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Genetic Organization and Diversity of the 3′ Noncoding Region of the Hepatitis C Virus Genome[1]

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The 3′ noncoding region (3′ NCR) of the hepatitis C virus (HCV) genome contained in viral particles was analyzed by an RNA linker ligation followed by reverse transcription-polymerase chain reaction. Sequence analysis of the amplified fragment from four strains, including different genotypes 1b, 2b, 3a, and 3b indicated that the 3′ NCR is composed of between 200 and 235 nts. The sequence of the 3′ NCR consists of a type-specific region (immediately following the termination codon), a poly(U) stretch, a C(U)n-repeat, and highly conserved region termed the core element. The poly(U) stretch and C(U)n-repeat regions varied in length and in sequence among different genotypes. Core elements having putative secondary structure consisted of 98 or 100 nts and were highly conserved in all genotypes. Most of the nt changes found in different genotypes did not affect the secondary structure of the core elements, suggesting that this region may play an important role in replication, stabilization of the HCV RNA, and/or packaging of the genome. Most of the HCV-1b strains carried two U residues at the 3′ end of the core element, while the minor HCV-1b strains had no U residues, demonstrating that there are two variants in type 1b strains. Amplification of the core element using linker-primed cDNA was comparable with that using the 3′ proximal core element-primed cDNA, indicating that the 3′ end of HCV genome was terminated by an OH group.

Hepatitis C virus (HCV) is the major causative agent of non-A, non-B hepatitis (*5*). It is an enveloped virus containing a positive strand RNA genome of approximately 9400 nucleotides (nt) in length. Its genome consists of a relatively long (341-nt) 5′ noncoding region (5′ NCR), a large open reading frame, which encodes 3010 ~ 3033 amino acid residues, and a short 3′ NCR followed by a homopolymer tail of A (*9*) or U residues (*6, 11, 14, 27*). By analogy with flaviviruses (*4*), this polypeptide can be divided into a 5′ structural region consisting of the putative core and envelope proteins and a 3′ region corresponding to nonstructural (NS1 to NS5) proteins. HCV shows substantial nt sequence diversity throughout the viral genome and has been classified into several genotypes to date (*2, 19, 21*). The 5′ NCR is highly conserved among all genotypes (*3, 19*) and it shares significant sequence and secondary structure homology with those of pestiviruses (*1, 9*), suggesting an important role for the 5′ NCR in both replication and polypeptide translational initiation (*26*). The polypyrimidine tract between nt 191 and 199 is an important site for translation initiation and is the binding site for the cellular protein p89 (*29*).

Contrary to the 5′ NCR, the sequence of the 3′-NCR consists of a short sequence (27–70 nt), which was largely diversified among different genotypes, and a homopolymer tail of U residues. Putative stem-loop structures have been identified in the region both proximal to the 3′ end of the coding region and the 3′ NCR of the genome in all HCV groups in spite of considerable primary sequence differences (*10*). Yoo *et al.* (*30*) transfected human hepatome cells with *in vitro*-transcribed putative full-length cDNA. They apparently detected the replication of the transfected genome in cells, although the 3′ end of the transcribed RNA was terminated by either 15 U or 15 A residues, since the poly(U) tail was believed to represent the 3′-terminus of the genome with those of picornavirus (*16*).

Recently, Tanaka *et al.* (*22*) published information about the sequence following the poly(U) tail of the 3′ terminus of the HCV type 1b genome. They synthesized a cDNA of negative strand genome RNA from a patient's

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liver followed by the addition of a poly(G) tail by terminal transferase to assist the subsequent PCR. The longest 3′ NCR comprising approximately 200 nt included a 98-nt sequence with a putative stem-loop structure at the 3′ end. They also determined the 3′ NCR of type 2a genome of the viral genome using an RNA ligation linker method (*23*).

Tick borne encephalitis virus (TBEV), a family of *Flaviviridae* that is close to HCV, has a 3′ NCR that varies in length from 350 to 750 nt. Wallner *et al.* (*28*) analyzed the 3′ end of various strains and showed that the size heterogeneity was restricted to a variable region following the termination codon, whereas the most 3′-terminal 100 nt form a highly conserved core element. In some strains, e.g., Neudoerfl/263 and 132, the poly (A) stretch, which is believed to be the 3′-terminus of the genome, was found between the termination codon and the core element. Therefore, HCV and flavivirus genomes now seem to share common sequence organization in the 3′ NCR besides the coding region (*4, 8*). We describe here the cloning of the 3′ NCR compared between four HCV viral genome subtypes, the structural organization of the 3′ NCR, and the 3′-terminus of the genome.

HCV samples were obtained from patients with chronic hepatitis as follows; plasma samples 1257 (HCV-1b), 1509 (HCV-1b), and 4149 (HCV-1b) were obtained from a Japanese patient, B15 (HCV-1b) and 42 (HCV-2b) from Brazilian patients, sample 865 (HCV-3a) from an Australian patient that was previously reported (*27*), EM22 (HCV-3a) from a Spanish patient, and B1 (HCV-3b) from a Bangladesh patient. Genotypes were determined based on the slot blot hybridization using type-specific probe (*21*) and the sequence analysis of a portion of NS5B regions (360 nt) including GDD motif (*7, 13, 19*).

RNA linker (GAUUGAGCAUCGAAUUCACGAUACUG-CCG-NH_2) with an amino group at the 3′ end was synthesized with an ABI DNA synthesizer using phosphoramidites. Forty nanograms of the linker RNA was phosphorylated with 13 U of T4 polynucleotide kinase (Takara) in the presence of 0.5 μM RNase-free ATP (Boeringer Manheim) in 10-μl volume. RNA was prepared from 500 μl of plasma as previously described (*7*) and dissolved in 5 μl of diethylpyrocarbonate-treated water. To avoid a contaminant RNase attack as much as possible, ligation reaction was carried out under lower concentrations of ATP (1.5 μM), and shorter reaction time (1 hr) at 17° than those of previous report (*17*). Under these conditions, 50–60% of the linker joined with the target RNA with only a slight degradation of RNA. The ligation reaction was carried out in a solution of 1.5 μl of RNA, 4 ng of phosphorylated RNA linker, 3.3 mM dithiothreitol, 1.5 μM ATP, 12 U of RNase inhibitor (Takara), and 1 U of RNA ligase (Takara) in 6 μl reaction volume at 17° for 1 hr. The ligation products were precipitated with ethanol after addition of 4 μl water, 1 μl of 2 M sodium acetate, 1 μg glycogen (Boeringer Manheim), and 25 μl ethanol. Ligated products were washed with 70% ethanol, preprecipitated, and dried at room temperature.

Ligation products were dissolved into 3 μl water and converted into cDNA using 4 ng of primer #3′-4 (Table 1) and 100 U SuperScript II in a 10-μl solution volume following the manufacturer's instructions (Gibco BRL).

Oligonucleotides used in this study are listed in Table 1. PCR was run on at 94° for 1 min, 55° for 45 sec, and 69° for 1.5 min in first 2 cycles using Taq start (Stratagene) in 12.5 μl volume followed by 90° for 1 min, 60° for 45 sec, and 69° for 1.5 min with 48 cycles. Type 1b cDNAs were amplified with a primer set of type 1b-specific NS5B coding region #3′-1 and the linker region #3′-3. Type 2b cDNAs were amplified with a nested PCR using primer set of type-specific NS5B region #3′-2 and the linker #3′-3 (first 35 cycles) and with a primer set of type-specific TSR #3′-5 and the linker (#3′-3). The whole 3′ NCR of HCV genomes with type 3a and type 3b could not be amplified by any primers and any PCR conditions we used. Therefore, PCR was carried out by dividing it into two portions with 11 overlapping nts: one was the region between TSR and the 5′ site of conserved region using a primer set of #3′-17 and #3′-10 primers and the other was the whole conserved region with the linker using a primer set of #3′-11 and #3′-3. Southern blot analysis was used to monitor whether or not the 3′ NCR was amplified and to estimate their sizes.

When RT-PCR products from four samples of type 1b were analyzed by polyacrylamide gel electrophoresis followed by Southern blot analysis using poly$(A)_{30}$ as a probe (*15*), fragments containing the homopolymer tail of A or U residues were obtained as major products. These fragments accompanied a substantial tailing background but their major bands were almost the same size with 260 nt in length. PCR products from samples 1509 and 4149 contained a minor component fragment which was 30–60 nt longer than the major component. Each band was excised and sequenced. Results are shown in Fig. 1. The major bands with 260 nt in length were composed of the expected sequence of the type 1b-specific primer (NS5B) followed by noncoding sequence (*11, 24*), homopolymer stretch of U residues, CU or C(U)n-repeat regions, and conserved region comprising 98 nt found by Tanaka *et al.* (*22*). We designate this region as a core element which was first proposed by Wallner *et al.* in the 3′ end of tick-borne encephalitis virus (*28*). These results demonstrate that they were clearly derived from HCV-1b RNA due to the presence of the sequence between the termination codon and the start position of the poly(U) stretch, which we named as a type-specific region in the 3′ NCR (TSR). The core sequences were identical or almost identical except one nucleotide change to that reported by Tanaka *et al.* (*22*). However, all these samples contained additional two U residues at the end of

TABLE 1

Oligonucleotide Used in This Study

Oligonucleotide	Sequence (5′-3′)	Genotype and position[a]
#3′-1	ATAGAATTCTACCTGCTCCCCAACCG(AG)TGAA	1b:9340-1963
#3′-2	ATAGAATTCTTACTCCCCGCTCGGTAGA	2b:9246-9264
#3′-3	TCGGCAGTATCGTGAATTCGATGC	Linker
#3′-4	CGGCAGTATCGTGAATTCGA	Linker
#3′-5	AACCCTAGCTACACTCCATAGC	2b:9438-9459
#3′-7	AGCCGTGACTAGGGCTAAGATGG	CE[b]:30-8
#3′-9	GATCTGCAGAGAGGCCAGTATCAGC	CE:94-70
#3′-10	AGCCGTGACTAGGGCTAAGATGG	CE:8-30
#3′-11	TTTTGGTGGCT CCATCTTAGCC	1b:CE:-4-18
#3′-12	TTAAGGTGGCTCCATCTTAGCC3′	3a, 3b CE:-4-18
#3′-17	TTAGGATCCATTTGCTGCTTTGCCTACTCCTACT	3a, 3b:9339-9365
#3′-31	AAACAGCTAGCCGTGAC	CE:100-84

[a] The sequence numbers of 1b, 2b, and 3a are in the nucleotide sequences of HCV-J (*11*), HCV-J8 (*14*), and HCV-K3a/650 (*27*), respectively.
[b] CE is the core element.

the core element. Direct sequence analysis of PCR products also showed the presence of two U residues at the 3′ end.

In contrast to the 260-nt fragment, longer fragments generated from type 1b samples did not have the two U residues at the 3′ end (Fig. 1). They differ in the 260-nt fragment by slightly longer TSR comprising four or five C residues at its 3′ end, long poly (U) stretch interrupted by CCGC sequence and C residues, and no typical C(U)n-repeat. These results demonstrate that there are two variants in HCV-1b in regard to the 3′ NCR. We tentatively named each variant HCV-1b2U and HCV-1b0U, respectively. Other variants of the core elements were also found (nt change at position 65 or 83), but they seem to be insignificant since these nt changes do not extend to other regions. The nucleotide change from A to G residue at position 65 can still maintain the base-pair formation with U residue (see Fig. 3), whereas the nucleotide change from U to C residue at position 83 abolishes the base-pair formation with A.

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Sample(Type)          TSR                                  poly(U)stretch and C(U)n-repeat
4149-2U(1b)     UGAACAGGGAGCUAAACACU CCAGGCCAAUAGGCCAUUCCC    (U)35CG(U)3C(U)5CUUU(U)5G(U)4CUCUUUUCC(U)4
1257(1b)        -------------------- --------------------//   (U)33----------------------------------------
B15(1b)         -------------------- --------------------//   (U)35--------------C--------------C-------
1509-2U(1b)     -------------------- --------------------//   (U)35--------------C------------C---------
4149-0U-1(1b)   ---------------C---- -----------------C---C   (U)14CCGC(U)33C(U)9C(U)27C(U)5
4149-0U-2(1b)   ---------------C---- ---------------------C   (U)14CCGC(U)33C(U)9CC(U)27C(U)5
1509-0U(1b)     -------------------- -----------------C---C   (U)13CCGC(U)21C(U)11C(U)10CC(U)24C(U)4C(U)5
42(2b)          UAGAGCGGCAAACCCUAGCU ACACUCCAUAGCUAGUUUC      (U)10G(U)39CC(U)3C(U)4CCUUC(U)7CCUC(U)4CUU
865(3a)         UGAGUGGUAAGCUAACACUC CAAUUCUG                 (U)10C(U)34CUCUUUCCUAACUUUCCUAUUAUCUUAUAUCCUUCUUAA
EM22(3a)        -------------------- --------                 (U)47//////----------------------------------------
B1(3b)          UGAGUGGUAUACACUCCAAU UCUG                     (U)52CUUUCCUAACUUUCCUAUCAUCUUAUAUCCUUCUUAA

Sample(Type)                                                 Core element
4149-2U(1b)     GGUGGCUCCAUCUUAGCCCUAGUCACGGCUAGCUGUGAAAGGUCCGUGAG CCGCAUGACUGCAGAGAGUGCUGAUACUGGCCUCUCUGCAGAUCAUGUUU
1257(1b)        ---------------------------------------------------- ----------------------------------------------------
B15(1b)         ---------------------------------------------------- --------------G-------------------------------------
1509-2U(1b)     ---------------------------------------------------- --------------G-------------------------------------
4149-0U-1(1b)   ---------------------------------------------------- ---------------------------------C---------------//
4149-0U-2(1b)   ---------------------------------------------------- ---------------------------------C---------------//
1509-0U(1b)     ---------------------------------------------------- --------------G-------------------------------------//
42(2b)          ---------------------------------------------------- ---------------------C-UA-----U-----------------//
865(3a)         ---------------------------------------------------- ----------------------------------------------------//
EM22(3a)        ---------------------------------------------------- ----------------------------------------------------//
B1(3b)          ---------------------------------------------------- ----U-----------------UA-------------------A--//
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FIG. 1. Nucleotide sequences of the 3′ NCR of the HCV genome. Nucleotide sequences were shown by each genotype group and characteristic regions such as TSR, the poly(U) stretch with a C(U)n-repeat regions, and the core elements. One or two major sequences from the PCR products are listed in each sample. Nucleotides identical to representative sequence are depicted as horizontal bars and the gaps introduced for the alignment are shown by skew bars. Sample numbers and their genotype are shown to the right. 2U and 0U in sample name indicate presence and absence of two U residues at the 3′ end, respectively.

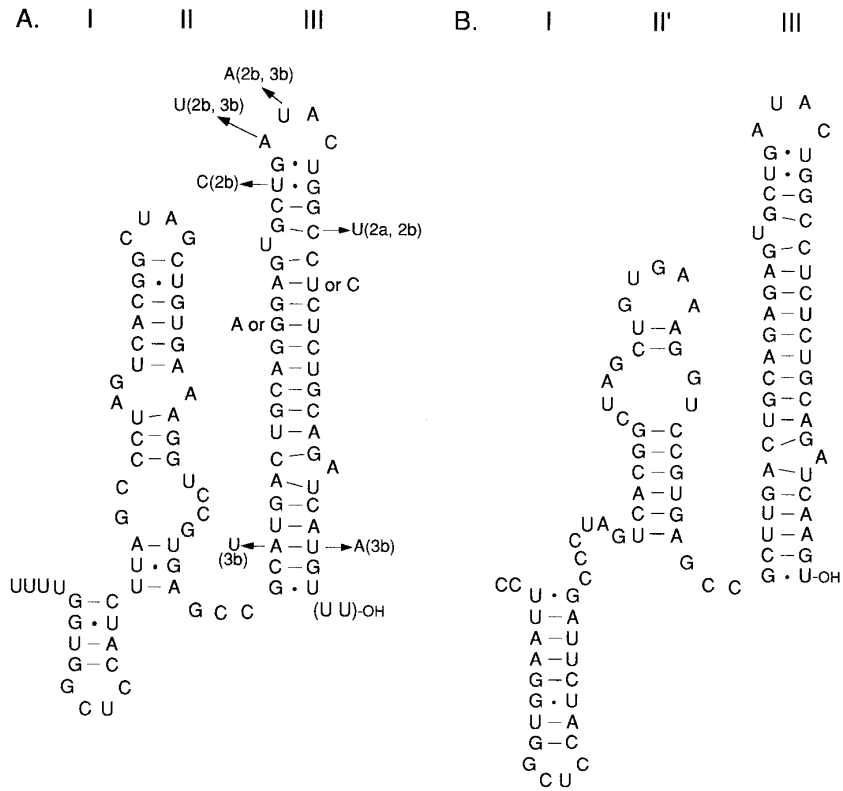

FIG. 2. Schematic representation of the predicted stem-loop structures within the core element found in the HCV-1b (left) and HCV-3b (right). Substituted nts found in different genotypes are indicated by the arrow. Two U residues at the 3′ end were found only in the HCV-RNA from major type 1b strain. We termed three hair-pin stem-loop structures I, II, and III. The stem-loop II potentially forms an alternative structure II′ which is likely to exist in the HCV-3b-RNA.

Other isolates such as type 2a, 2b, 3a, and 3b were very difficult to amplify by one-round PCR. After the second round of the PCR, several products appeared from type 2a and type 2b samples. The sequence of cloned cDNA of type 2b is shown in Fig. 1, which also contains TSR of type 2b (*14*), homopolymer tail which was interrupted by G residue, C(U)n-repeat, and the core element. In contrast to type 2b, all PCR products derived from type 2a RNA were not able to be cloned. Direct sequencing analysis indicated that the type 2a sequence was almost identical to that reported by Tanaka *et al.* (*23*), short poly(U) stretch, C(U)n-repeat, and the same core element as that of type 2b (data not shown). When the stems were named, in order from 5′ to 3′, I, II, and III, the nucleotide changes were found only in stem-loop III (Fig. 2). We precisely analyzed the 3′ end of type 2a and 2b, but no additional U residue was found.

The whole 3′ NCR of HCV-3a and HCV-3b genomes could not be amplified by any of the primers and by any of the PCR conditions we used. Therefore, PCR of the 3′ NCR was carried out by dividing it into two portions with 11 overlapping regions; one was from TSR (*18, 25, 27*) to the 5′ site of the core element and the second was the whole core element with the linker region. As shown in Fig. 1, both consist of TSR, poly(U) stretch, C(U)n-repeat, and the core element. Type 3b-derived sequence showed several nt changes in the stem-loop III of the core element (see Fig. 2). The core sequence obtained from two type 3a samples were the same as that of one of the three type 1b-2U. However, neither HCV-3a nor HCV 3b genome contained extra U residues at the 3′ end.

In type 3b sequence a U-A pair replaced the A-U pair of type 1b keeping the free energy of stem-loop III. However, HCV-3a and -3b RNAs have the potential to form a larger stem-loop I structure by the addition of four base-pairs at the 3′ end of the C(U)n-repeat region as shown in Fig. 3b. If the stem-loop I is strengthened, the neighboring stem structure is no longer a pair of the stem-loop II due to the alternation of base pair partners. Thus, two alternative structures are predicted with respect to middle stem loop, II or II′ as illustrated in Fig. 2. The failure of amplification of the whole 3′ NCR from HCV-3a and HCV-3b may attribute to a strong secondary structure of the core element.

When comparing these four HCV-1b RNA, the length of poly(U), the sequences of C(U)n-repeat and the core sequence are similar to each other despite the fact that these samples were collected from distant geographical regions and distinct from that of other isolates demon-

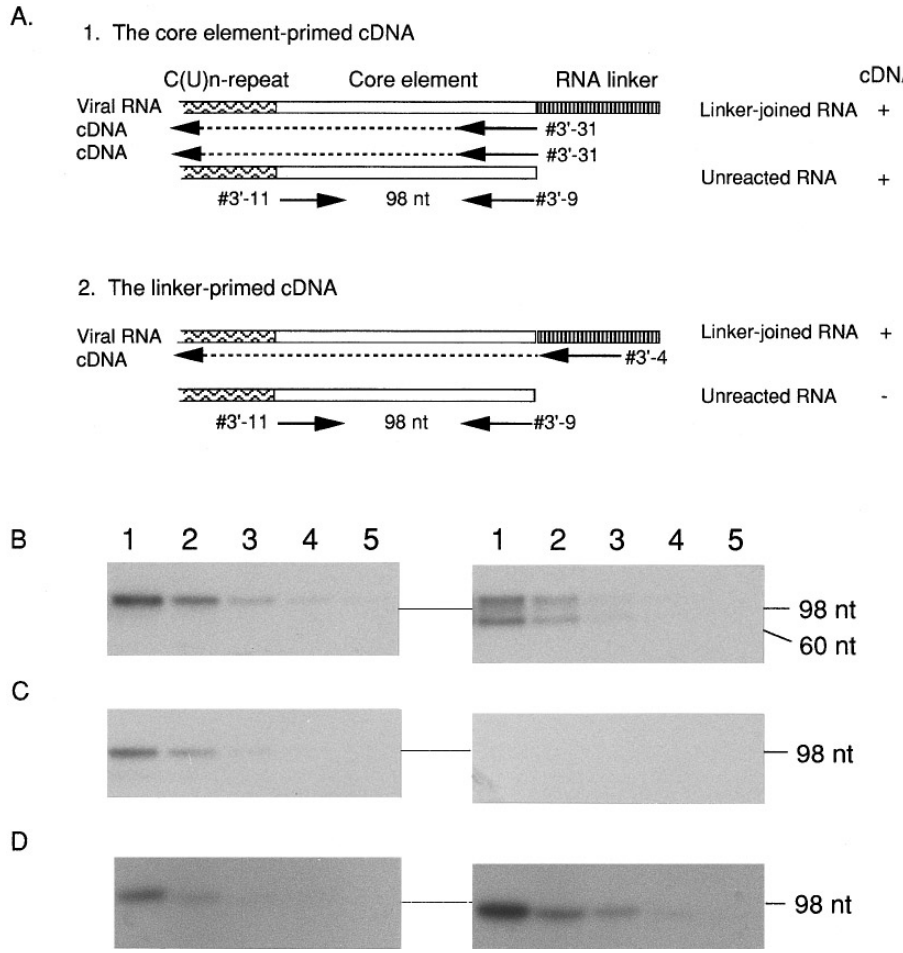

FIG. 3. Analysis of the 3′ end of HCV genome. (A) Strategy of the experiments. After ligation of HCV-RNA and the linker RNA, cDNA was synthesized from either proximal of the 3′ end of the genomic region (#3′-31) or the linker site (#3′-4) and PCR of the core element was carried out using primers #3′-11 and #3′-9. The ratio of the amplified fragment from the genome-primed cDNA and that from the linker primed cDNA would estimate whether or not the 3′ OH group of the HCV-RNA is free. (B) Southern blot analysis of PCR products of the core elements. cDNA was synthesized from ligation products of HCV-RNA (from sample B15) and the linker RNA using the primer #3′-31 (left) or #3′-4 (right) followed by PCR of the core element under the condition of 94° for 30 sec, 55° for 20 sec, and 72° for 10 sec, with 35 cycles in 50 μl reaction volume. The PCR product was then divided into five equal portions (10 μl each) and was subjected to additional PCR with 1–5 cycles. Amplified fragments (2 μl) were electrophoresed on 4% polyacrylamide gel, transferred on Hybond-N+ by alkali fixation method (*15*), and hybridized with ^{32}P-labeled oligonucleotide #3′-7. Lanes 1–5 are the product taken from cycles 40–36, respectively. (C) cDNA was also synthesized from nonligated HCV-RNA using the primer #3′-31 (left) or #3′-4 (right) followed by PCR of the core element. (D) Plasmid containing the 3′ NCR of sample at the *Eco*RV restriction enzyme site of T7 Blue (Novagen) was digested with *Bam*HI restriction enzyme and RNA was synthesized by T7 RNA polymerase. After treatment with DNase I twice, RNA was diluted and used for cDNA synthesis using the primer #3′-31 (left) or #3′-4 (right) followed by PCR of the core element. Lanes 1–5 are the products taken from cycles 35–31, respectively.

strating that these 3′ NCR sequences are also useful for genotyping. It is noted that the sequence analysis of 10 clones obtained from 4149 (type 1b), 10 clones from 42 (type 2b), and 9 from EM22 (3a) represented a number of different sequences in the poly(U) and the C(U)n-repeat regions; however, all clones seem to remain within the range of their respective type. The length of homopolymer was partly caused by PCR error, but variation of the C(U)n-repeat region is not likely to be generated by PCR error, because the cloned sequence is conserved during PCR. These results suggest that the C(U)n-repeat region is one of the most variable regions in the HCV genome.

Although the results obtained above suggest the presence of an OH group at the 3′ terminus, the question is still needed to be addressed whether all of the 3′ terminus of the HCV genome carries a free OH group. The possibility cannot be ruled out that the 3′-end was modified and only a small portion was cleaved at the sequence obtained above after the ligation reaction. Therefore, we attempted a qualitative analysis of the efficiency of the joining reaction between the linker RNA and the HCV RNA using B15 sample (HCV-2U type). If there were many unreacted HCV RNA as we expected, then the 3′ end of the HCV genome would be modified.

Figure 3A illustrates our strategy. After the joining reaction between the linker RNA and the HCV RNA, the reaction mixture was divided into two parts and cDNA was synthesized using two different primers; one annealed with a linker site and the other annealed with a sequence close to the 3′ terminal end of the core element. PCR was then carried out in the core element. If the 3′ terminus was modified, the overall yield of PCR product obtained using the linker-primed cDNA should be less than that obtained using the internally primed cDNA. Southern blot analysis of the PCR products obtained after 36–40 cycles from two different cDNAs (Fig. 3B) indicated that the both PCR products were linearly increased as much as 40 cycles. However, the PCR product from internally primed cDNA was a single component, whereas there were two components from the linker primed cDNA. No PCR product was obtained when the linker-primed cDNA was synthesized before the ligation reaction (Fig. 3C). Sequence analysis of these two amplified fragments revealed that the larger fragment contained the whole core element while the smaller one contained the region between only positions 22 and 31 of the core with primer sequences. This unexpected priming reaction was caused by the presence of slightly homologous sequence with that of the primer. Amplification of unexpected fragments suggests that the addition of nts to the 5′ terminus of cDNA may alter the conformational change of the secondary structure in the core elements. Comparing the two PCR products, amplification using linker primed cDNA was about twofold more effective than that using the internally primed cDNA. When the same experiment was performed using a control RNA which synthesized *in vitro* from cloned cDNA of the B15-3′ NCR with the linker, amplification products from the linker-primed cDNA were about three-times more than that from the internal primed cDNA (Fig. 3D). Taken together with these results, ligation reaction seems to be more than 100%. This discrepancy may be partly caused by additional nts at the 3′ end of the linker. Therefore, we can conclude that our ligation reaction is very effective and that the 3′ terminus of HCV genome contains a free OH group. We also did the same experiments using B1 samples (type 3b) and obtained the same results (data not shown).

Tanaka *et al.* reported the 3′ NCR of type 1b and type 2a (*22, 23*) and Kolykhalov *et al.* also determined the 3′ NCR of type 1a and type 2b (*14*). However, there was no report of the two extra U residues at the 3′ end of the core element in HCV-type 1b and 3′ NCR of type 3a and 3b. These discrepancies might be due to different ligation conditions or the presence of other different subtypes, since our samples used in this experiment were still small in number. Based on the Tanaka *et al.* report, most of the minus strand synthesis starts from the edge of the plus strand RNA. The question remains whether the HCV-1b2U genome primarily contains this sequence or whether two extra U residues are added postreplicationally. In all our tests thus far, neither type 1a nor type 2a RNA had an extra U residue at the 3′ end.

Little is known about the function of the 3′ NCR of HCV genome. Yoo *et al.* (*30*) transfected human hepatome cells (Huh7) with *in vitro*-transcribed HCV RNA that carried a homopolymer tail of either A15 or U15 at the 3′ terminus and apparently succeeded the replication of the transfected genome in cells. These results suggest a nonessential function for the C(U)n-repeat and the core element in the replication process. However, high conservation of the primary and secondary structure of the core elements and the presence of poly(U) stretch and C(U)n-repeat may play an important role in regulation of replication and/or packaging process.

Biological functions of poly(U) stretch, C(U)n-repeat, and the core elements remain to be elucidated.

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