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A Single Nucleotide Insertion in the 5′-Untranslated Region of Hepatitis C Virus Leads to Enhanced Cap-Independent Translation

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The 5′-untranslated region (5′-UTR) of hepatitis C virus (HCV) contains an internal ribosome entry site (IRES) that directs translation of the viral open reading frame (ORF). The 5′-UTR consists of 341 nucleotides (nt) in most strains, and multiple segments within this region are important for its IRES activity. Sequencing analysis of a full-length HCV cDNA clone derived from a Japanese HCV1b-positive patient showed the 5′-UTR was 342 nt long due to a nucleotide T insertion at position 207. The influence of this T insertion on the IRES activity in directing cap-independent translation was investigated. The IRES of the 5′-UTR342 was approximately five- and two- to sevenfold more active in directing luciferase expression in monocistronic and bicistronic expression systems, respectively, when compared with the IRES of the 5′-UTR341 of a previously reported HCV1b strain. In addition to the T insertion, another point mutation involving an A to C transition at position 119 was also present in the 5′-UTR342. Simultaneous comparison of the IRES activities in engineered constructs that contained each of the two mutations indicated that the insertion at position 207 is responsible for the enhanced IRES activity of the 5′-UTR342. Further determination of the abilities of the engineered 5′-UTRs harbouring A, G, or C insertions at the same position to initiate translation indicated that both T and non-T nucleotide insertions lead to enhanced cap-independent translation.

INTRODUCTION

Hepatitis C virus (HCV) is the major etiological agent of parenterally transmitted non-A, non-B hepatitis (Choo et al., 1989). The genome of HCV is a single-stranded, positive-sense RNA of ~9500 nucleotides (nt) that contains a single open reading frame (ORF) encoding a polyprotein of 3008–3037 amino acids (Clarke, 1997). Although the coding region of HCV RNA shows significant heterogeneity among different isolates (Bukh *et al.,* 1995), the 5′-untranslated region (5′-UTR) is highly conserved, indicating an important role for it in HCV RNA replication and/or translation. Since an internal ribosome entry site (IRES) in the 5′-UTR was first identified by Tsukiyama-Kohara *et al.* in 1992 (Tsukiyama-Kohara *et al.,* 1992), increasing evidence has demonstrated that initiation of translation of HCV is directed by a cap-independent manner analogous to that seen in picornaviruses.

Sequence analysis of a full-length HCV cDNA clone derived from a Japanese clinical specimen (unpublished data) showed that the 5′-UTR was 342 nucleotides long due to a nucleotide T insertion at position 207 (5′-UTR342), rather than 341 nucleotides in length, which is generally found in reported isolates. In view of its critical position, the influence of this T insertion on the ability of the 5′-UTR in directing cap-independent translation was investigated. Here we report that the IRES of the 5′-UTR342, which contains another mutation (A to C transition at position 119) in addition to the T insertion when compared with that of another reported HCV1b isolate (Tang *et al.,* 1994; Accession Number: D00832), has increased activity in directing cap-independent translation. By engineering the appropriate constructs containing each of the two mutations, we show that the increased IRES activity is solely mediated by the T insertion at position 207. Further determination of the IRES activity of the mutated 5′-UTRs harbouring A, G, or C insertions indicate that both T and non-T nucleotide insertions lead to enhanced cap-independent translation.

RESULTS

Distinct nucleotide sequence in the 5′-UTR of a HCV cDNA clone

A full-length cDNA clone of HCV was successfully constructed from a Japanese HCV1b-positive specimen (unpublished data). Sequence analysis of this clone revealed that the 5′-UTR was 342 nt long due to a T

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A

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                1
5'-UTR 341 GCCAGCCCCC TGATGGGGGC GACACTCCAC CATAGATCAC TCCCCTGTGA 50
5'-UTR 342 GCCAGCCCCC TGATGGGGGC GACACTCCAC CATAGATCAC TCCCCTGTGA

               51
5'-UTR 341 GGAACTACTG TCTTCACGCA GAAAGCGTCT AGCCATGGCG TTAGTATGAG 100
5'-UTR 342 GGAACTACTG TCTTCACGCA GAAAGCGTCT AGCCATGGCG TTAGTATGAG

              101
5'-UTR 341 TGTCGTGCAG CCTCCAGGAC CCCCCCTCCC GGGAGAGCCA TAGTGGTCTG 150
5'-UTR 342 TGTCGTGCAG CCTCCAGGCC CCCCCCTCCC GGGAGAGCCA TAGTGGTCTG

              151
5'-UTR 341 CGGAACCGGT GAGTACACCG GAATTGCCAG GACGACCGGG TCCTTTCTTG 200
5'-UTR 342 CGGAACCGGT GAGTACACCG GAATTGCCAG GACGACCGGG TCCTTTCTTG

              201
5'-UTR 341 GATCAAACCC GCTCAATGCC TGGAGATTTG GGCGTGCCCC CGCGAGACTG 250
5'-UTR 342 GATCAAACCC GCTCAATGCC TGGAGATTTG GGCGTGCCCC CGCGAGACTG

              251
5'-UTR 341 CTAGCCGAGT AGTGTTGGGT CGCGAAAGGC CTTGTGGTAC TGCCTGATAG 300
5'-UTR 342 CTAGCCGAGT AGTGTTGGGT CGCGAAAGGC CTTGTGGTAC TGCCTGATAG

              301
5'-UTR 341 GGTGCTTGCG AGTGCCCCGG GAGGTCTCGT AGACCGTGCA CC 342
5'-UTR 342 GGTGCTTGCG AGTGCCCCGG GAGGTCTCGT AGACCGTGCA CC
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B

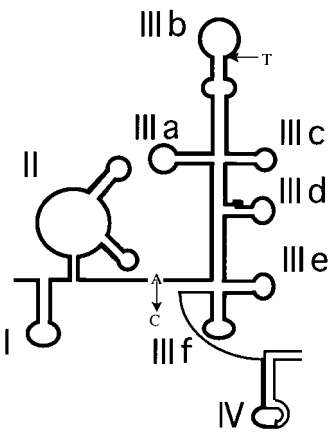


FIG. 1. (A) Alignment of the HCV 5′-UTR341 and the 5′-UTR342 sequences. Nucleotide differences between the 5′-UTR341 and the 5′-UTR342 are indicated. (B) Scheme of the secondary structure of the IRES of HCV, showing the locations of the two mutations which distinguish the IRES of the 5′-UTR342 from that of the 5′-UTR341. The secondary structure prediction and loop numbering are from Brown *et al.* (1992) and Honda *et al,* (1996).

insertion at position 207 that has not been reported previously. We analyzed 10 additional clones derived from three independent PCR reactions and found this T insertion in all 10 analyzed clones, suggesting that it was not artificially produced by the PCR procedure (data not shown). In addition to this T insertion, there was another mutation involving an A to C transition at position 119 compared with another HCV1b strain that has been described elsewhere (Tang *et al.,* 1994; Collier *et al.,* 1998) (Fig. 1).

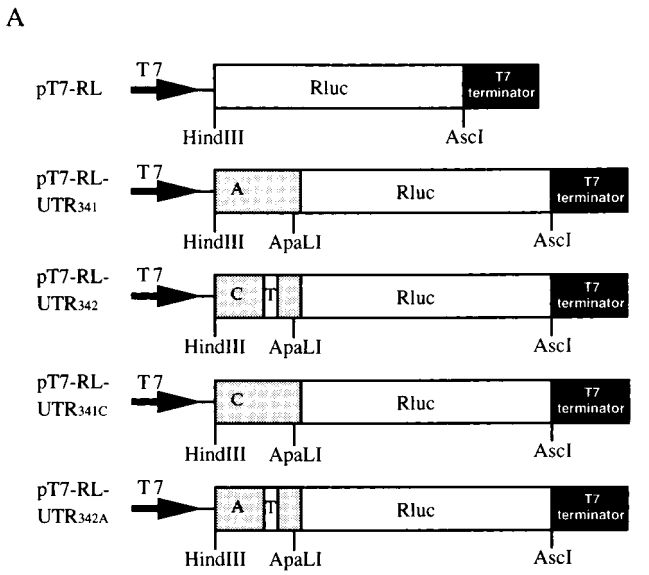
Enhanced translational efficiency of the 5′-UTR342 over 5′-UTR341 in monocistronic RNAs

Because the T insertion is close to the polypyrimidine tract, its influence on IRES activity was investigated. We compared the abilities of 5′-UTR342 and 5′-UTR341 in directing cap-independent translation. Because the position of HCV IRES relative to the initiation codon is critical for its activity (Jackson *et al.,* 1995), we designed the monocistronic test constructs by connecting a Renilla luciferase (Rluc) reporter gene directly to the initiating ATG to mimic the native HCV RNA (Fig. 2A). Varying amounts of these RNAs were *in vitro* translated in rabbit reticulocyte lysates in the presence of 120 mM KCl, which is the physiological salt concentration and allows HCV RNA translation in an IRES-dependent manner. The translation products were examined by luciferase assay. As shown in Fig. 2B, the Rluc activity of the translation product proportionally increased with the amounts of RNA templates and at all different amounts of RNA used in translation, the Rluc activity of T7-RL-UTR342 was consistently about fourfold higher than that of T7-RL-UTR341.

Given the fact that there was another nucleotide substitution (A to C) in addition to the T insertion in 5′-UTR342 when comparing with the sequence in 5′-UTR341, transcripts from two site-directed mutant constructs pT7-RL-UTR341C and pT7-RL-UTR342A, each harboring one of the mutations present in the 5′-UTR342 (Fig. 2A), were *in vitro* translated simultaneously. The translation level of Rluc from T7-RL-UTR342A was similar to that from T7-RL-UTR342, and Rluc from T7-RL-UTR341C was comparable to that from T7-RL-UTR341 (Fig. 2B). This result indicates that the T insertion at position 207 is the main contributor to the increased IRES activity, whereas the A to C substitution at position 119 has little, if any, effect on IRES-directed translation.

To investigate whether the translational enhancement by the single nucleotide insertion in the 5′-UTR342 of HCV was due to stabilization of mRNAs, we monitored the stability of these RNAs in reticulocyte lysates during *in vitro* translation, by primer extension study using a primer complementary to the 5′-UTR sequence. Primer extension was performed under the condition in which the primer-extended products reflected the amounts of RNA in a linear relationship within the range of RNAs amounts used (Ito *et al.,* 1998). Figure 3 showed that the kinetics of mRNA degradation were similar for T7-RL-UTR341 and T7-RL-UTR342, indicating that these two RNAs had comparable stabilities and that the translational enhancement effect by the single nucleotide insertion in the 5′-UTR342 was not due to RNA stabilization by this insertion.

To explore whether the enhanced translation activity of the 5′-UTR342 over 5′-UTR341 also was seen in intact cells, the monocistronic vector DNAs were transfected into HepT cells stably expressing the T7 RNA polymerase, Rluc activities in the lysates were determined 48 h after transfection. Consistent with the results from *in vitro* translation, the corrected Rluc activity from the pT7-RL-UTR342 transfected HepT cells was found to be six- to sevenfold higher than that from the pT7-RL-UTR341 and pT7-RL-UTR341C transfected HepT cells, while the Rluc activity from the pT7-RL-UTR342A transfectants was



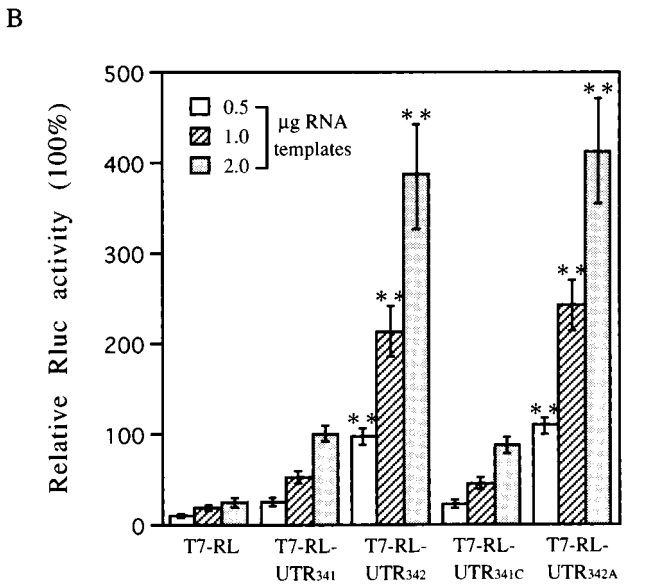


FIG. 2. Relative abilities of different 5′-UTRs to direct translation of monocistronic mRNAs *in vitro*. (A) Schematic diagrams of pT7-RL and its related plasmids used. Name and relevant features of the plasmids are indicated. pT7-RL contains the T7 promoter (large arrow), Rluc reporter genes (open box) and T7 terminator (closed box). pT7-RL-UTRs additionally contain their respective 5′-UTRs (shaded boxes) between T7 and Rluc. Restriction enzyme cleavage sites used for DNA manipulation are indicated underneath. (B) Relative Rluc activities of the translation products from various RNAs. Different amounts (0.5, 1, and 2 μg) of each RNA transcript were used in *in vitro* translation. Rluc activity of the translation product from 2 μg of T7-RL-UTR341 is arbitrarily taken as 100% and the Rluc activities of other RNAs are normalized to this. The columns and bars represent the means and standard deviations of two sets of triplicate studies. The asterisks indicate that the differences in translational level of these RNAs compared to that of the corresponding amount of T7-RL-UTR341 RNA are significant. $^{**}P < 0.01$.

comparable to that from the pT7-RL-UTR342 transfectants (Fig. 4). The Rluc activity from pT7-RL transfectants was extremely low, presumably due to inefficient initiation of cap-independent translation. Thus 5′-UTR342 mediates enhanced translation of monocistronic RNAs both *in vitro* and *in vivo*.

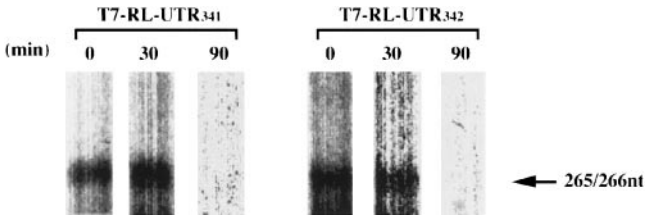


FIG. 3. RNA stability of T7-RL-UTR341 (left) and T7-RL-UTR342 (right) RNA. Two micrograms of each RNA were mixed with rabbit reticulocyte lysates. At 0, 30, and 90 min after *in vitro* translation, RNAs were harvested, and one-half of them were used as the templates for primer extension experiments using a 5′-UTR primer. Arrow indicates the primer-extended products (265 nt in length for T7-RL-UTR341 and 266 nt for T7-RL-UTR342 RNA, respectively), which correspond to the intact 5′ end of HCV RNA.

Increased IRES activity of the 5′-UTR342 compared with that of 5′-UTR341 in bicistronic RNAs

To confirm the results obtained with the monocistronic test system, we further compared the IRES activity of the 5′-UTR342 with that of the 5′-UTR341 in bicistronic constructs which contain the SV40 promoter followed by firefly luciferase (Fluc) reporter gene, the HCV 5′-UTR sequence preceding the Rluc reporter gene, and the SV40 late poly(A) signal (Fig. 5A). Four kinds of bicistronic vectors pSVGLRUTRs and the control vector pSVGLR, which lacks 5′-UTR between the two reporter cistrons, were transfected into COS-1 cells. The Fluc activity expressed from the upstream cistron was mea-

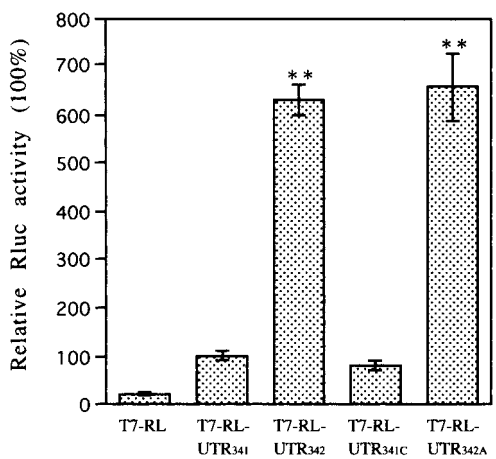


FIG. 4. Relative translational activities of the monocistronic mRNAs from different 5′-UTRs *in vivo*. Plasmids depicted in Fig.2A were transfected into HepT cells stably expressing T7 RNA polymerase. Relative Rluc activities in the lysates were determined at 48 h posttransfection. The columns and bars represent the means and standard deviations of two independent triplicate transfections. $^{**}P < 0.01$ compared with T7-RL-UTR341.

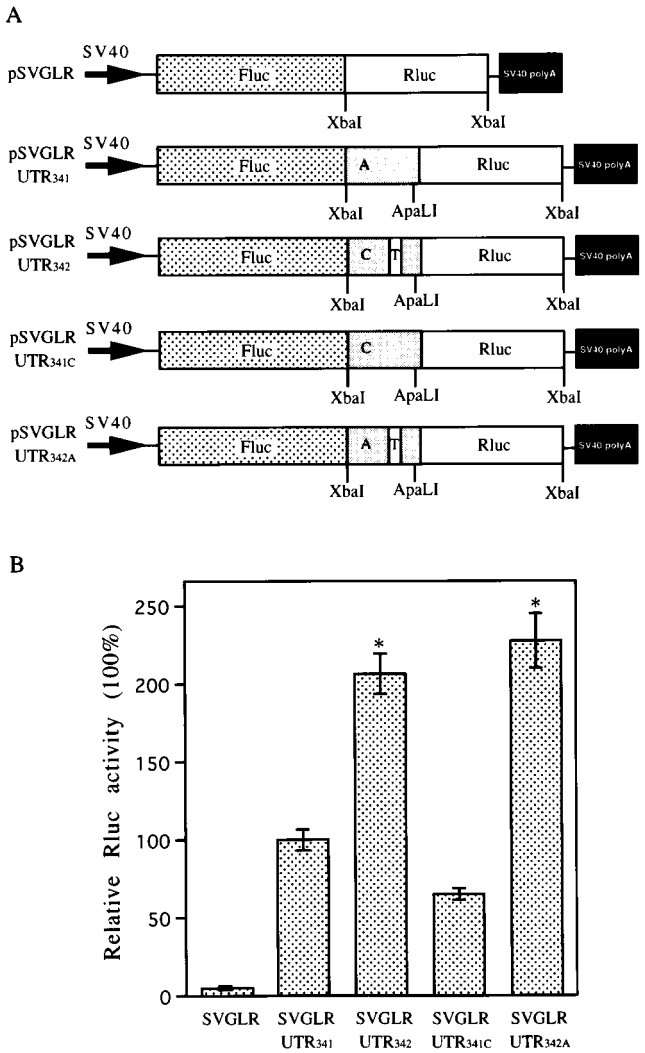


FIG. 5. Relative IRES activities of different 5′-UTRs in capped bicistronic RNAs. (A) Schematic diagrams of plasmids used. pSVGLR contains an SV40 promoter (large arrow), Fluc reporter gene (dotted box), Rluc reporter gene (open box), and SV40 late poly (A) (closed box). pSVGLRUTRs additionally contain the corresponding 5′-UTRs (shaded boxes) between the two luciferase reporter genes. (B) Plasmids were transfected into COS-1 cells. Relative Rluc activities in the lysates were determined at 48 h posttransfection. The columns and bars represent the means and standard deviations of three independent triplicate transfections. *$P < 0.05$ compared with SVGLRUTR341.

sured to normalize differences in the efficiencies of the transfections. As shown in Fig. 5B, expression of the second cistron (Rluc) from the control vector pSVGLR was negligible, and each inserted 5′-UTR significantly stimulated expression of this downstream reporter gene. The expression level of Rluc directed from the IRES of 5′-UTR342 was higher than that from the IRESs of 5′-UTR341 and 5′-UTR341C by approximately twofold, similar to the results from the monocistronic system although the level of enhancement was lower. Also, the Rluc activity from pSVGLRUTR342A was comparable to that from pSVGLRUTR342, confirming that the T insertion at position 207 is responsible for the enhanced IRES activity of 5′-UTR342. This result indicates that the enhanced IRES activity of 5′-UTR342 over 5′-UTR341 is not restricted to hepatocyte-derived cells and further suggests that factor(s) involved in increased IRES activity observed here is not cell-type specific.

In view that the bicistronic RNAs described above differ from the monocistronic RNAs in having a cap structure at the 5′ end and a poly(A) tail at the 3′ end, the less degrees of enhancement by the T insertion in pSVGLRUTRs constructs, relative to the monocistronic constructs pT7-RL-UTRs, may be due to the influence of 5′-cap and 3′-poly(A) on the IRES-dependent translation. To explore this possibility, we reconstructed the bicistronic constructs pT7GLRUTRs, which contain T7 promoter and terminator instead of the SV40 promoter and poly(A) in pSVGLRUTRs (Fig. 6A), and transfected them into HepT cells. Rluc activities in the lysates were determined 48 h after transfection. As shown in Fig. 6B, the translation efficiencies of the downstream cistron (Rluc) of pT7GLRUTR342 and pT7GLRUTR342A were found to be approximately sevenfold higher than those of pT7GLRUTR341 and pT7GLRUTR341C, being fully consistent with the results obtained with the monocistronic constructs pT7-RL-UTRs. Thus the T insertion in 5′-UTR342 significantly enhances the cap-independent translation efficiency in both monocistronic and bicistronic systems.

Effect of insertions with different nucleotides on the IRES-directed translational efficiency

To investigate whether insertions with other nucleotides (A, G, or C) have the same cap-independent translational enhancement as the T insertion, we constructed the monocistronic vectors pT7-RL-UTR342(207A), pT7-RL-UTR342(207G), and pT7-RL-UTR342(207C) which contain A, G, and C insertion at position 207, respectively (Fig. 7A). By transient transfection of these constructs into the HepT cells, the activities of these mutated 5′-UTRs in directing cap-independent Rluc expression were compared with that of the naturally variant 5′-UTR342(207T). As shown in Fig. 7B, the Rluc activities of the HepT cells transfected with pT7-RL-UTR342(207A), pT7-RL-UTR342(207G), and pT7-RL-UTR342(207C) were approximately four-, four- and sixfold higher than that of pT7-RL-UTR341 respectively, and the Rluc activity of the pT7-RL-UTR342(207T) transfectant was consistently about sevenfold higher than that of pT7-RL-UTR341. Thus, both the T and non-T nucleotide insertions (A, G, or C) at position 207 of 5′-UTR enhance the cap-independent translation although the degrees of enhancement are different.

DISCUSSION

In this study, we have shown that a single nucleotide insertion in the 5′-UTR of HCV derived from a Japanese

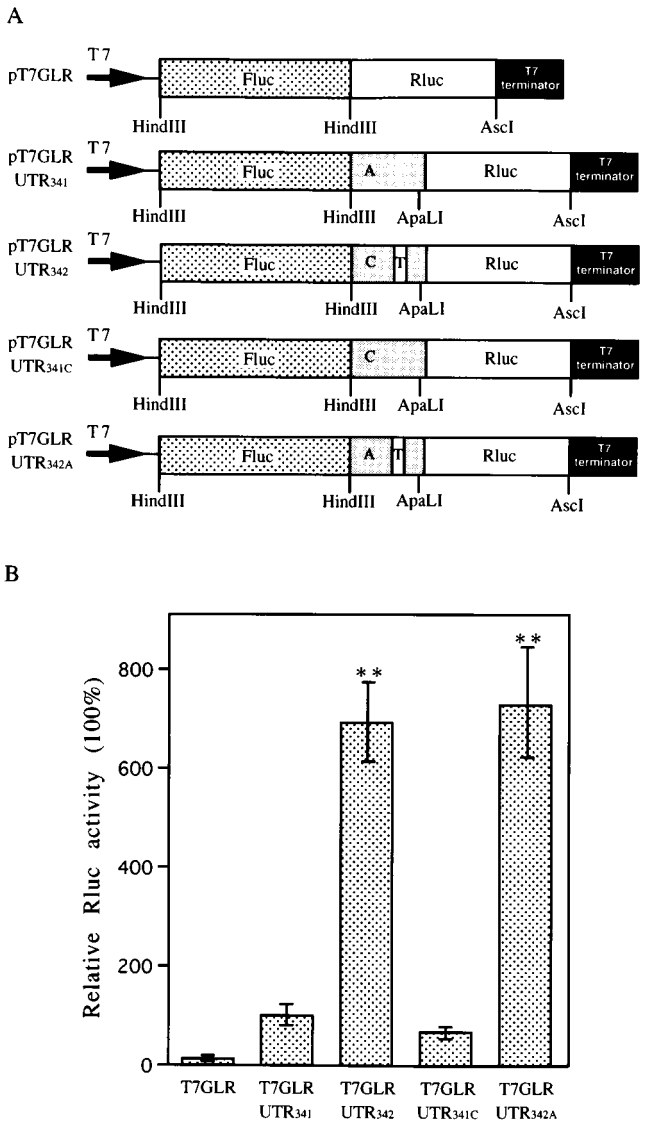


FIG. 6. Relative IRES activities of different 5′-UTRs in uncapped bicistronic RNAs. (A) Schematic diagrams of plasmids used. pT7GLR contains a T7 promoter (large arrow), Fluc reporter gene (dotted box), Rluc reporter gene (open box), and T7 terminator (closed box). pT7GLRUTRs additionally contain the corresponding 5′-UTRs (shaded boxes) between the two luciferase reporter genes. (B) Plasmids were transfected into HepT cells. Relative Rluc activities in the lysates were determined at 48 h posttransfection. The columns and bars represent the means and standard deviations of three independent triplicate transfections. $^{**}P < 0.01$ compared with T7GLRUTR341.

clinical specimen induced an enhanced cap-independent gene expression in monocistronic and bicistronic expression systems. This enhancement was not due to RNA stabilization by the single nucleotide insertion. There are several examples demonstrating that single mutation can alter IRES function. A single substitution in the FMDV-IRES enhanced the degree of internal ribosome entry by 1.5- to 5-fold (Martinez-Salas *et al.,* 1993) and a point mutation of the IRES element in the c-myc 5′-UTR derived from patients with multiple myeloma exhibited increased expression of c-myc protein (Paulin *et al.,* 1996, 1998; Stoneley *et al.,* 1998).

Translation is one of the processes in viral replication and the contribution of IRES activity to viral replication ability has been reported for poliovirus (Kawamura *et al.,* 1989). Therefore, the high viremia (10^9 copies/ml plasma) of the subject from whom the HCV isolate containing the 5′-UTR342 was derived can be explained, at least partially, by the enhanced IRES activity of the 5′-UTR342.

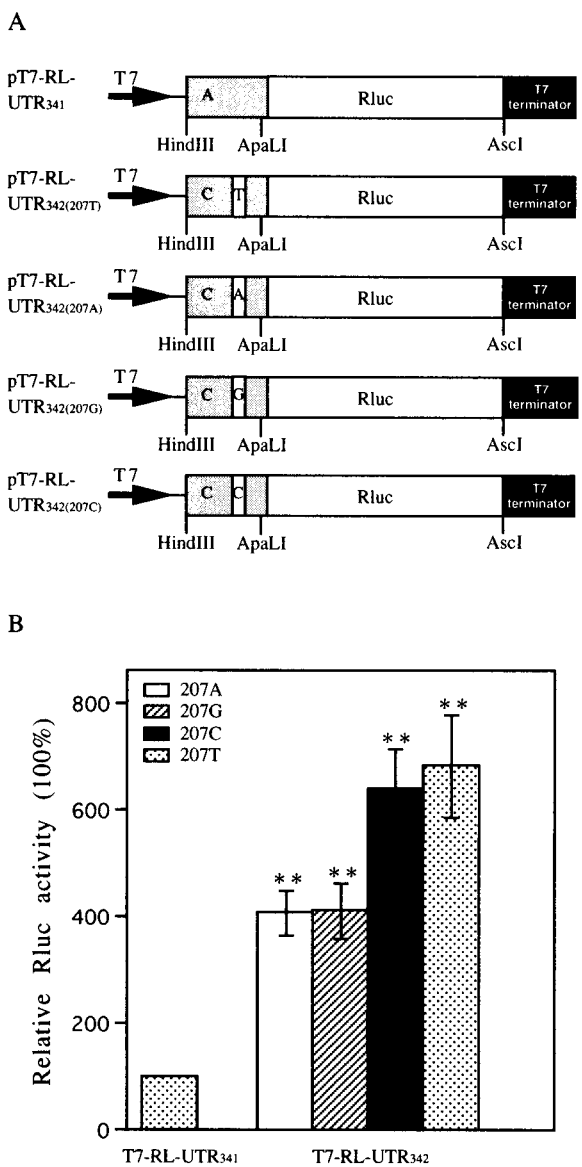


FIG. 7. Effect of insertions with different nucleotides in the 5′-UTR on cap-independent translational activity. (A) Schematic diagrams of plasmids used. Name and relevant features of the plasmids are indicated. Instead of the T insertion in 5′-UTR342(207T), pT7-RL-UTR342(207A), pT7-RL-UTR342(207G), and pT7-RL-UTR342(207C) contain the 5′-UTRs with the insertions of A, G, and C, respectively. (B) Plasmids were transfected into HepT cells. Relative Rluc activities in the lysates were determined at 48 h posttransfection. The columns and bars represent the means and standard deviations of two independent triplicate transfections. $^{**}P < 0.01$ compared with T7-RL-UTR341.

It is still the subject of much debate whether the core sequence of HCV is absolutely required for its IRES activity (Tsukiyama-Kohara *et al.,* 1992; Wang *et al.,* 1993; Reynolds *et al.,* 1995). A general consideration is that the core sequence that constitutes part of the domain IV structure is not obligatory for translation but may be necessary to modulate translation during virus replication. In the present study, we observed efficient expression of the reporter genes fused directly to the initiator AUG, consistent with the observation reported by Tsukiyama-Kohara *et al.,* (1992) and Wang *et al.,* (1993),. Because we do not have any data regarding the effect of T insertion on the translational efficiency of the IRES element containing core sequence, the trivial possibility that inclusion of the core sequence affect the translational enhancement by this T insertion can not be ruled out completely.

The fact that the bicistronic transcript of pSVGLRUTRs differs from that of pT7GLRUTRs in having a cap structure at the 5′ end and a poly (A) tail at the 3′ end may contribute to the difference in the degree of enhancement between pSVGLRUTRs (twofold) and pT7GLRUTRs (sevenfold). Additionally, it was reported that relative IRES activity is different in cell types used for transfection due to various distribution of host factors involved in cap-independent translation initiation (Kamoshita *et al.,* 1997; Collier *et al.,* 1998). This may also partially explain this observed difference in the magnitude of enhancement between these two bicistronic reporter systems in which different cell lines, COS-1 and HepG2, were used.

The molecular basis for the increase in IRES activity mediated by the single nucleotide insertion is not known, but the inserted residue is within the putative binding site of pyrimidine tract binding protein (PTB) (Ali and Siddiqui, 1995) which was thought to be a translation factor (Hellen *et al.,* 1993; Witherell *et al.,* 1993; Hellen *et al.,* 1994; Witherell and Wimmer, 1994) although whether PTB is essential for internal initiation of translation of HCV virus RNA is still controversial (Kaminski *et al.,*1995; Reynolds *et al.,* 1995), suggesting that this insertion may facilitate the interaction of the IRES with cellular factors needed for initiation of translation. Recently, results from enzymatic footprinting analyses showed that eIF3 protected C204, A214, A215 U216, and U212 in domain III of HCV IRES from cleavage by RNases ONE and V1, respectively, suggesting that the binding site for the translation initiation factor eIF3 on the IRES element of HCV is within this apical region of domain III (Sizova *et al.,* 1998). The T insertion at position 207 presented here is centered in this region, it therefore could be speculated that the affinity of eIF3 for the IRES element in the 5′-UTR342 might be higher than that for the IRES element in the 5′-UTR341. However, proof of this hypothesis requires more stringent tests such as UV cross-linking analysis and UV competition assay.

MATERIALS AND METHODS

Plasmids construction

Monocistronic vectors, which contain a T7 promoter, the 5′-UTR342 derived from a full-length cDNA clone or the 5′-UTR341, originated from another HCV genotype 1b-positive serum, the Rluc gene amplified from pRL-TK (Promega) and a T7 terminator (see Fig. 2A), were constructed using pGEMEX-1 vector (Promega), which had been modified by deletion of all of the T7 gene 10. Briefly, cDNA containing the 5′-UTR was amplified by PCR with a sense primer containing a *Hin*dIII site at the 5′ end (5′-cccaagcttGCCAGCCCCCTGATGGGGGC-3′) and an antisense primer (5′-GGTGCACGGTCTACGAGACC-3′). The Rluc gene was amplified with a sense primer containing an *Apa*LI site (5′-accgtgcaccATGACTTCGAAAGTTTATGA-3′) and an antisense primer containing an *Asc*I site (5′-ttggcgcgccTTATTGTTCATTTTTGAGAA-3′). These PCR products were digested with *Hin*dIII and *Apa*LI or *Apa*LI and *Asc*I, respectively, purified by agarose gel electrophoresis, and ligated with pGEMEX-1 digested at *Hin*dIII/*Asc*I sites that had been artificially introduced between the T7 promoter and T7 terminator. The resultant vectors are termed pT7-RL-UTR342 and pT7-RL-UTR341. The site-directed mutants pT7-RL-UTR341C, which differs from pT7-RL-UTR341 by an A to C substitution at position 119, and pT7-RL-UTR342A, which differs from pT7-RL-UTR342 by a C to A substitution at position 119, were generated by subcloning the PCR fragments containing the corresponding mutations and the Rluc fragment into the pGEMEX-1 vector with the same procedure (Fig. 2A). Additionally, vector pT7-RL which contains the Rluc reporter gene immediately downstream of the T7 promoter without any 5′-UTR sequence was also constructed as a control. Similarly, pT7-RL-UTR342(207A), pT7-RL-UTR342(207G), and pT7-RL-UTR342(207C), which contain A, G, and C insertion at position 207 (Fig.7A), were constructed by subcloning the PCR fragments containing the respective mutations and the Rluc fragment into the modified pGEMEX-1 vector. The authenticity of all PCR products were verified by sequencing each of the resulting fragments in these plasmids.

To construct the bicistronic vectors pSVGLRUTRs and pSVGLR (Fig. 5A), all the UTRs-linked or nonlinked Rluc fragments with *Xba*I sites at both ends were made by PCR with pT7-RL-UTRs or pT7-RL as the templates, digested with *Xba*I and inserted into the *Xba*I site of pGL3-Control vector (Promega). To generate pT7GLRUTRs and pT7GLR (Fig. 6A), the Fluc gene was amplified from pGL3-Control vector by PCR and inserted into the *Hin*dIII site of pT7-RL-UTRs and pT7-RL. The correct orientations and sequences of these constructs were confirmed by nucleotide sequencing.

Cell culture

The cell lines COS-1 and HepG2 were purchased from the American Type Culture Collection (ATCC). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO BRL) supplemented with 10% fetal calf serum and 50 units/ml penicillin and streptomycin in a 5% CO_2 humidified atmosphere.

The cell line HepT, which stably expresses T7 RNA polymerase, was established by transfecting HepG2 cells with the pAM8–1 vector (kindly provided by Dr. Nakanishi, Osaka University), which contains CAG promoter followed by T7 RNA polymerase gene and RSV-LTR promoter followed by the puromycin resistance gene in two reversely arranged expression units. The culture conditions for HepT cells were the same as for HepG2 cells except for the presence of 5 μg/ml puromycin (Sigma).

In vitro RNA transcription and translation

Plasmids were linearized by digestion with *Asc*I and used as templates for run-off RNA synthesis with T7 RNA polymerase according to the protocol supplied by the manufacturer (Boehringer Mannheim). After transcription, 10 units of RQ DNaseI (Promega) was added to the reaction mixture to digest DNA templates. The mixture was extracted with phenol-chloroform and RNA was precipitated with ethanol-7.5 M ammonium acetate. The transcripts were purified with NucTrap Probe Purification Columns (Stratagene) to remove unincorporated nucleotides and then quantified by spectrophotometric reading. We also compared the RNA amount among the transcripts by agarose gel electrophoresis and ethidium bromide staining.

In vitro translation was carried out with nuclease-treated Rabbit Reticulocyte Lysate (RRL, Promega). Translation reactions (50 μl) were programmed with 0.5, 1, or 2 μg of RNA, 35 μl of lysate, 20 units of RNase Inhibitor (RNasin, Promega), 20 μM amino acid mixture in the presence of 120 mM KCl and carried out at 30°C for 90 min. After the reaction was stopped by adding RNase A, 2.5 μl of the reaction mixture was used for luciferase assay.

Primer extension

Two micrograms of various RNAs were incubated with rabbit reticulocyte lysates under the same conditions for *in vitro* translation described above. Total RNA was extracted from the lysates at 0, 30, and 90 min after the reaction using TRIZOL Reagent (GIBCO BRL), and one-half of the sample RNA was analyzed by primer extension using a ^{32}P-end-labeled primer (5′-AACACTACTCG-GCTAGCAGT-3′) complementary to the 5′-UTR of HCV RNA as previously described (Ito *et al.,* 1998).

Transfection

COS-1 and HepT cells (when expression from the T7 promoter was desired) were seeded onto 35-mm-diameter tissue culture dishes 24 h before transfection. Seven micrograms of each plasmid DNA were used for transfection by a calcium phosphate method (Profection Mammalian Transfection System, Promega). For transfection with the monocistronic constructs of the pT7-RL-UTRs series, pGL3-Control vector was cotransfected at a molar ratio of 10:1 to normalize the transfection efficiency. Triplicate wells were transfected with each construct, and each experiment was performed at least twice. The cells were harvested after 48 h, and cell lysates were assayed for luciferase activity as described below.

Luciferase assay

Cell lysates were prepared from transfected cells, centrifuged briefly, and 20 μl of the supernatants were used for luciferase assays with the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Luciferase activities were measured using a BLR-301 Luminometer (Aloka). The Rluc activity directed from the IRES of 5′-UTR341 was arbitrarily designated as 100%, and the Rluc activities from the IRES of other 5′-UTRs were normalized to this value.

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