TWO *CIS*-ACTING ELEMENTS IN NEGATIVE RNA STRAND OF HEPATITIS C VIRUS INVOLVED IN SYNTHESIS OF POSITIVE RNA STRAND *IN VITRO*

L. YE, K.A. TIMANI, L. YE*, L. KONG, X. YANG, Q. LIAO, J. WU

State Key Laboratory of Virology, Ministry of Education, College of Life Sciences, Wuhan University, Wuhan, Hubei 430072, P.R. China

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Summary. – Sequences at the 3'-ends of both positive and negative strands of Hepatitis C virus (HCV) RNA harbor *cis*-acting elements required for RNA replication. However, little is known about the properties of the negative RNA strand as a template for the synthesis of positive RNA strand. In this study, a purified recombinant HCV RNA-dependent RNA polymerase (RdRp) was used to investigate the synthesis of positive RNA strand using the 3'-terminal region of negative RNA strand ((–)3'T RNA) as template. A mutagenesis analysis was performed to evaluate the role of the 3'-proximal stem-loop and the first 3'-cytidylate (3'C) of the negative RNA strand in the synthesis of the positive RNA strand. A negative RNA strand of wild type (*wt*) HCV as template was able to direct the synthesis of a full-length positive RNA strand. Deletion of the 3'-proximal stem-loop resulted in an approximately 90% decrease in RNA synthesis. Disruption of the 3'-proximal stem-loop structure by nucleotide substitutions led to a 70–80% decrease in RNA synthesis. However, the restoration of the stem-loop by compensatory mutations in the stem region restored also the RNA synthesis. Likewise, the deletion or substitution of the first 3'C by guanylate (G) led to a 90% decrease in the RNA synthesis; while the substitution by adenylate (A) or uridylate (U) resulted in a 60–80% decrease in the RNA synthesis only. These findings demonstrate that the 3'-proximal stem-loop and the first 3'C of the negative RNA strand of HCV are two *cis*-acting elements involved in the synthesis of the positive RNA strand.

Key words: Hepatitis C virus; RNA; positive RNA strand; negative RNA strand; RNA-dependent RNA polymerase; RNA synthesis; *cis*-acting element

Introduction

HCV, a major pathogen of non-A, non-B transfusion-associated hepatitis causes often development of malignant chronic disease, such as liver cirrhosis and hepatocellular carcinoma (Choo *et al*., 1989; Honghton, 1996). It is estimated that over 170 million people are infected with HCV worldwide. At present, there is no vaccine or effective treatment for HCV. Understanding of HCV replication will help the development of a HCV vaccine and possible anti-HCV strategies.

HCV (the *Hepatitis C virus* species, the *Hepacivirus* genus, the *Flaviviridae* family) possesses a single-stranded positive-sense RNA genome of approximately 9.6 kb. The genomic RNA consists of 3 parts: a 5'-untranslated region (5'-UTR), a single ORF and a 3'-untranslated region (3'-UTR) (Bartenschlager and Lohmann, 2000). The ORF encodes a polyprotein of 3010 amino acids, which is cleaved by cellular and viral proteases into at least 10 structural and non-structural proteins. As the non-structural protein NS5B provides RdRp activity it is a key enzyme involved in virus genome replication (Behrens *et al*., 1996). The structure of HCV NS5B and its RdRp activity have been studied in detail using recombinant

*Correspondig author. E-mail: linbaiye@whu.edu.cn; linbaiye@hotmail.com; fax: +8627-68764763.

Abbreviations: A = adenylate; 3'C = 3'-cytidylate; G = guanylate; HCV = Hepatitis C virus; IRES = internal ribosome entry site; RdRp = RNA-dependent RNA polymerase; (–)3'T RNA = 3'-terminal region of the negative RNA strand; U = uridylate; 3'-UTR = 3'-untranslated region; 5'-UTR = 5'-untranslated region

full-length or C-terminally deleted NS5B proteins (Lohmann *et al*., 1997, 1998; Yanashita *et al*., 1998; Bressanelli *et al*., 1999, 2002; Ferrari *et al*., 1999; Tomei *et al*., 2000). NS5B is capable of primer-independent (*de novo*) initiation of RNA synthesis on an RNA template. However, the mechanism of this process is so far unknown. Although different HCV genotypes and isolates vary considerably regarding their polypeptide-coding sequences, the sequences of the 5'-UTR and 3'-UTR are relatively well conserved. The conservation is likely to maintain RNA structures that are required for HCV RNA replication and translation (Clark, 1997).

The replication of HCV RNA genome comprises two steps: the synthesis of negative RNA strand on the positive RNA strand as template and subsequent synthesis of positive RNA strand on the newly synthesized negative RNA strand as template. Therefore 3'-terminal regions of both positive and negative RNA strands are assumed to be important for the initiation of replication of HCV genome. The HCV 3'-UTR has been extensively studied *in vitro* and *in vivo* (Tanaka *et al*., 1996; Cheng *et al*., 1999; Kolykhalov *et al*., 2000; Friebe and Bartenschlager, 2002; Yi and Lemon, 2003). However, the function of 3'-terminal region of negative RNA strand ((–)3'T RNA) is not clear. The last 220 nucleotides, where the initiation of synthesis of positive RNA strand presumably takes place, are predicted to fold into five stabile stem-loops (Schuster *et al*., 2002; Smith *et al*., 2002). Oh *et al*. (1999) have reported that the region of nts 122–239 (the numbering starting from the 3'-end) of negative RNA strand contains a *cis*-acting sequence required for the synthesis of positive RNA strand *in vitro*. Reigadas *et al*. (2001) have found out that the first three nucleotides and the first 45-nucleotide stretch are involved in highly efficient synthesis of positive RNA strand.

The highly conserved 5'-UTR of positive RNA strand is involved in viral RNA replication. It consists of two distinct RNA elements, a short 5'-proximal RNA element (nt 1–43) and a long element of internal ribosome entry site (IRES) (nts 44–341). The IRES is required for initiation of viral translation. The short 5'-proximal RNA element is important for optimal translation as well (Friebe *et al.*, 2001). In addition, the 5'-proximal RNA element is also important for viral RNA replication. The 5'-proximal stem-loop (nts 5–20) is essential for RNA replication *in vivo* (Luo *et al.*, 2003). However, the mechanism involved is unknown. It is possible that (i) the 5'-proximal stem-loop itself is important or (ii) the 3'-terminal element on complementary negative negative RNA strand is important for initiation of synthesis of positive RNA strand. Corresponding to the 5'-proximal stem-loop of 5'-UTR (nt 5–20), the 3'-terminal element on complementary negative RNA could also form a stabile stem-loop, which is the first stem-loop on negative RNA strand (starting from the 3'-end) (Schuster *et al*., 2002; Smith *et al*., 2002).

In this study, we examined the importance of the 3'-proximal stem-loop and first 3'C of the negative RNA strand in the synthesis of positive RNA strand using a purified recombinant HCV NS5B.

Materials and Methods

Plasmid construction. pBRTM/HCV1-3011 plasmid carrying full-length ORF of HCV genotype 1a (Grakoui *et al*., 1993) and pUCRT/T_7-5'-UTR plasmid containing the sequences of 5'-UTR and a part of the core protein gene of HCV genotype 1a (Luo, 1999) were used. Standard recombinant DNA technologies were employed for plasmid construction (Sambrook and Russell, 2001). The DNA sequence encoding NS5B was PCR amplified from pBRTM/HCV1-3011 plasmid using NS5B forward and reverse primers (Table 1). The reverse primer was designed to introduce a deletion of 21 C-terminal amino acids of NS5B. The PCR product was digested with *Bam*HI/*Nhe*I and cloned into the same sites of the prokaryotic expression vector pETHis-2.9 to form the recombinant plasmid pET5BΔ21. The latter contained a 6-His tag at the 5'-end of NS5B gene to facilitate the protein purification.

Table 1. Sequences of the primers used in this study

Primer	Nucleotide sequence (5'–3')
NS5B forward	GCAGGGATCCTCAATGTCTTATACCTGG (*BamH*I)
NS5B reverse	AATATGCTAGCGCGGGGCCGGGCATGAGA (*Nhe*I)
(–)3'T forward	GTACGGTACCCGGGCTGAGCCCAGGTCC (*Kpn*I)
(–)3'T reverse	GTCGCTGCAGCCAGCCCCCTGATGG (*Pst*I)
T7 promoter	GAATTGTAATACGACTCACTATAGGG
(–)3'-Δ5-20	CCAGACACTCCACCATAGATCACTCCCC
(–)3'-MSI-1	GCCACTTTTTTGATGGGGGCGACACTCCACCATAG
(–)3'-MSI-2	GCCAGCCCCCTGATTTTTTGGACACTCCACCATAGATCACTCCCC
(–)3'-MSI-3	GCCACGGGGGTGATCCCCCGGACACTCCACCATAGATCACTCCCC
(–)3'-MSI-4	GCCAGAAAAATGATTTTTTCGACACTCCACCATAGATCACTCCCC
(–)3'Δ1	CCAGCCCCCTGATGGGGGCG
(–)3'-C/U	ACCAGCCCCCTGATGGGGGCG
(–)3'-C/A	TCCAGCCCCCTGATGGGGGCG
(–)3'-C/G	CCCAGCCCCCTGATGGGGGCG

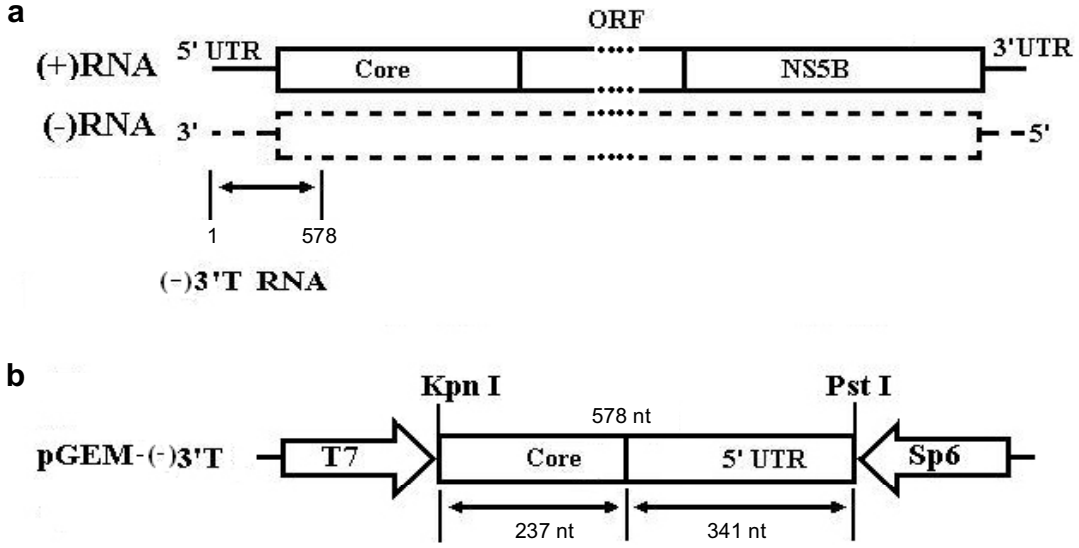


Fig. 1

Scheme of construction of pGEM-(–)3'T plasmid

A. A scheme of HCV (+)RNA and (–)RNA with the 3' terminal region of negative RNA strand ((–)3'T RNA).
B. The (–)3'T RNA region (578 nts) cloned into pGEM-3Zf (+) plasmid to acquire a recombinant plasmid, pGEM-(–)3'T. The inserted sequence was flanked by T7 and Sp6 RNA polymerase promoters.

The DNA sequence corresponding to (–)3'T RNA (Fig. 1A), was PCR amplified from pUCRT/T_7-5'-UTR plasmid with the (–) 3'T forward and reverse primers (Table 1). The PCR product was digested with *Kpn*I/*Pst*I and cloned into pGEM-3Zf(+) plasmid to obtain the recombinant pGEM-(–)3'T plasmid. The inserted sequence was 578 nts long, including sequences of 5'-UTR and a part of the core protein gene (Fig. 1B).

Expression and purification of recombinant NS5B protein. Escherichia coli strain BL21 (DE3) was transformed with the recombinant pET5BΔ21 plasmid. The cells were cultured in 200 ml of the 2×YT medium at 37°C until A_{600} reached 0.6–0.8. To induce gene expression IPTG (Sigma) was added to a final concentration of 1.0 mmol/l and cultivation continued for another 6 hrs. The cells were harvested by centrifugation, resuspended in 10 ml of the buffer A (20 mmol/l Tris-HCl pH 8.0, 500 mmol/l NaCl, 1% Triton X-100, and 1mmol/l phenylmethylsufonyl fluoride), lysed by ultrasonic treatment, and centrifuged at 15,000 × g for 20 mins. The supernatant was collected, loaded onto a 4 ml Ni-NTA affinity column and chromatographed (Qiagen). The column was washed with the buffer A containing 20 mmol/l imidazole in the first step and 60 mmol/l in the second step. The retained proteins were eluted with the buffer A containing 200 mmol/l imidazole. The eluate was dialyzed against the buffer B (20 mmol/l Tris-HCl pH 8.0, 500 mmol/l NaCl, and 5 mmol/l dithiothreitol) overnight at 4°C to remove imidazole. The purified NS5B protein was concentrated using polyethylene glycol 6,000 and stored at -80°C in 10% glycerol. The identity of NS5B protein was determined by Western blot analysis with a rabbit polyclonal anti-NS5B antibody (kindly presented by prof. G. Luo, Kentucky University, Lexington, KY, USA). Proteins were assayed according to Bradford (1976).

Preparation and purification of RNA templates. The *wt* (–)3'T RNA was transcribed from *Pst*I-linearized pGEM-(–)3'T with T7 RNA polymerase (Takara, Japan) (Fig. 1). (–)3'T RNAs with various mutations at the 3'-end (Figs. 4 and 5) were transcribed from the corresponding PCR-amplified fragments containing the T7 RNA polymerase promoter in correct orientation. These PCR reactions were preformed with pGEM-(–)3'T plasmid as template. The T7 promoter primer was used as common forward primer, while the corresponding reverse primer introduced various mutations. To determine whether a sequence/structure of the first stem-loop of (–)3'T RNA (from the 3'-end) is important for RNA synthesis, one deletion and four substitution mutations were introduced into the first stem-loop by reverse primers (–)3'-Δ5-20, (–)3'-MSI-1, (–)3'-MSI-2, (–)3'-MSI-3 and (–)3'-MSI-4, respectively. The first stem-loop was deleted by removing a stretch of nts 5-20 ((-)3'T RNA-Δ5-20). The first stem-loop structure was disrupted by substitution of the stretch of CGGGGG at nts 5–10 (from the 3'-end) with CAAAAA ((–)3'T RNA-MSI-1) or by substitution of the stretch of CCCCCG at nts 15–20 with AAAAAC ((–)3'T RNA-MSI-2). The first stem-loop structure was retained in the case of changing the GC base pairs of the stem to CG base pairs ((–)3'T RNA-MSI-3) or substituting the base-paired GC nucleotides of the stem by base-paired UA nucleotides ((–)3'T RNA-MSI-4). All these mutated RNA templates are shown in Fig. 4B. To determine whether the first 3'C of (–)3'T RNA (Fig. 5A) is important for RNA synthesis, the reverse primers (–)3'ΔC, (–)3'-C/U, (–)3'-C/A, and (–)3'-C/G introduced a C deletion and C substitutions by U, A and G, resulting in mutated RNA templates designated (–)3'T RNA-ΔC, (–)3'T RNA-C/U, (–)3'T RNA-C/A and (–)3'T RNA-C/G, respectively (Fig. 5A).

After transcription with T7 NA polymerase, DNA templates were digested with RQ_1 RNase-free DNase (Promega) for 30 min at

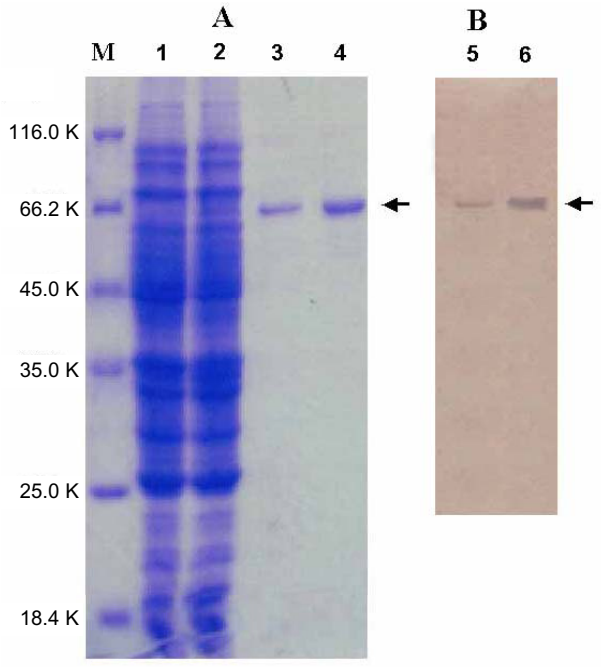


Fig. 2

Expression and purification of NS5B protein

A. SDS-PAGE. Protein size marker (lane M); cell lysate before IPTG induction (lane 1); cell lysate after IPTG induction (lane 2); NS5B protein eluted with 200 mmol/l imidazole (lane 3); purified NS5B protein after dialysis and concentration (lane 4). M_r values given in K.
B. Western blot analysis. The same sample as on lane 2 (lane 5); the same sample as on lane 4 (lane 6). Arrowheads indicate the positions of the NS5B protein.

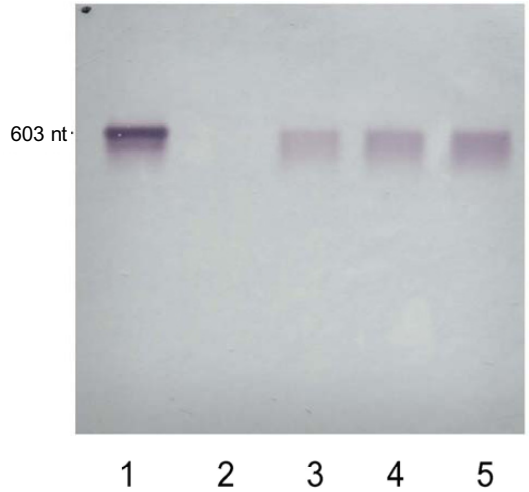


Fig. 3

Northern blot analysis of RNA synthesis from wt (–)3'T RNA

Control RNA (100 ng) transcribed from *Kpn*I-linearized pGEM-(–)3'T with Sp6 RNA polymerase (lane 1); negative control, reaction mixture without NS5B protein (lane 2); 2, 4 and 6 μg/ml (–)3'T RNA used in RdRp assay (lanes 3–5).

37°C. RNA transcripts were extracted with phenol-chloroform, ethanol-precipitated and analyzed by electrophoresis in 1.5% agarose gel in the presence of formaldehyde. The RNAs were recovered from the gel and purified using the RNaid Kit (Qbiogene). The concentration of purified RNA was assayed on the basis of A_{260}.

RdRp assay was performed in a mixture (50 μl) containing 20 mmol/l HEPES pH 8.0, 1.5 mmol/l $MnCl_2$, 100 mmol/l ammonium acetate, 1 mmol/l DDT, 500 μmol/l GTP, 250 μmol/l each of CTP, ATP and UTP, 40 U of RNasin (Biostar, Canada), 2–6 μg/ml RNA template, and 300 ng of purified NS5B protein (RdRp). After 2 hrs at 30°C, the reaction was stopped with 100 mmol/l EDTA. The RNA products were extracted with phenol-chloroform, ethanol-precipitated, air-dried and subjected to Northern bolt analysis. In RdRp assay for *wt* (–)3'T RNA template, different template concentrations (2, 4 and 6 μg/ml) were tested. In RdRp assay for mutated templates, only one template concentration (4 μg/ml) was used.

Northern blot analysis. A digoxigenin-labeled (–)3'T probe was transcribed from *Pst*I-linearized pGEM-(–)3'T plasmid with T7 RNA polymerase by adding 50 μmol/l digoxigenin-UTP (Roche) to the transcription mixture. The RNA probe was purified as described above, except that phenol-chloroform extraction was omitted. For Northern blot analysis, the dried RNA products were dissolved in DEPC-treated bidistilled water, mixed 1:1 with the 2× denaturing buffer (0.025% SDS, 0.025% bromophenol blue, 0.025% xylene cyanol, and 0.5 mmol/l EDTA pH 7.5 in 95%formamide), incubated at 85°C for 10 mins, chilled on ice for 10 mins, and subjected to agarose gel/formaledehyde electrophorsesis (see above). The gel was then capillary-blotted onto a Hybond nylon membrane in 20×SSC buffer and the blot was further processed in a standard way (Sambrook and Russell, 2001). The hybridization and immunological detection were performed using the DIG Easy Hyb Granules (Roche) and DIG Nucleic Acid Detection Kit (Roche), respectively according to the manufacturers' instructions. Briefly, after hybridization at 68°C for 6 hrs, the blot was washed first at low stringency (2×SSC and 0.2% SDS, 25°C) and then at high stringency (0.1×SSC and 0.1% SDS, 68°C), blocked for 30 mins (at room temperature), and incubated with a reagent containing anti-digoxigenin-AP (Roche) for 30 mins, both steps at room temperature. After a 2-fold washing with a buffer (0.1 mol/l maleic acid pH 7.5, 0.15 mol/l NaCl, and 0.3% Tween 20), hybridization bands were visualized with the BCIP/NBT solution. The intensities of the bands were determined using the KODAK ID 3.5 software.

Results

Expression and purification of recombinant NS5B protein

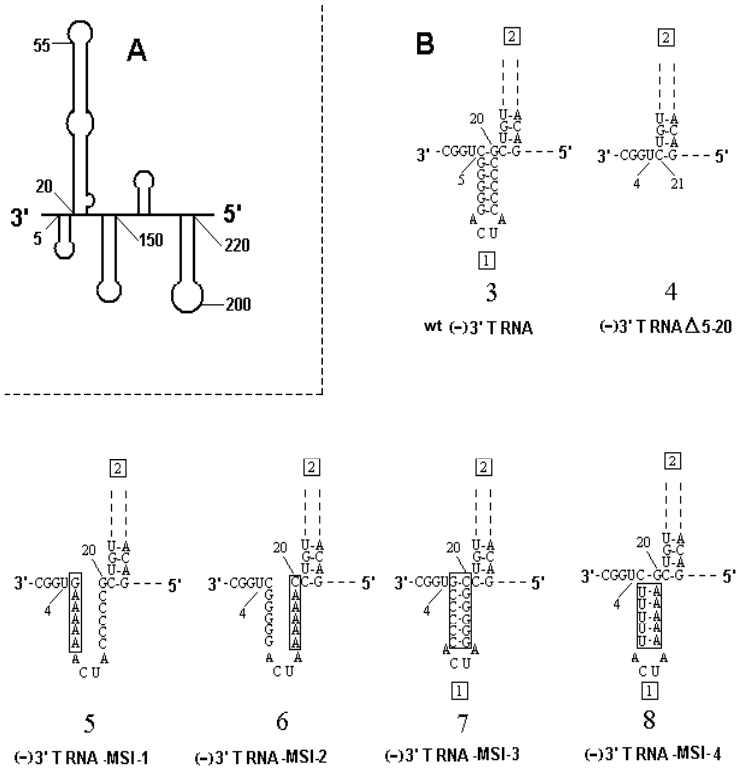
NS5B protein was expressed from pET5BΔ21 plasmid in *E. coli* after IPTG induction (Fig. 2, lane 2). As full-length native NS5B is not soluble, 21 hydrophobic amino acids from its C-terminus were removed to increase its solubility. And to facilitate the purification of NS5B, a 6-His tag was fused to its N-terminus. In this way a soluble NS5B protein of 66 K was purified by the Ni-NTA affinity chromatography to homogeneity (Fig. 2A, lanes 3 and 4). The protein was confirmed to be HCV NS5B by Western blot analysis using rabbit polyclonal NS5B antibodies (Fig. 2B).

RNA synthesis from wt (–)3'T RNA template by NS5B

To assess suitability of the NS5B purified from *E. coli* for RNA synthesis Northern blot analysis of products of RdRp assays was carried out (Fig. 3). The purified NS5B protein was able to synthesize an RNA product identical in size (603 nt) with the (–)3'T RNA transcribed from *Kpn*I-linearized pGEM-(–)3'T with Sp6 RNA polymerase. This indicates that the RNA synthesized RNA from the (–)3'T RNA template was a full-length product. In addition, with the increase in concentration of the template also the amounts of RNA products increased. Namely, with 2, 4 and 6 μg/ml RNA templates the intensities of the bands were 23.8%, 36.2% and 50.4%, respectively, of that of the control 100 ng RNA, indicating that the RNA synthesis was dose-dependent on the template concentration.

Effect of the first stem-loop of (–)3'T RNA on RNA synthesis

To determine the sequence and/or structural requirements of the first stem-loop for RNA synthesis, we introduced a number of mutations into the first stem-loop by nucleotide deletions and substitutions (Fig. 4B). The mutations distinctly influenced the synthesis of the positive RNA strand (Fig. 4C). When the first stem-loop was deleted in the mutated template (–)3'T RNA-Δ5-20, the RNA synthesis decreased by 92.7% as compared with that from *wt* (–)3'T RNA (Fig. 4C, lanes 3 and 4). When the secondary structure of the first stem-loop was disrupted by nucleotide substitutions in (–)3'T RNA SI-1 and (–)3'T RNA SI-2, the RNA synthesis



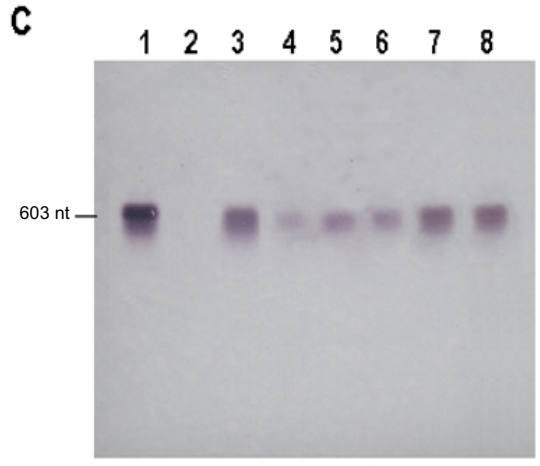


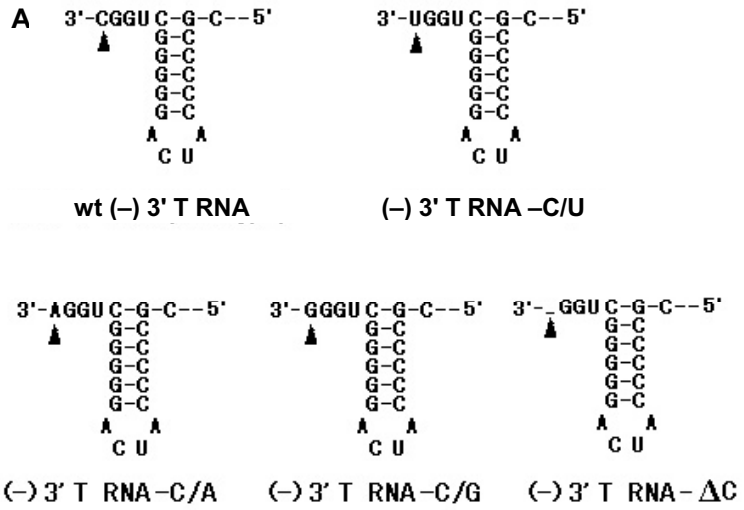
Fig. 4

Effect of the first stem-loop structure of (–)3'T RNA template on RNA synthesis

A. Secondary structure of the first 220 nts from the 3'-end of (–)3'T RNA (Schuster *et al*., 2002; Smith *et al*., 2002).

B. Sequences and structures of *wt* and mutated first stem-loop RNA elements. The first stem-loop structure in *wt* (–)3'T RNA (3); the first stem-loop deleted by removing nts 5–20 and resulting in a mutated RNA template (–)3'T RNA-Δ5-20 (4); the first stem-loop structure disrupted by substituting the stretch of CGGGGG (nts 5–10) with CAAAAA and resulting in (–)3'T RNA-MSI-1 (5); the first stem-loop structure disrupted by substituting the a stretch of CCCCCG (nts 15–20) with AAAAAC and resulting in (–)3'T RNA-MSI-2 (6); the side of the stem nucleotides switched (but the stem-loop structure retained) in (–)3'T RNA- MSI-3 (7); the GC base pairs in the stem substituted by UA base pairs (but the stem-loop structure retained) in (–)3'T RNA-MSI-4 (8). Open boxes refer to the sequences in the stem where the nucleotide substitutions occurred.

C. Northern blot analysis of RNA products from (–)3'T RNAs with various mutations at the first stem-loop. Control RNA, 100 ng (lane 1); negative control, reaction mixture without NS5B (lane 2), RNA products from *wt* (–)3'T RNA, (–)3'T RNA-Δ5-20, (–)3'T RNA-MSI-1, (–)3'T RNA-MSI-2, (–)3'T RNA-MSI-3, and (–)3'T RNA-MSI-4 (lanes 3–8, respectively).



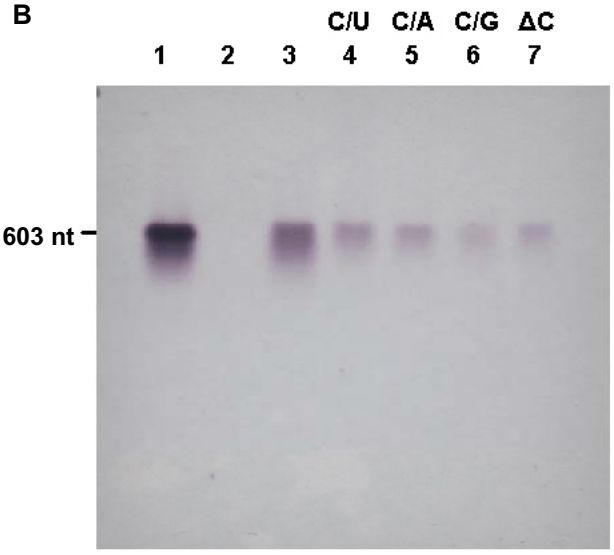


Fig. 5

Effect of the first 3'C of (–)3'T RNA on RNA synthesis

A. The structures of the first 21 nucleotides of *wt* and mutated (–)3'T RNAs. The first 3'C in *wt* (–)3'T RNA was substituted by U, A and G and deleted in (–)3'T RNA-C/U, (–)3'T RNA-C/A, (–)3'T RNA-C/G and (–)3'T RNA-ΔC, respectively. Arrowheads indicate the first 3'C and mutated nucleotides.

B. Northern blot analysis of RNA products from *wt* and mutated (–)3'T RNAs. Control, *wt* (–)3'T RNA (100 ng, lane 1); negative control, reaction mixture without NS5B protein (lane 2); RNA products from *wt* (–)3'T RNA, (–)3'T RNA-C/U, (–)3'T RNA-C/A, (–)3'T RNA-C/G and (–)3'T RNA-ΔC, respectively (lanes 3–7).

decreased by 74.3% and 78.4%, respectively (Fig. 4C, lanes 5 and 6). However, restoration of the stem-loop by compensatory mutations in the stem with different nucleotides restored the RNA synthesis too. When the first stem-loop structure was restored in (–)3'T RNA SI-3 and (–)3'T RNA SI-4, the RNA synthesis reached 91.3% and 86.5% of that with *wt* (–)3'T RNA (Fig. 4C, lanes 7 and 8). These results indicated that it is the secondary but not primary structure of the first stem-loop that plays an important role in the RNA synthesis. Although the mutations in the first stem-loop structure influenced the RNA synthesis, the size of RNA products from these mutated templates was not altered.

Effect of the first 3'C of (–)3'T RNA on RNA synthesis

Mutagenesis analysis was also used to investigate the role of the first 3'C of (–)3'T RNA in RNA synthesis. The first 3'C was deleted or substituted by U, A and G, respectively in the mutated (–)3'T RNA templates (Fig. 5A). The RNA syntheses from these mutated RNA templates decreased dramatically as compared with that from *wt* (–)3'T RNA (Fig. 5B). Namely, when the C was deleted or substituted by G the RNA synthesis decreased by 90.8% or 87.1%, respectively of the *wt* control (Fig. 5B, lanes 7 and 8). When the C was substituted by U or A, the RNA synthesis decreased by 66.2% or 75.9%, respectively of the *wt* control (Fig. 5B, lanes 4 and 5). These results indicated the first 3'C is essential for high efficiency of synthesis of positive RNA strand and plays an important role in initiation of RNA synthesis.

Discussion

The enzymatic activities of HCV NS5B have been studied in detail by using its recombinants. *In vitro*, the enzyme is able to initiate RNA synthesis by primer-dependent, copy-back and *de novo* (primer-independent) mechanisms. In this study, the positive RNA strand synthesized from (–)3'T RNA was a full-length product, suggesting that the initiation of synthesis of positive RNA strand from (–)3'T RNA template belongs to the *de novo* mechanism. This result is consistent with those of other reports. Although some of them indicate that 2–3 products of different size are generated from the negative RNA strand, a full-length product is predominant

(Oh *et al*., 1999; Reigadas *et al*., 2001; Piccininni *et al*., 2002).

To ensure that the RNAs are synthesized in appropriate amounts and have correct termini, the *de novo* initiation requires the 3'-end of template harboring *cis*-acting elements for replication. Luo *et al*. (2003) have reported that the 5'-proximal stem-loop structure of 5'-UTR is essential for the HCV RNA replication *in vivo*. It is possible that its complementary RNA element (the 3'-proximal stem-loop structure of the negative RNA strand) can affect the synthesis of positive RNA strand. In this study, when 3'-proximal stem-loop was deleted or its secondary structure was disrupted, the RNA synthesis decreased by 70–95%. However, the restoration of the stem-loop by compensatory mutations in the stem region was able to restore the RNA synthesis. These findings indicate that the 3'-proximal stem-loop of (–)3'T RNA is a *cis*-acting element for the synthesis of positive RNA strand. Thus, the effect of 5'-proximal stem-loop structure of 5'-UTR on HCV RNA replication *in vivo* may be attributed to its complementary RNA element on the negative RNA strand affecting the synthesis of positive RNA strand. Whereas the deletion of 3'-proximal stem-loop of (–)3'T RNA and disruption of its secondary structure resulted in dramatic decrease in the synthesis of positive RNA strand *in vitro*, the deletion or disruption of 5'-proximal stem-loop structure of 5'-UTR abolished the replication of subgenomic of HCV RNA in Huh 7 cells (Luo *et al*., 2003). This discrepancy may be largely due to two reasons. Firstly, in RdRp assay *in vitro*, the amounts of RNA templates and NS5B protein were high enough to facilitate the detection of the RNA product. In addition, the RNA templates and RNA products were very stabile in the *in vitro* system because of the usage of an RNase inhibitor (RNasin). However, in the experiments *in vivo*, the amount of the RNA template transfected into the cell is much less than that used *in vitro* and the RNA templates and products are easy to be degraded inside the cells. Therefore, a distinct decrease in RNA synthesis *in vitro* might be reflected in a similar effect on RNA replication *in vivo.* Secondly, besides the effect on the synthesis of positive RNA strand, the 5'-proximal stem-loop of 5'-UTR is important for optimal translation and perhaps also for RNA stability. Thus its mutation can affect viral RNA replication, translation and stability. These multiple effects can lead finally to the absence of RNA replication *in vivo.*

It has also been verified that the presence of 3'C favors the *de novo* initiation of RNA synthesis by viral RdRp polymerase (Kao *et al*., 2000; Zhong *et al*., 2000; Shim *et al*., 2002). In most of HCV genotypes and isolates, the first 3'-nucleotide of negative RNA strand of HCV is C. Therefore, we also investigated the role of the first 3'C in (–)3'T RNA in the synthesis of positive RNA strand. When the first 3'C was deleted or substituted by U, A and G, respectively, the RNA synthesis decreased dramatically, indicating that the first 3'C plays an important role in the synthesis of positive RNA strand. Butcher *et al*. (2001) and Bressanelli *et al*. (1999) have proposed a *de novo* initiation model from their work on bacteriophage Φ6 polymerase, whose structure is highly similar to that of HCV NS5B. In that model, 3'C provides the specificity of template, as steric hindrance within the binding pocket would prevent a larger adenine or guanine base from entering and electrostatic interaction would not favor the uracil base. The model has been further supported by a recent report proposing three NTP binding sites in the structure of HCV NS5B (Bressanelli *et al*., 2002).

Kao *et al*. (2000) have proposed that the template for *de novo* initiation by HCV NS5B requires a stable stem structure, a single-strand sequence at the 3'-end and C (as an initiation nucleotide) in this sequence. Their results came from the study on several RNA sequences unrelated to HCV RNA. Interestingly, we found that the 3'-proximal sequence/structure of negative RNA strand including the 3'-proximal stem-loop structure and the first 3'C satisfied these requirements well. Here we show that the first stem-loop and the first 3'C of negative RNA strand are two *cis*-acting elements for the synthesis of positive RNA strand. Hence our results support their proposal. The 3'-proximal sequence/structure of positive RNA strand, however, do not possess these two elements and do not satisfy all the requirements, which may lead to a weak template activity of 3'-UTR *in vitro* (Tanaka *et al*., 1996; Reigadas *et al*., 2001; Piccininni *et al*., 2002; Kim *et al*., 2002). Since the synthesis of positive RNA strand on the negative RNA strand as template is a necessary step in HCV RNA replication, these two *cis*-acting elements in negative RNA strand may represent useful targets for developing drugs inhibiting viral replication.

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