The 5′ untranslated region of cucumber mosaic virus RNA4 confers highly competitive activity on heterologous luciferase mRNA in cell-free translation

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Abstract The cell-free translation of virion RNAs of several tripartite RNA viruses has shown that RNA4, a subgenomic RNA, is more competitive than other virion RNAs. Recently, the 3′ untranslated region (UTR) of alfalfa mosaic virus (AMV) RNA4 was identified to be a competitive determinant. In this study, we observed that the RNA4 of cucumber mosaic virus (CMV), another tripartite RNA virus, was also found to be a strong competitor in translational competition among CMV virion RNAs. To identify the competitive determinant of CMV RNA4, we constructed various chimeric luciferase mRNAs containing RNA4 and/or vector-derived UTRs. The relative translations of luciferase-containing mRNA in the presence of a competitor mRNA showed that the 5′ UTR, not the 3′ UTR, substantially contributed to the highly competitive activity of CMV RNA4.

Key words: Cucumber mosaic virus; Competitive determinant; 5′ Untranslated region; mRNA competition; Translation efficiency

1. Introduction

Competition among various mRNAs for limiting translation components occurs during development [1,2] and virus infection [3,4]. Translational competition has also been observed in cell-free translation of such tripartite plant RNA viruses as brome mosaic virus (BMV) and alfalfa mosaic virus (AMV) [5,6]. These viruses contain three genomic RNAs (RNAs 1, 2 and 3) and one subgenomic RNA (RNA4). The RNA4 encoding virus coat protein (CP) is generated from the 3′ half of the RNA3 during virus RNA replication [7,8]. In BMV and AMV, RNA4 was found to be highly competitive in cell-free translation when translated in high concentration of virion RNAs [5,6]. Recently, Hahn et al. (1997) reported that the 3′ untranslated region (UTR) of AMV RNA4 was important for the highly competitive translational activity [9]. The 3′ UTR of AMV has serial stem-loop structures with a high frequency of AUG triplets. Particularly, the GAUG at 20 nt downstream of translation termination codon was critical in the competitive activity of RNA4 [9]. However, it has not been determined whether the 3′ UTR can confer competitive advantage on heterologous genes.

Cucumber mosaic virus [7] is another representative member of tripartite plant RNA viruses. The 3′ UTRs of CMV and BMV have different RNA features capable of forming tRNA-like structures [7,8] with a relatively low frequency of AUG triplets compared with that of AMV [9]. Therefore, the competitive mechanism of AMV RNA4 cannot be directly applicable to the cases of CMV and BMV RNAs. Since translation initiation is generally the rate-limiting step in protein synthesis, and since some initiation factors, thought to be critical in mRNA discrimination, exist at relatively low concentrations, mRNA with either higher affinity or lower requirement for the discriminatory initiation factor(s) will be more competitive than other mRNAs [10–12]. These proposed mechanisms suggest that 5′ UTRs rather than 3′ UTRs in most mRNAs are more likely to play an important role(s) in mRNA competition for translational components.

In this study, we attempted to determine whether the 5′ UTR and/or 3′ UTR of CMV RNA4 can confer competitive advantage on heterologous luciferase RNA during competitive translation.

2. Materials and methods

2.1. Virus strain and RNA extraction

Kor-CMV (Korean isolate) used in this study was described in the previous paper [13]. Kor-CMV RNAs were extracted with phenol from virus particles and then ethanol precipitated.

2.2. Reverse transcription and polymerase chain reaction (RT-PCR)

The first strand cDNA was synthesized by avian myeloblastosis virus reverse transcriptase (Poscochem., Korea). The reverse transcription reaction was performed at 40°C for 3 h with 10 μg of virion RNAs in a 50-μl reaction volume which contained 500 μM dNTP, 100 pmol of 3′ MS primer [GACA(CCCGGG:*Sma*I)(ACGCGT:*Mlu*I)GGTCTCCTTTTGGAGG], RNasin (0.5 U/μl), reverse transcriptase (1.0 U/μl) and reaction buffer supplied from the manufacturer. Then, the reaction mixture was extracted with phenol/chloroform and precipitated with ethanol. The resulting pellet was dissolved in 20 μl water. Five μl of the obtained cDNA mixture was amplified with Dynazyme (GENOMED). PCR for the amplification of RNA3 cDNA was carried out in a 50-μl reaction volume containing additional 100 pmol of 3′ MS primer, 100 pmol of 3-5′ primer [AGC(GGATCC:*Bam*HI)(TAATACGACTCACTATA:T7 promoter)GTAATCTTACCATCTGTGTGT], 500 μM dNTP, 5 units of Dynazyme and reaction buffer supplied by the manufacturer. PCR was performed in a thermal cycler (Perkin Elmer Cetus) for 20 cycles with each cycle of reaction at 94°C for 1 min, 60°C for 0.5 min and 72°C for 3.5 min. The amplified product was digested with both *Bam*HI and *Sma*I and then subcloned into pUC18. The resulting plasmid was designated as pT7Kor3 (Fig. 2A).

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Abbreviations: CMV, cucumber mosaic virus; UTR, untranslated region; BMV, brome mosaic virus; AMV, alfalfa mosaic virus; PCR, polymerase chain reaction; CP, coat protein; ORF, open reading frame; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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2.3. Plasmid construction

The full-length cDNA of RNA4 was obtained from pT7Kor3 by PCR with 3′ MB primer [GACA(GGATCC:*Bam*HI)(ACGCGT:*Mlu*I)GGTCTCCTTTTGGAGG] and 4-5′ primer [AGC(GGATCC:*Bam*HI)(TAATACGACTCACTATA:T7 promoter)GTTATTGTCTACTGACTATATAGAGAGTT]. The PCR-amplified product was digested with *Bam*HI and then subcloned into pUC18, which is designated here as pT7Kor4 (Fig. 2A). The insert cDNA of pT7Kor3 was ligated into the site between *Bam*HI and *Sac*I of pSELECT-1 (Promega), which results in pSELECTKor3. The insert cDNA of pT7Kor4 was subcloned between *Pst*I and *Mlu*I sites of pSELECTKor3 and given the designation of pSELECTKor4. The pSELECTKor4 was used as template of oligonucleotide-directed mutagenesis for the insertion of *Afl*II site by KOR4-5MUT [GATTCAGATTTGTC CAT)CTTAAG:*Afl*II)ACTCGACTCAATTCT] at the 5′ flanking region and *Apa*I site by KOR34-3MUT [GGGAGGGTTCTGGAA(GGGCCC:*Apa*I)GAATCATACTGGGAG] at the 3′ flanking region of CP open reading frame (ORF). The pSELECTKor3 was used as template for the insertion of *Afl*II site by KOR3-5MUT [ACCTTGGAAAGCCAT(CTTAAG:*Afl*II)CCTCGGGAAATCTAAC] at the 5′ flanking region of 3a ORF, which is located at the 5′ half of RNA3 (Fig. 2A), and *Apa*I site by KOR34-3MUT at the 3′ flanking region of CP ORF. These double mutations were introduced by essentially following the methods of Mutant-Express® Km (Takara, Japan) except that ampicillin repair was used instead of kanamycin. The mutated plasmids were designated as pSELECTKor4MUT and pSELECTKor3MUT, respectively. To construct pT7Kor3MUT, the mutated cDNA insert of pSELECTKor3 was subcloned between *Bam*HI and *Sma*I sites of pUC18. After pSELECTKor4MUT was digested with *Xba*I and *Sac*I, the mutated full-length cDNA clone of RNA4 was transferred to pUC18, which results in pT7Kor4MUT (Fig. 2B). The firefly luciferase ORF was amplified from pSP-LUC$^+$ vector (Promega) by 5′ primer of GCA(CTTAAG:*Afl*II)ATGGAAGACGCCAAAAACAT and 3′ primer GCA(GGGCCC:*Apa*I)AATTACACGGCGATCTTTC, and then directly inserted into pGEM-T vector (Promega) in T7 to SP6 orientation, which results in pGEM-LUC. To construct pT7Kor4MLUC, the luciferase ORF of pGEM-LUC was ligated into the site between *Afl*II and *Apa*I of pT7Kor4-MUT. On the other hand, the luciferase ORF of pGEMLUC was inserted between *Sac*II and *Apa*I sites of pBluescript-KS (Stratagene), which results in pBlueKSLUC. To construct pBlueKSLUCT34, the cDNA of 3′ UTR of RNA4, the same with the 3′ terminal sequence of RNA3, was generated by *Apa*I and *Sma*I digestion of pT7Kor3, and then inserted between *Apa*I and *Kpn*I sites downstream of luciferase ORF of pBlueKSLUC. All the names of the plasmids resulting from experiments in this section are given by the author.

2.4. In vitro transcription and RNA preparation

VL-LUC-VT (Fig. 2C) was generated as capped transcripts by in vitro transcription from *Pvu*II-linearized pBlueKSLUC. L4-LUC and L4-LUC-T34 (Fig. 2C) were generated from *Apa*I-linearized and *Mul*I-linearized pT7Kor4MLUC, respectively. VL-LUC-T34 (Fig. 2C) was generated from *Mlu*I-linearized pBlueKSLUCT34. Kor-CMV RNA3 and RNA4 were generated from *Mlu*-linearized pT7Kor3 and pT7Kor4, respectively. All mRNAs used in the competitive translation were obtained as capped transcripts by mMESSAGEmMACHINE kit (Ambion). After DNaseI treatment, the reaction mixture was precipitated with ammonium acetate and ethanol after phenol/chloroform extraction. The resulting transcripts were passed through Sephadex G50 spun column (Sigma), and then precipitated again with ammonium acetate. The resulting pellet was dissolved in RNase-free water, and then measured by spectrophotometry. The integrity of transcripts was ascertained by agarose gel electrophoresis.

2.5. In vitro translation and luciferase assay

In vitro translation was performed with wheat germ lysate (Promega) basically according to the manufacturer. For radiolabelled protein products, 0.8 mCi/ml of [^{35}S]methionine (>1000 Ci/mmol, Amersham) and 80 μM of amino acids minus methionine were used, while only 80 μM of amino acids mixture complete (Promega) was used for unlabelled translation products. The concentrations of potassium acetate and transcripts used were indicated in the figure legends. Translation mixture containing radiolabelled protein products was analyzed by exposing X-ray film (Fuji) after running 12% SDS-PAGE. The translation level of unlabelled luciferase was indirectly measured with MicroLumat LB 96 P luminometer (EG and G BERTHOLD) by mixing 5 μl of translation reaction mixture with 50 μl of luciferase assay reagent (Promega).

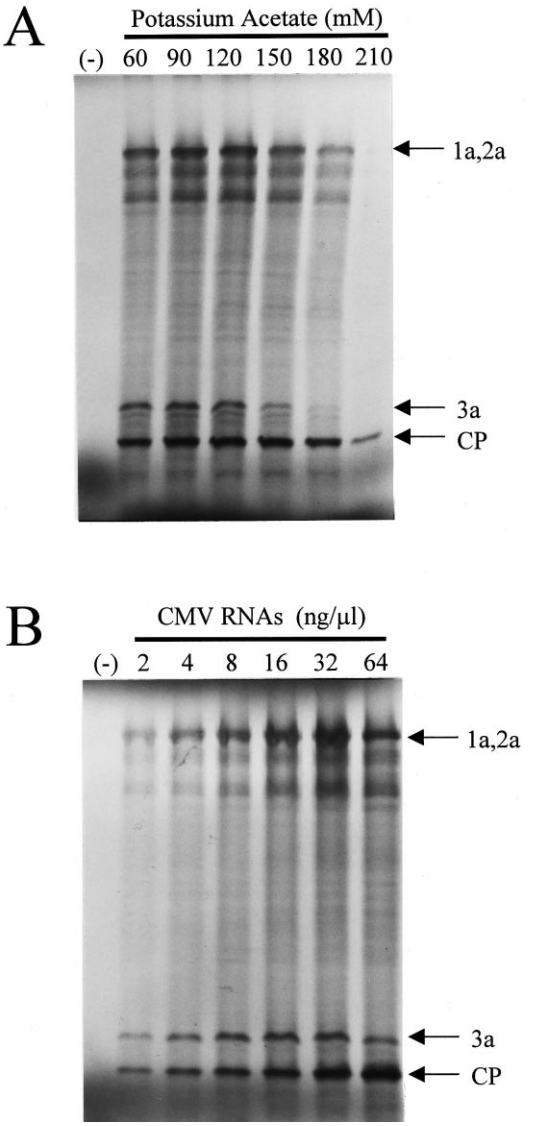

Fig. 1. In vitro translation of CMV virion RNAs. The translation mixture was separated on 12% SDS-PAGE. The corresponding positions of full-length translation products are indicated at the left of the respective panel. The 1a, 2a, 3a and CP were synthesized from CMV RNAs 1, 2, 3 and 4, respectively. A: The same amount of CMV virion RNAs (10 ng/μl) was translated with the indicated concentration of potassium acetate for 60 min at 25°C in wheat germ extracts. (–) means that no RNA was added at the translation reaction in condition of 60 mM potassium acetate. B: The indicated amounts of CMV virion RNAs were translated with 90 mM potassium acetate for 60 min at 25°C in wheat germ extracts. (–) means that no exogenous RNA was added in the translation reaction.

3. Results and discussion

3.1. Translation properties of virion RNAs

CMV RNAs contain RNAs 1, 2, 3 and 4. Since ionic strength affects in vitro translation [14], we investigated the optimal concentration of potassium acetate in wheat germ extract (Fig. 1A). The translation products from virion RNAs appeared at three positions in 12% SDS-PAGE. The

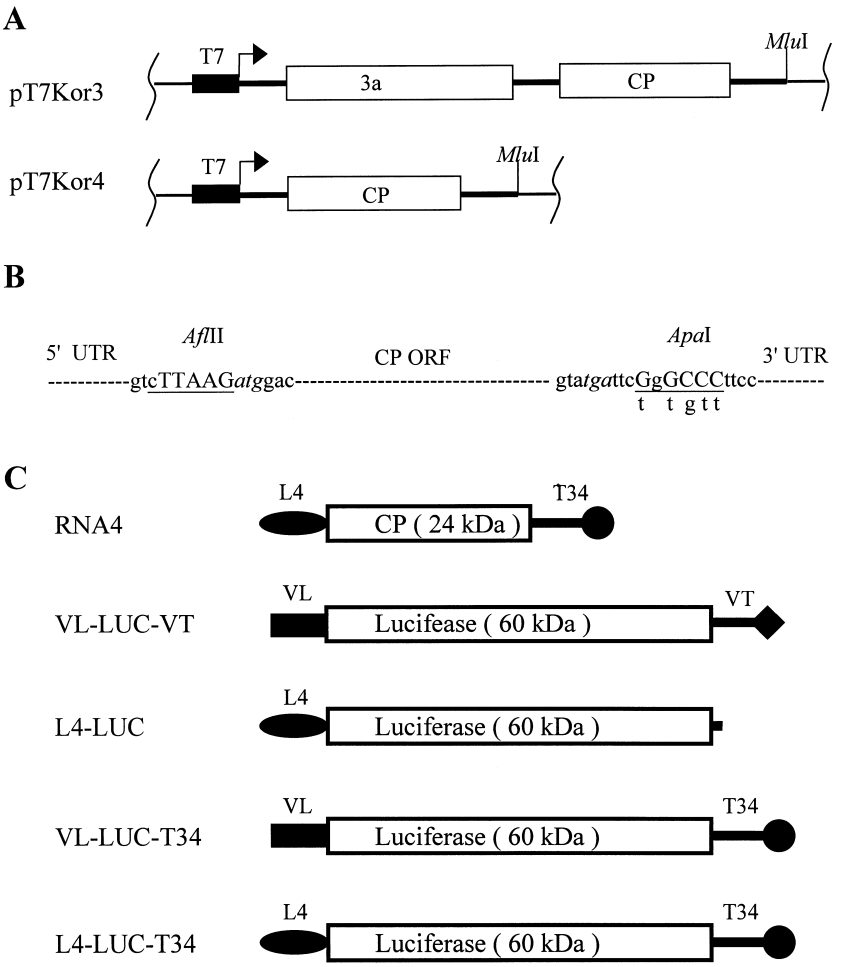

Fig. 2. Schematic diagram of plasmid and chimeric mRNA constructs. A: Both pT7Kor3 and pT7Kor4 contain bacteriophage T7 promoters, which is indicated with T7, to initiate transcription at the first nucleotide of the respective virus RNA. B: The corresponding sense DNA sequence of RNA4 is indicated with small letters, while the mutated sequence from RNA4 is represented by capital letters. The translation initiation and termination sites are italicized as *atg* and *tga*, respectively. C: Schematic diagram of RNA4 and chimeric mRNA constructs. L4 and VL mean RNA4-derived 5′ UTR and vector-derived 5′ UTR, respectively. T34 and VT mean RNA4-derived 3′ UTR and vector-derived 3′ UTR (about 230 nt), respectively.

1a (110 kDa) and 2a (97 kDa) proteins from RNAs 1 and 2 were not separated from each other, and they appeared to be one band in 12% SDS-PAGE. The three protein bands of translation products reached the maximum around 90–120 mM potassium acetate and decreased gradually with the increase of salt concentration (Fig. 1A). Interestingly, the translation product from RNA3 gradually decreased above 120 mM and was radically reduced at 180 mM, while that from RNA4 was slightly reduced even at 180 mM (Fig. 1A).

A highly competitive environment can be easily achieved by increasing the concentration of mRNA mixture in cell-free translation. When the concentration of virion RNAs was gradually increased up to 64 ng/μl, the only protein band that also increased up to 64 ng/μl was the translation product from RNA4 (Fig. 1B). The translation product from RNA3 seemed to reach a plateau around 16 ng/μl and decreased evidently in the highly competitive environment (64 ng/μl) of virion RNAs (Fig. 1B). The result indicates that CMV RNA4 is a strong competitor and that CMV RNA3 is a weak one.

It has been reported that the translation efficiency of a strong competitor does not decrease radically in high salt concentration, and that of a weak competitor decreases rapidly in high salt concentration in cell-free translation [15,16]. These observations coincide with our result; CMV RNA4 showing good translation at high ionic strength is a strong competitor, while CMV RNA3 of poor translation at high ionic strength is a weak competitor (Fig. 1A and B).

3.2. Full-length cDNA clone, RNA3, RNA4 and chimeric luciferase transcripts

For the detailed investigation of the highly competitive activity of CMV RNA4, we obtained a full-length RNA4 cDNA clone by PCR from the RNA3 cDNA that had been cloned by RT-PCR from virion RNAs. The complete sequence of Kor-CMV RNA3 had been determined previously from several cDNA partial clones [13], so that we could design PCR primers for the correct 5′ and 3′ termini of Kor-CMV RNA3 and RNA4. PCR primers for the amplification of full-length cDNAs of RNA3 and RNA4 were also designed to contain a T7 promoter sequence in 5′ primers and a unique *Mlu*I sequence in 3′ primers (see Section 2). Thus, the transcripts corresponding to RNA3 and RNA4 were obtained by in vitro transcription from *Mlu*I-linearized DNA templates of pT7kor3 and pT7Kor4, respectively (Fig. 2A).

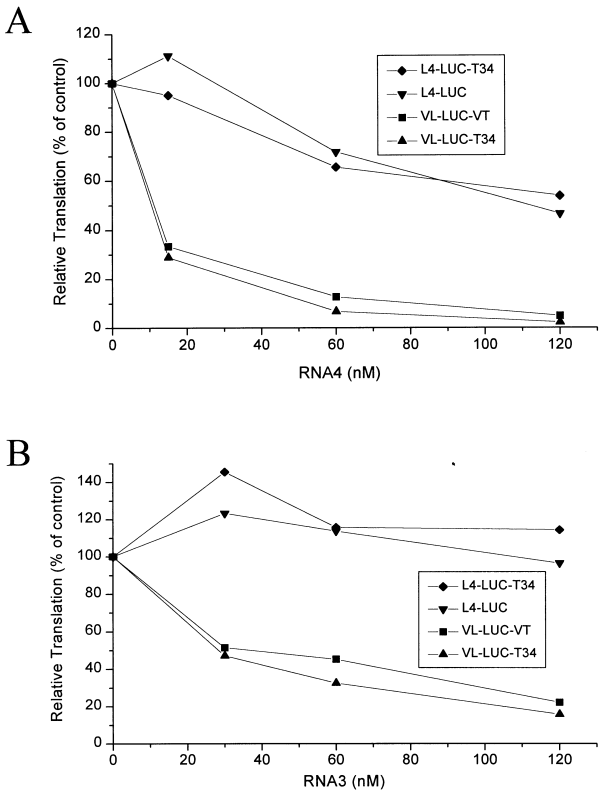

Fig. 3. Competitive translation of RNA4 (or RNA3) and respective chimeric mRNA construct containing luciferase ORF. The respective luciferase mRNA construct (3.75 nM) was translated in the presence of RNA4 (A) or RNA3 (B) with its amount increasing. The translation level chimeric luciferase mRNA in the absence of competitor mRNA was normalized to 100%. Translation was performed at 25°C with 90 mM potassium acetate for 60 min in wheat germ extract.

To replace CP open reading frame (ORF) with luciferase ORF, we inserted an *Afl*II site upstream of the translation initiation codon and an *Apa*I site downstream of the translation termination codon of RNA4 (Fig. 2B). From the insertion of the *Afl*II site, the original Kozak context [17,18] was changed from GUCA(+1)UGG to AAGA(+1)UGG, but the sequence at position −3, which should be purine for efficient translation, was maintained (guanine to adenine).

In general, mRNA can be divided into three functional regions corresponding to 5′ UTR, coding region and 3′ UTR. If any part of these functional regions could make RNA4 highly competitive, it would be possible to confer competitive advantage on heterologous RNA by transferring the unique RNA feature. Therefore, we designed four luciferase-containing mRNA constructs with or without and/or vector-derived UTR(s) (Fig. 2C). The L4-LUC transcript devoid of 3′ UTR could be simply obtained from *Apa*I-linearized template of pT7KorMLUC containing the 3′ UTR cDNA of RNA4 (Fig. 2B and C).

3.3. Competitive determinant of RNA4

The competitive activity of a test mRNA can be measured by monitoring the relative translation levels in cell-free translation on different concentrations of other mRNA, as a competitor [15,19]. The concentration of the test mRNA should be under the half-saturated condition of maximum protein synthesis in competitive translation [15]. In this study, the luciferase-containing mRNA constructs were used at a concentration of 3.75 nM, corresponding to 0.075 pmol or about 50 ng in 20 μl reaction volume, and this concentration was well under the half-saturated condition of maximum protein synthesis (data not shown).

We measured the translation levels of the four RNA4-derived constructs containing luciferase ORF in the presence of CMV RNA4, a strong competitor (Fig. 3A). The mRNA constructs (L4-LUC-T34 and L4-LUC) with the 5′ UTR and RNA4, regardless of the presence of 3′ UTR, showed a gradual decrease of translation in the competitive environment. On the other hand, the mRNA constructs (VL-LUC-VT and VL-LUC-T34) with vector-derived 5′ UTR were inhibited very sharply with the increase of RNA4 (Fig. 3A). To exclude any possibility of unknown effect(s) by RNA4-encoded CP and to reconfirm the strong competitive abilities of chimeric luciferase mRNAs containing the 5′ UTR of RNA4, we titrated the translations of the four mRNA constructs again with RNA3 (Fig. 3B), a weak competitor (Fig. 1B). The result showed that the translation levels of mRNA constructs (L4-LUC-T34 and L4-LUC) containing the 5′ UTR of RNA4 did not decrease in broad concentrations of RNA3, while the translation levels of mRNA constructs (VL-LUC-VT and VL-LUC-T34) containing vector-derived 5′ UTR gradually decreased (Fig. 3B). The previously proposed structure of the 5′ UTR of CMV RNA4 [20] might be involved with the degree of an affinity and/or with the requirement for a discriminatory initiation factor(s) that is thought to play an important role(s) in mRNA discrimination during competitive translation.

The translation of a test mRNA may be inhibited in various degrees according to the competitive activity of added other mRNA. The higher the competitive activity of the added mRNA, the higher the inhibition of translation of the test mRNA [15,19]. Our result is that RNA4, rather than RNA3, intensely inhibited the translation of chimeric luciferase mRNA (Fig. 3A and B). This confirms that RNA4 is a strong competitor while RNA3 is a weak one. Likewise, if the competitive activity of the added mRNA is sufficiently weaker than that of the test mRNA, the translation level of the test mRNA may not be reduced significantly [15,19]. It is also coincidental with our result that the translation level of chimeric luciferase mRNAs containing RNA4-derived 5′ UTR did not decrease in the presence of RNA3, a weak competitor (Fig. 3B).

These results indicate that the 5′ UTR, not the 3′ UTR, substantially contributes to the highly competitive activity of CMV RNA4. Moreover, the 5′ UTR alone was shown to confer highly competitive activity on heterologous luciferase RNA in mRNA competition. The interesting feature of the 5′ UTR of CMV RNA4 might be used to express heterologous gene at highly competitive environment, e.g. *Xenopus* oocytes, as well as to elucidate mechanism(s) of mRNA competition.

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