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Properties of Hepatitis C virus minigenome containing mutated 5'UTR region and luciferase transgene

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Summary. – Sequences at the 3'UTR of Hepatitis C virus (HCV) negative-strand (-)RNA play an important role in the initiation of positive-strand (+)RNA synthesis. However, the underlying mechanism in cellular context is still unclear. In this report, we designed several cDNA-based HCV-like minigenomes containing different mutations at the 5'UTR of (+)RNA. These (+)RNAs transcribed from the minigenomes *in vitro* were transfected into HCV replicon cells for producing (-)RNAs with deletions of different stem loops (SL) at the 3'-end. The results showed that expression of the antisense transgene from minigenome increased, when the minigenome containing deletion of SL-C1+D1+E1 at the 3'-end of (-)RNA was transfected into the HCV replicon cells compared to that of the full minigenome. The expression of the transgene from minigenome decreased using other mutant minigenomes containing deletions SL-A1, SL-A1+B1, and SL-A1+B1+C1 at the 3'-end of (-)RNA. Finally, the transgene from SL-C1+D1+E1 of (-)RNA using CMV promoter-driven minigenome was expressed at higher level than full minigenome in HCV replicon cell lines. These results indicated that the region of (-)RNA interacting with HCV replicase may locate in the SL-C1+D1+E1 region of (-)RNA.

Keywords: Hepatitis C virus; minigenome; RNA dependent RNA polymerase; replication

Introduction

HCV is the major pathogen of chronic hepatitis that in some cases leads to liver cirrhosis and hepatocellular carcinoma (Drazan, 2000) There are more than 170 million HCV-infected individuals worldwide and at present, there is no vaccine or effective antiviral drug against this disease. The current treatment for HCV infection including interferon-α in combination with ribavirin has limited efficacy (Reddy *et al*., 2001). HCV is a member of the family *Flaviviridae* with the single-stranded positive-sense RNA genome of about 9600 nts in length (Brass, Moradpour, and Blum, 2006). It contains a single ORF flanked by two UTRs. The ORF encodes a large polyprotein precursor that is further cleaved into structural (C, E1, E2) and nonstructural (NS2, NS3, NS4, NS5) proteins (Locatelli *et al*., 2001). The protein NS5B, viral RNA-dependent RNA polymerase(RdRp), plays a key role in the viral RNA replication (Shirota *et al*., 2002).

Early studies of the molecular aspects of HCV life cycle and development of alternative antiviral approaches were impeded by the lack of an efficient cell culture system (Blight *et al.,* 2000)**.** The replicon system described by Lohmann *et al.* (1999) was a great breakthrough in the propagation of HCV in cell culture. In this system, culture-adapted mutations within the NS proteins are required for an efficient replica-

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Abbreviations: CMV = cytomegalovirus; CRE = cis-acting replication element; EMCV = Encephalomyocarditis virus; HCV = Hepatitis C virus; IRES = internal ribosome entry site; LCMV = Lymphocytic choriomeningitis virus; luc, Rluc, fluc = luciferase, firefly luciferase, Renilla luciferase, respectively; NS = nonstructural; RdRp = RNA dependent RNA polymerase; RVFV = Rift valley fever virus; SL = stem loop; HDV Rz = Hepatitis D virus ribozyme

Table 1. Oligonucleotides used for PCR

Plasmid	Oligonucleotide	Sequence 5'-3'
pT7/5U rFBURz	5'UTR-F	tataagcttTAATACGACTCACTATAGCCAGCCCCCGATTGGGGGC
	5'UTR-R	tgctctagaTTTGGTTTTTCTTTGAGGTT
	Fluc-F	Ctctctagaatggaagacgccaaaaacata
	Fluc-R	Ctctctagattacacggcgatctttccgccc
	IRES-F	cgcggatccCAGACCACAACGGTTTCCCTC
	Ires-R	ctctctagaATTATCGTGTTTTCAAAGG
	3'UTR-F	cgcggatccGGAAACTTGGGGTCCCACCC
	3'UTR-R	ataggcgccagcgaggaggctgggaccatgccggccACTTGATCTGCAGAGAGGCC
	HDVRz-top	CGCCGGCTGGGCAACATTCCGAGGGGACCGTCCCCTCGGTAATGGCGAATGGGACCg
	HDVRz-bottom	aattcGGTCCCATTCGCCATTACCGAGGGGACGGTCCCCTCGGAATGTTGCCCAGCCGG
pT7/5UΔ45 rFBUrz	F5'UTR-Δ45	atactcgagTAATACGACTCACTATAtgtgaggaactactgtcttcac
pT7/5UΔ124 rFBUrz	F5'UTR-Δ124	atactcgagTAATACGACTCACTATAcctcccgggagagccatagtggtc
pT7/5UΔ227 rFBUrz	F5'UTR-Δ227	atactcgagTAATACGACTCACTATAtgggcgtgcccccgcgagactg
	R-HDVRz	ctcgtcatcGGTCCCATTCGCCATTACC
pCMV/5U rFBURz	F5'UTRfull	atactcgagTCTGGCTAACTGCCAGCCCCCGATTGGGGGC

The restriction enzyme digestion site is underlined.

tion (Lohmann *et al*., 1999). In particular, the involvement of 5'UTR and 3'UTR in viral replication was investigated (Fang *et al*., 2000; Friebe *et al*., 2001). It was reported the replicon containing full-length genotype of 2aJFH1 (Japanese fulminant hepatitis) genome that could replicate efficiently and produce viral particles (Cai *et al*., 2005). These systems and later-developed JFH1-based chimeras facilitate many aspects of HCV research (Targett-Adams and McLauchlan, 2005; Cagnon *et al*., 2004). However, there are also limitations for the use of HCV sub-genome and full-length replicons in the investigation of viral replication mechanism or viral pathogenicity, because of their strict genotype-specificity.

Recently, application of the synthetic minigenomes has provide a new tool for studying cis-acting replication elements (CREs) and host cofactors involved in the viral replication, maturation and packaging of many positive or negative single-stranded RNA viruses, such as Lymphocytic choriomeningitis virus (LCMV) and Rift Valley fever virus (RVFV) (Cornu and de la Torre, 2001; Ikegami *et al*., 2005). The construction of HCV-like minigenome consisted of the antisense sequence of a reporter gene and internal ribosome entry site (IRES) from Encephalomyocarditis virus (EMCV) flanked by the 5'- and 3'-end regions of HCV (Zhang *et al*., 2005). These authors also showed that the minigenome RNA could act as a functional template for the replication complex to produce (-) strand of HCV minigenome RNA that directed expression of luciferase (luc) in the cells expressing HCV RdRp. Based on the understanding of HCV replication, (-) strand of the minigenome RNA not only acts as a template for expression of the reporter gene, but also as a template for synthesis of (+)RNA (Fig. 1). Thus, we hypothesized that the role of different mutated HCV 5'UTR regions in virus replication can be investigated by means of the expression level of transgene in the minigenome.

In this study, we used several HCV minigenomes that produced (-)RNA containing different SLs at the 3'-end under T7 and cytomegalovirus (CMV) promoter. According to the expression level of a transgene carried by these minigenomes in HCV replicon cells, the properties of HCV minigenomes containing mutated 5'UTR in cellular context were determined.

Materials and methods

Cells. The human hepatoma cell line Huh7.5 was maintained in DMEM supplemented with 10% fetal calf serum (Invitrogen), 2 mmol/l L-glutamine, penicillin 100 IU/ml, and streptomycin 100 μg/ml at 37°C in a 5% CO_2 atmosphere. The cell line Huh7.5/BB7 was established by electroporation of the Huh7.5 cells with BB7 replicon RNA that was obtained by the transcription of pHCVrep1b BB7 plasmid and following selection in medium containing 500 μg/ml of G418. The cells were frozen in aliquots after 1 month of cultivation.

Plasmid constructs. For construction of the HCV-like minigenome pT7/5'UTR-rFluIR ES-3'UTR, a fragment containing HCV cDNA (1 to 377 bp) with the T7 promoter directly coupled at the 5'-end was amplified by PCR with primers 5'UTR-F and 5'UTR-R. The PCR products were digested with *Hin*dIII and *Xba*I and cloned into *Hin*dIII/*Xba*I-digested pUC18 to generate PT7/5'UTR. The EMCV IRES sequence was amplified from pIRES2-EGFP with primers IRES-F and IRES-R. As mentioned above, PCR products were digested with *Bam*HI and *Xba*I and cloned into *Xba*I/*Bam*HI-digested pT7/5'UTR to generate pT7/5'UTR-rIRES. The 3'-end of NS5B coding region(9067–9371 bp) fused to 3'UTR of HCV was amplified by PCR using primers 3'UTR-F and 3'UTR-R digested

with *Bam*HI and *Nar*I, and cloned into *BamH*I/*Nar*I digested pT7/5'UTR-rIRES, generating pT7/5'UTR-rIRES-3'UTR. Then, the annealed oligonucleotides containing the partial sequence of the HDV Ribozyme, HDVRz-top and bottom, was inserted into pT7/5'UTR-rIRES-3'UTR digested with *Nar*I/*Eco*RI, generating pT7/5'UTR-rIRES-3'UTR-HDV Rz. The luciferase gene was amplified from pGL-3 (Promega) using primers Fluc-F and Fluc-R, digested with *Xba*I and inserted into *Xba*I-digested pT7/5'UTR-rIRES-3'UTR-HDVRz, generating pT7/5'UTR-rFluIRES-3'UTR-HDVRz, abbreviated to pT7/5'UrFI3'URz. The inserted sequences of these constructs were confirmed by nucleotide sequencing.

To construct serial HCV minigenomes containing the different truncated 5'UTR, the deletion mutant of HCV minigenome was amplified from pT7/5UrFI3UR by forward primers F-5'UTR Δ45, or Δ124, or Δ227 and reverse primer R-HDVRz, then digested with *Xho*I and *Eco*RV, and cloned into *Sal*I/*Sma*I-digested pUC18, respectively. Thus, the corresponding HCV minigenome constructs were generated. Because *Sma*I was positioned at the 131 bp and 315 bp of the fragment 5'UTR, pT7/5UrFI3UR was digested by *Sma*I and ligated with T4 ligase and finally, pT7/5$U_{\Delta 131/315}$rFI3UR was generated. To construct pCMV/5UrFI3URz and pCMV/5$U_{\Delta 131/315}$rFI3UR, the HCV minigenome was amplified from pT7/5UrFI3UR and pT7/5$U_{\Delta 131/315}$rFI3UR by primers F-5UTRfull and R-HDVRz, digested with *Xho*I and *Eco*RV, and cloned into *Xho*I/*Eco*RV-digested pcDNA3.1(-), then pcDNA3.1(-)/5UrFI3UR and pcDNA3.1(-)/5$U_{\Delta 131/315}$rFI3UR were generated and abbreviated to pCMV/5UrFI3URz and pCMV/5$U_{\Delta 131/315}$rFI3UR.

In vitro transcription. To produce the RNA minigenome containing a minimal or no overhang at 3'-end, the plasmids were linearized at *Eco*RI site that located immediately downstream of the HDV ribozyme. These fragments were used as the templates for runoff RNA synthesis with T7 RNA polymerase according to the manufacturer's protocol (Takara). After transcription reaction was completed, 10 U of DNase I (Takara) were added to the reaction mixture to digest DNA templates. The mixture was extracted with phenol-chloroform and RNA was precipitated with isopropanol. The purity and integrity of the transcription products were determined by 3% agar gel electrophoresis in TAE buffer.

Transfection. 1 μg of transcript RNA generated *in vitro* from the DNA templates and linearized with *Eco*RI was mixed with 400 μl of a suspension 10^7 Huh-7.5/BB7 cells/ml in a cuvette with a gap width of 0.2 cm (Bio-Rad). After one pulse at 950 μF and 240 V with a Gene pulser system II (Bio-Rad), the cells were immediately transferred into 4 ml of complete DMEM. Aliquots of the cell suspension were seeded in culture dish and harvested at various time points. To determine a luciferase activity, the cells were washed three times with PBS and scraped off the plate into 350 μl of ice-cold lysis buffer (1% Triton X-100, 25 mmol/l glycylglycine, 15 mmol/l $MgSO_4$, 4 mmol/l EGTA, 1 mmol/l dithiothreitol) 48 hrs after transfection. For the DNA transfection, 1 μg of each reporter vector and 0.01 μg of phRluc vector (Promega) were co-transfected into Huh7.5/BB7 cells with Lipofectamine Transfection Reagent (Invitrogen) according to the manuals.

Luciferase assay. Cell lysates were prepared from the transfected cells as described (Fan and Wood, 2007) and 20 μl of the supernatants were used for luciferase assays with Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The luciferase activities were measured using a TD-20/20 luminometer (Promega). The Renilla luciferase (Rluc) activity from a co-transfected phRLuc Control vector was simultaneously measured to normalize the transfection efficiency.

RT-PCR for HCV minigenomic (-)RNA. Total RNA was extracted from the transfected cells with Trizol reagent (Invitrogen), precipitated with isopropanol, and treated with RNase-free DNase I (Takara). The DNA-free RNA was extracted with phenol-chloroform and precipitated with ethanol. Detection of (-)RNA from the minigenome was carried out by a two-step RT-PCR. First, total RNA (2 μg) was incubated with 2 pmol of luc-R primer (5'-TTA CACGGCGATCTTTCCGC-3') for 5 mins at 70°C. Then, it was kept at 42°C with 5x RT buffer and NTPs (10 pmol), DTT, RNase inhibitors, SuperScript™ II Reverse Transcriptase (Invitrogen) and DEPC-treated water for 60 mins. Next, RT was inactivated by

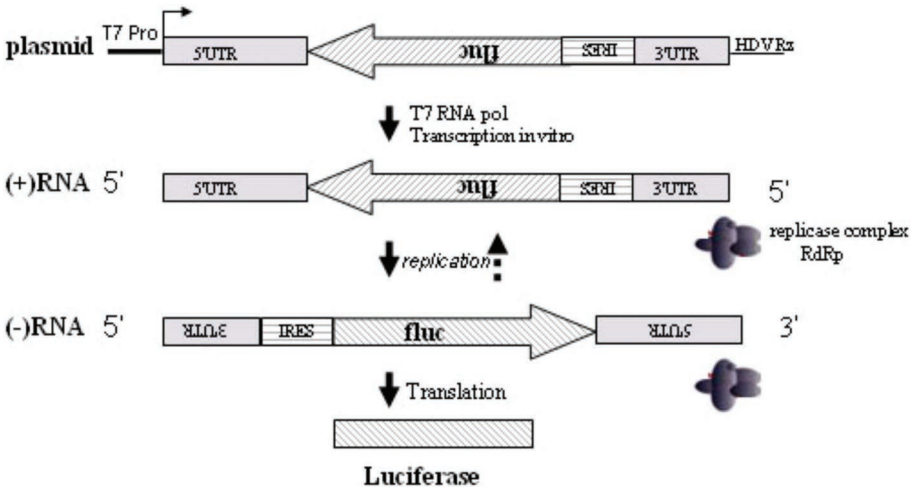

Fig. 1

Plasmid constructs with HCV minigenome cDNA and luciferase transgene and their transcription, replication, and expression

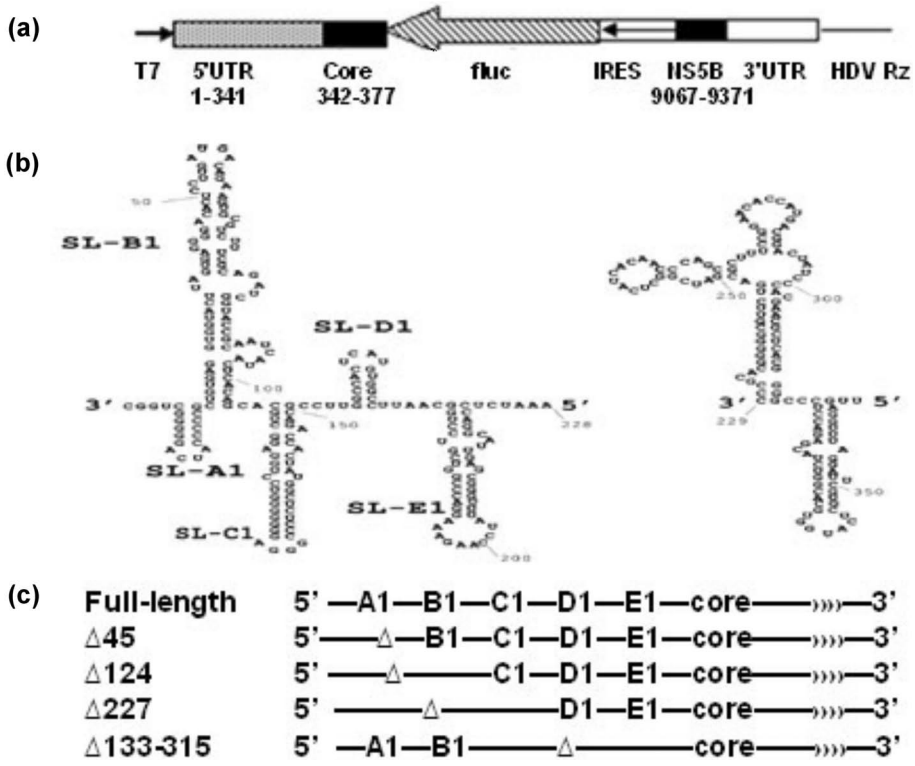

Fig. 2

5'UTR region of HCV (+)RNA with various deletions

(a) Full-length minigenome construct consists of the antisense sequence of luciferase gene and IRES flanked upstream by 5'UTR (nts 1–377) and downstream by 3'UTR containing NS5B coding region (nts 9067–9371) together with 3'UTR of HCV cDNA. (b) The secondary structure of 3'-terminal region of (-)RNA adopted from Schuster *et al.* (2002). (c) The minigenome constructs with different deletions of 5'UTR region. Δ45 = SL-A1; Δ124 = SL-A1+B1, Δ227 = SL-A1+B1+C1+D1; Δ131-315 = SL-C1+D1+E1. 5'UTR or 3'UTR with deletions are shown in boldface.

incubation for 15 mins at 70°C and the cDNA products were used as the template of PCR reaction. The 5 μl cDNA, luc-F'5'-CGT GGATTACGTCGCCAGTC-3' and luc-R primers were added into 2x Master PCR Buffer (New England Biolabs). The PCR conditions were 94°C, 30 secs; 55°C, 40 secs; 72°C, 55 secs for 30 cycles.

Results

Construction of plasmids with HCV minigenome and their transcription in vitro

To construct CMV-like minigenome, the CRE was amplified using PCR and ligated into pUC18 vector. The minigenome construct consists of the antisense sequence of firefly luciferase gene, IRES of EMCV flanked upstream by 5'UTR (1–377 nts), downstream by the HCV sequence containing NS5B coding region (9067–9371 nts) and 3'UTR of HCV cDNA (1–9605 nts). The cassette was positioned precisely at T7 transcription start site and followed by self-cleaving HDV ribozyme to ensure authentic 5'- and 3'-ends and was signed as pT7/5UrFI3URz (Fig. 2a). If the constructed minigenome cDNA is successfully used as a template for the replication complex, the firefly luciferase gene encoded by synthesizing (-)RNA should be expressed in HCV replicon cells. In agreement with the anticipated results, luciferase activity was readily detected, when the minigenome RNA transcribed *in vitro* from minigenome cDNA was transfected into the Huh7.5/BB7 cells harboring autonomously replicating HCV subgenome (Figs. 3, 4).

Effect of deletions in 5'UTR on the luciferase expression in cells transfected with HCV (+)RNA

To understand respective roles of several SLs of the 3'-end (-)RNA, we designed appropriate minigenome constructs. Based on the recognition of the secondary structure of the 3'-end, the mutant cDNA-based minigenomes that

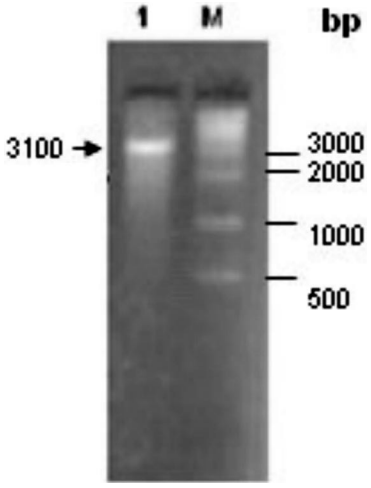

Fig. 3

***In vitro* transcript of plasmid with HCV minigenome cDNA**

Agarose gel electrophoresis of minigenome RNA transcript product (lane 1) and RNA size markers (lane M).

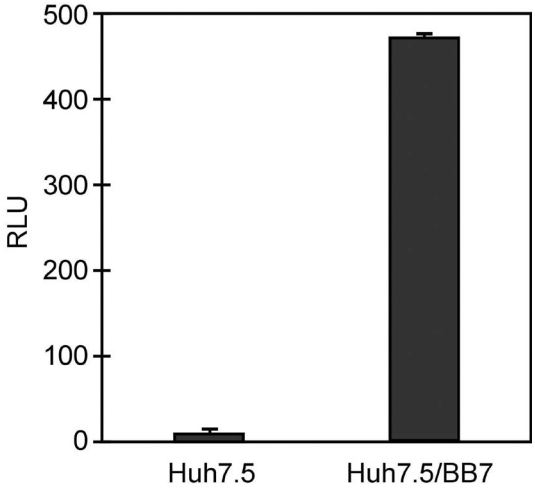

Fig. 4

Luciferase expression in cells transfected with HCV (+)RNA transcribed *in vitro*

The luciferase activity was detected in Huh7.5/BB7 cells. RLU = relative luciferase unit.

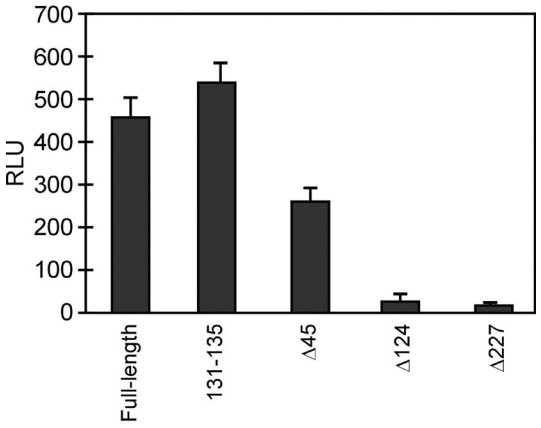

Fig. 5

Effects of the deletions in 5'UTR on luciferase expression in cells transfected with HCV (+)RNA

Huh7.5/BB7 cells were transfected with minigenome RNAs transcribed *in vitro* and RLU in the lysates were determined at 48 hrs post transfection. The minigenome containing the deletion of SL-C1+D1+E1 (Δ131-315) exhibited the highest luciferase activity. For other deletions see legend to Fig. 2. The columns and bars represent the means and standard deviations of three independent triplicate transfections.

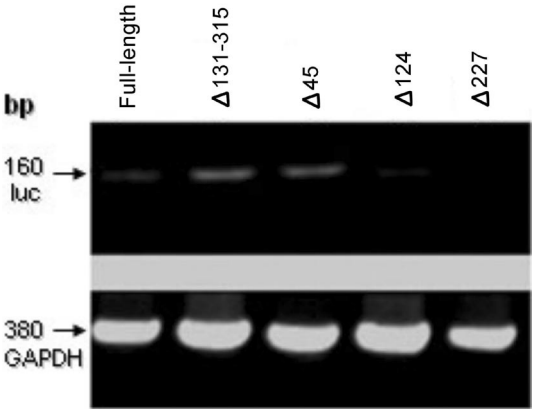

Fig. 6

Effects of deletions in 5'UTR on HCV (-)RNA levels in cells transfected with HCV (+)RNA

Huh7.5/BB7 cells were transfected with the minigenome RNA full-length or containing the deletions Δ131-315, Δ45, Δ124, Δ227, described in legend to Fig. 2. GAPDH was used as a system control.

contained deletions of SL-A1 (Δ45), SL-A1+B1 (Δ124), SL-A1+B1+C1+D1 (Δ227), SL-C1+D1+E1 (Δ131-315) were constructed (Fig. 2b,c). These mutants were transcribed *in vitro* into the corresponding minigenome RNAs that were transfected into the Huh7.5/BB7 cells. The luciferase activity assay showed that the transcripts could be used as a template to produce (-) strand using the functional replication components. The minigenome containing the deletion of SL-C1+D1+E1 exhibited the highest luciferase activity (Fig. 5).

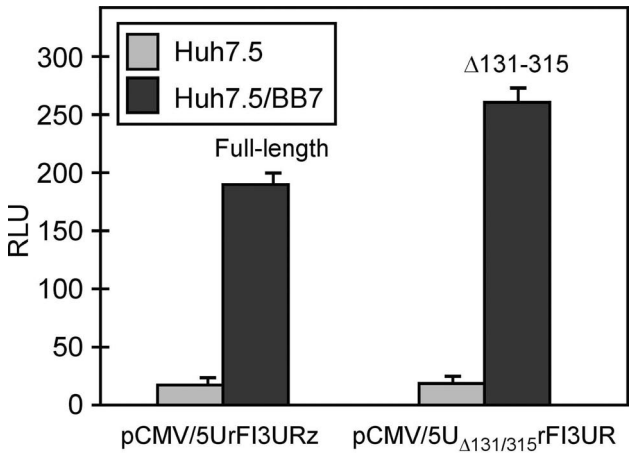

Fig. 7

Effect of the deletion in 5'UTR on luciferase expression in cells transfected with a plasmid containing HCV minigenome cDNA driven by CMV promoter

Huh7.5 and Huh7.5/BB7 cells were transfected with pCMV/5UrFI3URz and pCMV/5U$_{\Delta 131/315}$rFI3UR, respectively, and the luciferase activities were detected at 48 hrs post-transfection. RLU = relative luciferase unit. The columns and bars represent the means and standard deviations of three independent triplicate experiments.

Effect of deletions in 5'UTR on HCV (-)RNA levels in the cells transfected with HCV (+)RNA

To determine the levels of (-)RNA synthesis of these mutant minigenomes in transfected cells, we used RT-PCR employing the luciferase gene as a target gene. In agreement with the luciferase activity assay, the amount of (-)RNA in the cells transfected with minigenome containing the deletion of SL-C1+D1+E1 was much higher than with minigenomes containing deletions of SL-A1, SL-A1+B1 or SL-A1+B1+C1+D1 (Fig. 6).

Effect of a deletion in 5'UTR on luciferase expression in the cells transfected with plasmid containing HCV minigenome cDNA driven by CMV promoter

To investigate whether the transcripts from RNA polymerase II-driven HCV minigenome were able to act as a functional RNA template for synthesizing (-) strand RNA, we constructed a CMV-driven minigenome, in which the cDNA-based minigenome was cloned into the eukaryotic expression vector pcDNA3.1 downstream of the CMV promoter. To ensure that both 5'- and 3'-ends were similar to those found in the viral genome in natural infection, we positioned the 5'-end of the minigenome immediately after transcription start site of CMV promoter and the self-cleaving HDV ribozyme downstream at the 3'-end of the minigenome. The plasmid DNA constructs containing minigenome RNAs were transfected into the HCV replicon cells with Lipofectamine 2000 and detected luciferase activity indicated the ability of template minigenome RNA to synthesize a negative strand. We constructed two CMV-driven minigenomes: the first one contained full 5'UTR and the second one a deletion between Δ131-315 bp of 5'UTR in (+)RNA corresponding to the deletion of SL-C1+D1+E1 in 3'UTR of (-)RNA. Compared with the control Huh-7.5 cells, the firefly luciferase activities in Huh7.5/BB7 cells transfected with both DNA constructs were consistently higher. In addition, luciferase activity in the cells transfected with pCMV/5U$_{\Delta 131/315}$rFI3URz was higher than that in the cells transfected with pCMV/5U rFI3URz (Fig. 7). The results showed that the polymerase II-driven RNA transcripts were functional as the template for (-)RNA synthesis.

Discussion

Currently, there is no vaccine able to prevent HCV infection and conventional therapies are not able to eliminate the virus from majority of patients (Wu *et al.*, 2005). Thus, understanding of the molecular aspects of HCV life cycle is important for the development of alternative antiviral approaches and novel vaccination strategies. RNA replication is the key step in HCV life cycle, however, the molecular mechanism of HCV replication is not well understood (Takahashi *et al.*, 2005). It is generally accepted that HCV (+)RNA is transcribed into (-)RNA inside the cell that in turn serves as a template for production of viral (+)RNA progeny (Shi and Lai, 2001).

The major issue of HCV replication is the identification of CREs in RNA genome. Recently, it has been shown that CREs of HCV RNA may reside at various positions in the genome, but are mainly located at the UTRs. The highly conserved 5'UTR is composed of four distinct domains. It has been shown that the first 125 nt (domains 1 and 2) of the 5'UTR are essential for HCV RNA replication in HuH7 cells. The 3'UTR has a tripartite structure that is composed of a highly variable region immediately downstream of the polyprotein stop codon, a polypyrimidine [poly(U/UC)] tract of variable length, and a highly conserved 98-nt-long RNA part designated as X-tail (Reigadas *et al.*, 2001). In addition, the NS5B coding region may also harbor the functional CREs. In addition, better understanding of the HCV CREs provides an opportunity to construct CMV-like minigenome that will facilitate the analysis of CREs and trans-acting proteins in CMV replication.

It was clearly demonstrated that HCV replication consists of the synthesis of a new strand from the 3'-end of (+) or (-) strand, what plays an important role in the initiation of RNA synthesis (Reigadas *et al.*, 2001; Kim *et al.*, 2002). The role of

3'UTR of (+)RNA in binding of the replicase complex and initiating (-)RNA synthesis was previously explained (Yi and Lemon, 2003). In addition, the role of different regions of 3'UTR was also elucidated. Ventura *et al.*, 2005 utilized the recombinant RdRp to investigate the template and binding properties of mutated and deleted fragments derived from the 3'-end (-)RNA *in vitro* (Astier-Gin *et al.*, 2005). These results showed that several domains of 341 nts region of (-)RNA 3'-end interacted with the HCV RdRp during *in vitro* RNA synthesis, in particular the region located between nts 219–239. However, a little is known about the role of several SLs of the 3'-end (-)RNA.

Recently, the secondary structure of the 3'-end (-)RNA has been determined (Schuster *et al.*, 2002). It was found that 341 nts from the 3'-end (-)RNA folded into six SLs. With the exception of the short SL-A1 stem loop located at the very end, other structures differed from those of (+)RNA. Therefore, 3'-end (-)RNA domain is distinctly different from its corresponding antisense sequence in 5'UTR and also their respective roles in the initiation of RNA synthesis are likely to be different. Due to the lack of a reliable cell culture system for HCV replication, the replicon system is used as a surrogate for HCV-infected cell lines. Since translation and replication are linked in this system, the role of 3'-end (-)RNA in the initiation of (+)RNA synthesis cannot be directly assessed (Zhang *et al.*, 2007). In this study, we exploited HCV-like minigenome for the examination of sequences at the 3'-end (-)RNA. These mutants were designed according to the newly established secondary structure of 3'-end (-)RNA. The results of luciferase activity assay showed that the deletion close to the 3'-end (-)RNA affected synthesis of (+)RNA. The mutant minigenome containing deletion of SL-C1+D1+E1 located at the 3'-end (-)RNA severely reduced the synthesis of (+)RNA. The interpretation of these results is based on the assumption that the kissing loop between the 5'- and 3'-end of RNA genome may be necessary for the binding of replicase complex to initiate RNA synthesis (Friebe *et al.*, 2005). The mutant minigenomes contained deletion of SL-A1, SL-A1+B1, or SL-A1+B1+C1+D1 at the 3'-end of (-)RNA, where the interaction between HCV RNA genome occurs, displayed impairment of the replicase complex. As a result, the (-)RNA synthesis was reduced to such an extent, that the level of luciferase activity was lower than the luciferase activity with minigenome containing full length 5'UTR. For the mutant minigenome including the deletion of SL-C1+D1+E1, the kissing loop between the ends of virus genome may be formed correctly and the replicase complex binding domain located at (-)RNA may be deleted. Thus, the (+)RNA synthesis from the (-) strand was decreased and the luciferase activity was enhanced compared with the minigenome construct with a full length 5'UTR. Hence, SL-C1+D1+E1 at the 3'-end of (-)RNA may be the important region for the binding of RdRp.

It was reported for other positive-strand RNA viruses that the full-length viral RNA transcripts produced in the nucleus by polymerase II are efficiently transported into cytoplasm in the functional form (Almazan *et al.*, 2000; Khromykh *et al.*, 2001). Further, we examined whether the transcripts from RNA polymerase II driven HCV minigenome can act as a functional RNA template for synthesis of (-)RNA. We constructed the CMV promoter-driven minigenomes containing full length 5'UTR and the mutant minigenome containing deletion of Δ SL-C1+D1+E1 at 5'UTR. The results showed that the mutant minigenome had a lower ability to act as the functional template for synthesis of the (+)RNA. The mutant minigenome that produces minigenome RNA containing Δ SL-C1+D1+E1 located at the 3'-end of the (-)RNA enhanced the transgene expression in cellular system. Nevertheless, using HCV-like minigenome driven by RNA polymerase II will facilitate the CREs research of HCV RNA.

Summing up, we exploited HCV-like minigenomes to study the role of different sequences located at the 3'-end of (-)RNA. We believe that the results of this study significantly extend the understanding of molecular mechanism of HCV replication.

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