pTZ-TOX$\alpha\beta$, pTZ-TOX, pTZ-GUS$\alpha\beta$, pTZ-G12 and pGEM-GUS were translated in rabbit reticulocyte lysate. Polypeptide products were fractionated by polyacrylamide gel electrophoresis [9,10]. The results show that the translation of different reporter genes was considerably enhanced in a cell-free system in the presence of the 5′-$\alpha\beta$-sequence (Fig. 2A, B and C).

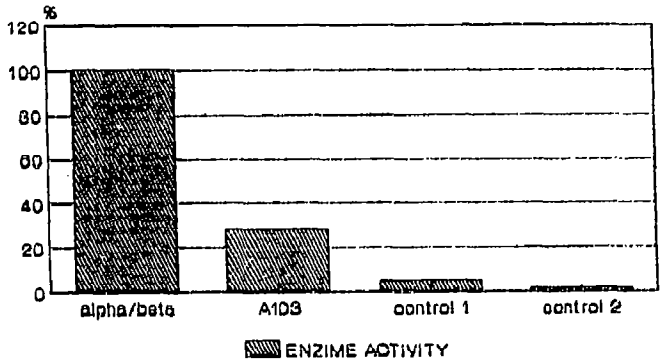

Fig. 3. Relative translation levels of the GUS-gene containing transcripts in rabbit reticulocyte lysate. The transcripts were synthesized using linearized plasmids pTX-GUS$\alpha\beta$ (alpha/beta), pTZ-G12 (control 1), pGEM-GUS (control 2) and pA103-GUS (A103) containing 53 nt leader sequence adjacent to the initiation codon of PVX coat protein gene (D. Zelenina et al., unpublished).

To quantitate these data more accurately samples were withdrawn from translation probes of the transcripts from pTZ-GUS$\alpha\beta$, pTZ-G12 and pGEM-GUS and analyzed for GUS activity. Non-capped transcripts containing $\alpha\beta$-sequence were significantly

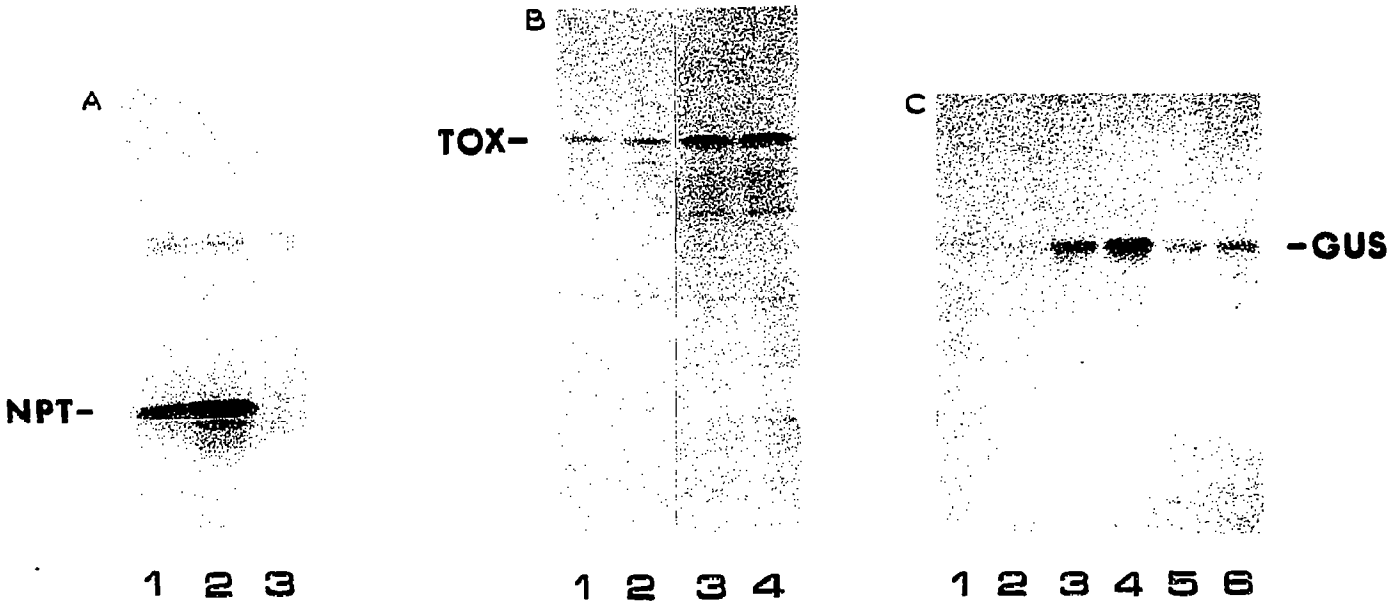

Fig. 2. Cell-free translation of chimeric transcripts in rabbit reticulocyte lysate (autoradiograms of gradient 8–20% gels [12]): (A) Lane 1 = control NPT II transcript, 20 μg/ml; lane 2 = $\alpha\beta$-containing transcript, 20 μg/ml; lane 3 = endogenous translation. (B) Lane 1 = control RNA transcript of *B. thuringiensis* TOX gene (20 and 40 μg/ml, respectively); lanes 2 and 3 = $\alpha\beta$-containing RNA transcript of *B. thuringiensis* TOX gene (20 and 40 μg/ml, respectively). (C) Lanes 1 and 2 = control RNA transcript of GUS gene (46 nt leader) (20 and 40 μg/ml, respectively); lanes 3 and 4 = $\alpha\beta$-containing RNA transcript of GUS gene (20 and 40 μg/ml, respectively); lanes 5 and 6 = control RNA transcript of GUS gene (12 nt leader) (20 and 40 μg/ml, respectively).

more active than those containing the artificial non-physiological 5'- leaders (Fig. 3).

The difference in GUS activity between the transcripts from pTZ-GUS$\alpha\beta$ and control pTZ-G12 varied significantly in different rabbit reticulocyte lysate preparations. Differences between these two transcripts in lysates from different sources varied from 3-fold induction to 14-fold induction (data not shown).

Translation of the transcripts with the artificial 5'-leaders (from pTZ-G12 and pGEM-GUS) was highly dependent on the presence of a cap structure (m^7GpppG) at the 5'-terminus. The effect of the $\alpha\beta$-leader on the in vitro expression of GUS gene was quantitatively similar to the effect of mRNA capping (data not shown). It is worth mentioning that the capping of the $\alpha\beta$-containing PVX-specific mRNAs resulted in further several-fold increase of in vitro translation [9].

To further investigate the translational enhancement activity of the $\alpha\beta$-sequence, we have used protoplast transient-expression assay. The activity of GUS was detected in protoplasts electroporated with pRTGUS or pRTGUS$\alpha\beta$, but not with pRT103. Electroporation with pRTGUS$\alpha\beta$ resulted in approximately 3-fold higher GUS activity than that with pRTGUS (data not shown). This observation suggests that the $\alpha\beta$-sequence substantially stimulates translation of naturally capped transcripts produced in plant cell nuclei.

The initiation codons in two control $\alpha\beta$-lacking constructs (pTZ-G12 and pRTGUS) containing the artificial leaders (Fig. 1) were situated within the local nucleotide context ACCAUGG. This context was shown to be several-fold more effective than the environments in which pyrimidines replace the purines in position –3 [11]. It should be noted that the start codons of $\alpha\beta$-containing transcripts from pTZ-GUS$\alpha\beta$ and pRTGUS$\alpha\beta$ are preceded by pyrimidine in position 3 (Fig. 1), as in the case of natural PVX genomic leader [6]. Thus, the observed effect of enhancement by $\alpha\beta$-sequence seems to be more pronounced taking into account nonfavorable AUG of $\alpha\beta$-carrying messengers.

Our data demonstrate that a synthetic derivative of the 5'-untranslated sequence of PVX RNA confers translational enhancement on different reporter genes despite the fact that the length and the primary structure of these genes and nonviral spacers upstream from their initiation codons were significantly varied.

Acknowledgements: We thank Dr. R. Töpfer for providing us with the pRT 101 to 105 plasmids.

REFERENCES

[1] Gallie, D.R., Sleat, D.E., Watts, J.W., Turner, P.C. and Wilson, T.M.A. (1987) Nucleic Acids Res. 15, 8693–8711.
[2] Jobling, S.A. and Gehrke, L. (1987) Nature 325, 622–625.
[3] Dolph, P.D., Racaniello, V., Villarmin, A., Palladino, F., Schneider, R. (1988) J. Virol. 62, 2059–2066.
[4] Ruiz-Linares, A., Bouloy, M., Girard, M. and Cahour, A. (1989) Nucleic Acids Res. 17, 2463–2476.
[5] Carrington, J.C. and Freed, D.D. (1990) J. Virol. 64, 1590–1597.
[6] Smirnyagina, E.V., Morozov, S.Yu., Rodionova, N.P., Miroshnichenko, N.A., Solovyev, A.G., Fedorkin, O.N. and Atabekov, J.G. (1991) Biochimie 73, 998–1011.
[7] Jobling, S.A., Cuthbert, C.M., Rogers, S.G., Fraley, R.T. and Gehrke, L. (1988) Nucleic Acids Res. 16, 4483–4498.
[8] Töpfer, R., Matzeit, V., Gronenborn, B., Schell, J. and Steinbiss, H.-H. (1987) Nucleic Acids Res. 15, 5890.
[9] Morozov, S.Y., Miroshnichenko, N.A., Zelenina, D.A., Fedorkin, O.N., Solovyev, A.G., Lukasheva, L.I. and Atabekov, J.G. (1990) Biochimie 72, 677–684.
[10] Laemmli, U.K. (1970) Nature 27, 680–685.
[11] Kozak, M. (1989) Mol. Cell. Biol. 9, 5073–5080.