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The 3′-terminal untranslated region of alfalfa mosaic virus RNA 4 facilitates the RNA entry into translation in a cell-free system

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In order to understand the role of the 3′-terminal untranslated region (3′-UTR) of alfalfa mosaic virus (AlMV) RNA 4 in viral RNA translation we have constructed the RNA derivatives differing in the length of their 3′-terminal portions and expressed them in a wheat germ extract. The result shows that the removal of the 3′-UTR from AlMV RNA 4 causes a lagged RNA translation in the cell-free system as compared with the translation of the full length RNA 4, thus suggesting the involvement of the 3′-UTR in the translation initiation pathway.

Wheat germ cell-free system; Translation initiation; Alfalfa mosaic virus RNA 4; 3′-UTR

1. INTRODUCTION

The ability of the virus to efficiently capture the host translation machinery once it has entered the host cell is known. The 3′-untranslated regions (3′-UTR) of viral RNAs seem to be important in the control of translation, but the real mechanism of the action is less well understood. The addition of the tobacco mosaic virus (TMV) 3′-UTR to chimeric mRNA constructs was shown to increase their expression up to 100-fold in vivo both in plant and animal cells [1–3]. Though 3′-UTRs can protect viral RNAs from nucleolytic degradation, the effect of stimulating protein synthesis in vivo could not be explained only in terms of mRNA stability.

AlMV RNA 4 is the messenger RNA for AlMV coat protein. In this monocistronic RNA the coat protein cistron of 660 nucleotides in length is flanked by a 5′-leader sequence of 39 nucleotide residues and a 3′-terminal non-coding region of 182 nucleotide residues [4]. In many plant viral RNA, e.g. TMV RNA, the 3′-terminal non-coding region ends with a tRNA-like structure capable of being aminoacylated by one of the aminoacyl-tRNA synthetases [5]. The AlMV 3′-UTR sequence does not arrange into a typical tRNA-like fold, but still has a highly developed secondary (and probably tertiary) structure bearing some similarities with the 'tails' of other plant viral RNAs [6].

As an approach to understand the role of the 3′-UTR we have compared the translational efficiencies of the viral mRNA constructs differing in their 3′-terminal parts in a wheat germ cell-free system. We have shown that the removal of the 3′-terminal tail from AlMV RNA 4 leads to a lagged mRNA translation. The results suggest that the viral 3′-UTR is involved in the translation initiation pathway.

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2. MATERIALS AND METHODS

2.1. *Materials*

Wheat germ S30 extract was from BBL Ltd, Latvian Biotechnology, Riga, Latvia. T7 polymerase was isolated from an overproducer *E. coli* strain and purified by S. Aksenovich at the Institute of Protein Research, Pushchino, Russia. [^{14}C]UTP with a specific activity of 51 mCi/mmol was from the Isotope, St. Petersburg, Russia. [^{14}C]Leu with a specific activity of 319 mCi/mmol was purchased from Amersham, UK. The pT7-2-42 plasmid containing a DNA copy of AlMV RNA 4 under the control of T7 promoter was kindly provided by Dr. K. Langereis [7,8].

2.2. *Transcripts*

The tr792, tr831 and tr964 RNA transcripts were obtained from the pT7-2-42 plasmid linearized with *Bst*XI (position 792 in AlMV RNA 4), *Pfl*MI (position 831) and *Sma*I (position 964), respectively. In vitro transcription was carried out as described in [9]. Radiolabeled transcripts were synthesized in the same reaction mixture but with UTP concentration reduced to 1.5 mM and [^{14}C]UTP added to 1.25 μCi per 100 μl. The transcripts were isolated by chloroform deproteinization and subsequent precipitation with 3 M LiCl. The RNA was dissolved in water to a concentration of 1 mg/ml.

2.3. *Cell-free translation system*

The P100 fraction was prepared from the wheat germ S30 extract according to the procedure of W. Kudlicki (personal communication; see also [10]). 5 ml of the wheat germ extract was centrifuged for 4 h at 47,000 rpm at 4°C in a Ti50 rotor, Beckman. The ribosomal pellet, retaining all other components of the protein-synthesizing machinery, was resuspended in 500 μl of buffer A: 20 mM HEPES, pH 7.5, 5 mM $Mg(OAc)_2$, 50 mM KOAc, 3 mM dithiothreitol, 10% glycerol. The translation mixture contained 7.5 μl of the P100 suspension, 25 pmol (or as denoted in the text) of unlabeled AlMV RNA, 1 μg creatine

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phosphokinase in a 50 μl volume of 24 mM HEPES buffer, pH 7.5 with 70 mM potassium acetate, 1.5 mM magnesium acetate, 100 μM ethylene diamine tetraacetate, 8 mM creatine phosphate, 1 mM ATP, 0.2 mM GTP, 3 mM dithiothreitol, 10 μM [^{14}C]Leu, and 50 μM of each of the other 19 amino acids. Incubation was done at 26°C. 5 μl samples of the translation mixture were taken at different times and the amount of [^{14}C]Leu incorporated into the synthesized protein was determined by measuring the radioactivity of the material precipitated in hot trichloroacetic acid. The results were recalculated to 50 μl of the reaction volume in pmol. The size of the polypeptides synthesized was determined by electrophoresis through 10 to 25% polyacrylamide gels run in a denaturing buffer [11].

2.4. *RNA decay assay*

The RNAs (75 pmol) labeled with [^{14}C]UTP (10,000 cpm/μg) were incubated in 150 μl of the wheat germ translation mixture at 26°C. 20 μl samples were removed after 0, 5, 10, 20, 40 and 60 min of incubation and diluted with 80 μl of ice-cold 20 mM Tris-HCl buffer, pH 8 with 5 mM KCl and 0.15 M NaCl. The samples were adjusted to 20 mM Tris-HCl buffer, pH 8.0, 2 mM ethylene diamine tetraacetate, 0.5% sodium dodecyl sulfate and extracted with 150 μl of deproteinization mixture consisting of 70 μl phenol, 67.2 μl chloroform and 2.8 μl isoamyl alcohol [12]. The deproteinized samples were precipitated by 70% ethanol with 100 mM ammonium acetate and then analyzed by electrophoresis in 5% polyacrylamide gels in the presence of 8 M urea with subsequent autoradiography [13].

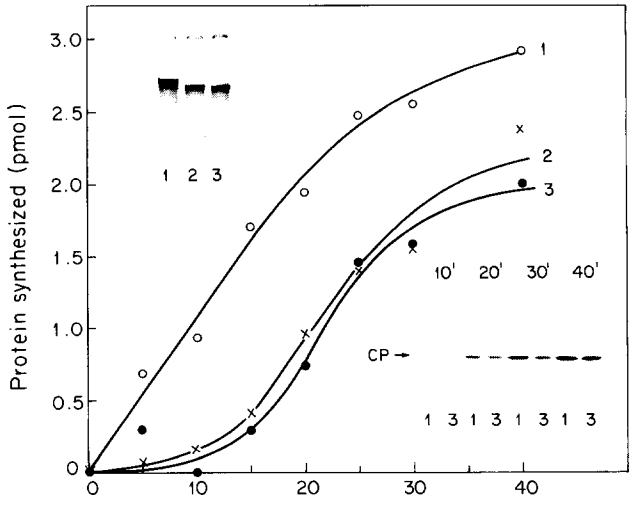

Fig. 2. Time course of protein synthesis in the wheat germ cell-free system programmed with L-UTR RNA (1), M-UTR RNA (2) and S-UTR RNA (3). Top inset: electrophoresis of RNAs differing in 3′-UTR length: (1) L-UTR RNA (tr 964), (2) M-UTR RNA (tr 831) and (3) S-UTR RNA (tr 792). Bottom inset: electrophoretic pattern of the products synthesized in the reactions with L-UTR RNA (1) and S-UTR RNA (3). CP, AlMV coat protein.

3. RESULTS AND DISCUSSION

To test the hypothesis that the 3′-UTR of AlMV RNA 4 may participate in protein synthesis, we compared the efficiency with which the derivatives of the AlMV RNA 4 differing in the 3′-terminal UTR length were translated in a wheat germ cell-free system. Three variants of viral mRNA were produced from plasmid pT7-2-42 containing a full copy of AlMV RNA 4 with additional 17 nucleotides downstream. Transcript 964, 'L-UTR RNA', contained a full-sized (long) 3′-UTR; transcript 831, 'M-UTR RNA', contained a 71% reduced (medium-sized) 3′-terminal UTR; and the transcript 792, 'S-UTR RNA', contained a 93% shortened (short) 3′-terminal UTR. Electrophoretic analysis of the products of RNA synthesis with T7 RNA polymerase has demonstrated that each of the three transcripts has a different mobility and represent the major band in the electrophoretic pattern (see below, Fig. 2, inset at the top). The main attention in this study was focused on the comparison of the L-UTR RNA and the S-UTR RNA, i.e. the transcripts with the full-sized 3′-UTR and virtually without the 3′-UTR, respectively.

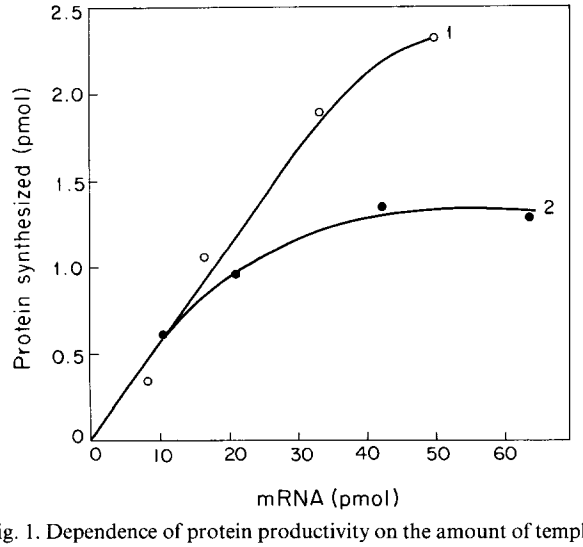

Fig. 1. Dependence of protein productivity on the amount of template RNA. The graph shows the amount of protein synthesized after 20 min of incubation as a function of the amount of the L-UTR RNA (1) and the S-UTR RNA (2) in the wheat germ cell-free system.

The effective use of cell-free translation systems based on the wheat germ extract represented a problem: while the effect on translation of 3′-UTR tails of eukaryotic mRNAs has been observed in vivo, it was absent or weak in a wheat germ S30 cell-free system [14]. In this study the *P100* fraction-based cell-free translation system was chosen as a greater amount of mRNA can be used in this case without the inhibiting effect which was typically observed in the S30 extract-based cell-free translation system. This probably permitted us to increase the translational difference between the full-sized and truncated mRNAs in the wheat germ cell-free system.

It is seen in Fig. 1 that at saturating amounts of mRNA in the incubation mixture (50 pmol) the L-UTR RNA with the full-sized 3′-UTR (reaction 1) provides a 75% higher level of protein synthesis than the truncated S-UTR RNA (reaction 2) in the *P100* fraction-based cell-free system.

The data of Fig. 2 show the amount of the protein synthesized as a function of the incubation time in the

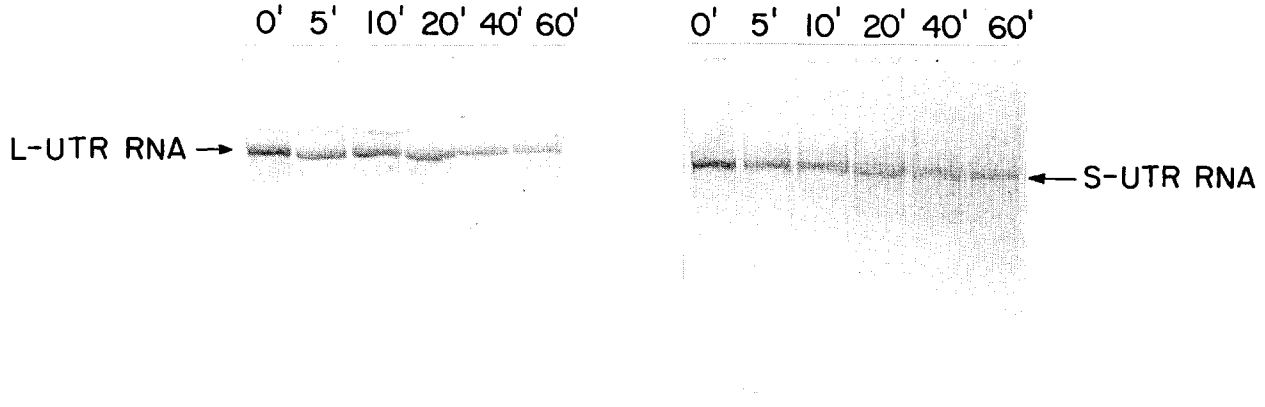

Fig. 3. Time course of the mRNA degradation during translation in the reactions with L-UTR RNA and S-UTR RNA, as revealed by gel electrophoresis and autoradiography.

reactions with the L-UTR RNA (reaction 1), the M-UTR RNA (reaction 2) and the S-UTR RNA (reaction 3). The kinetic curves indicate that there is a lag at the outset of translation in the cases of the shortened AlMV RNA 4. The result was reproduced many times in independent experiments. The lag lasted from 5 to 10 min. No such lag is manifested by the L-UTR RNA containing the full-sized 3'-UTR of the viral RNA 4. The electrophoresis autoradiogram (inset at the bottom of Fig. 2) shows the quality and the yield of the protein synthesized in the reactions 1 and 3. As seen, the product of AlMV RNA 4 translation is a homogeneous polypeptide with a molecular mass corresponding to that of the coat protein of AlMV. The amount of the protein synthesized by each time point was apparently higher in the reaction with the full-sized RNA 4 than with the truncated RNA during all the translation period.

Fig. 3 shows the degradation course of the RNAs differing in the 3'-UTR length as a function of the incubation time in the *P100* fraction-based translation system. The electrophoretic analysis of [^{14}C]RNAs demonstrates that the lag in the translation of the truncated S-UTR RNA was not associated with its preferential degradation. Over the course of a 40-min incubation approximately the same level of degradation was seen in both cases.

Thus, the experiments show that the removal of the 3'-terminal tail from AlMV RNA 4 results in a translational lag at the beginning of the protein synthesis course in the wheat germ cell-free system. This suggests that the presence of the structured 3'-UTR in the viral RNA is important for the viral RNA expression at the initiation phase of translation.

It is possible that the 3'-UTRs of plant virus RNAs take part in the attraction of initiation factors and/or ribosomal subunits and thus facilitate a quick entry of the RNA into the translation process. If this is the case, the 3'-UTR-carrying mRNAs have a preference over other mRNAs in initiation ability, and under in vivo conditions with competition between different mRNAs they should be predominantly involved in the initiation process. The effect of in vivo enhanced translation of the TMV UTR-carrying mRNAs [1–3] can be explained in the same terms.

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