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Structure of the 3′ Terminus of the Hepatitis C Virus Genome

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Hepatitis C virus (HCV), a positive-strand RNA virus, has been considered to have a poly(U) stretch at the 3′ terminus of the genome. We previously found a novel 98-nucleotide sequence downstream from the poly(U) stretch on the HCV genome by primer extension analysis of the 5′ end of the antigenomic-strand RNA in infected liver (T. Tanaka, N. Kato, M.-J. Cho, and K. Shimotohno, Biochem. Biophys. Res. Commun. 215: 744–749, 1995). Here, we show that the novel sequence is a highly conserved 3′ tail of the HCV genome. We repeated primer extension analyses with four HCV-infected liver samples and found the 98-nucleotide sequence in all the samples. Furthermore, experiments in which RNA oligonucleotide was ligated to the 3′ end of the HCV genome existing in infectious serum revealed nearly identical 3′ termini with no extra sequence downstream from the 98-nucleotide sequence, suggesting that this sequence is the tail of the HCV genome. This tail sequence was highly conserved among individuals and even between the two most genetically distant HCV types, II/1b and III/2a. Computer modeling predicted that the tail sequence can form a conserved stem-and-loop structure. These results suggest that the novel 3′ tail is a common structure of the HCV genome that plays an important role in initiation of genomic replication.

Hepatitis C virus (HCV), a major causative agent of non-A, non-B viral hepatitis, has about 9.5 kb of positive-stranded RNA genome with remarkable sequence diversity among individual isolates (for a review, see reference 3). Little is known about the molecular mechanisms of replication of the virus, including genomic replication and virion formation. Development of such studies has been limited by the low efficiency of virus replication in cell culture (19) and by the difficulty in synthesizing an infectious full-length RNA construct (23). Our aim was to study how the HCV genome replicates. Precise information about the complete genomic sequence, especially in the 3′-terminal region, was needed to develop an experimental system for studying the genomic replication of HCV, because the genomic replication of positive-strand RNA viruses is initiated from the 3′-end region, where specific replication mechanisms involve primary and secondary structures. Most previous reports, including ours, described a poly(U) stretch as the 3′-end structure of the HCV genome (4, 5, 9, 14, 15, 20, 22), and one group reported a poly(A) stretch instead of poly(U) (8).

Identification of the 3′ terminus of the HCV genome by primer extension analyses. Recently, we found a novel 98-nucleotide sequence downstream of the poly(U) stretch on the type II/1b HCV genome in infected liver tissue (21). The sequence was designated the 3′ X tail. To confirm whether the 3′ X tail is truly the 3′ end of the genome, primer extension-anchored PCR analysis was performed on three independent liver samples (samples B, C, and D), in addition to the previous sample (sample A) (Fig. 1 and 2A). These liver samples were the noncancerous portion of resected hepatoma tissue infected with type II/1b HCV. Prior to the reverse transcription (RT) reaction, the 3′ termini of the RNA molecules were chemically modified (7) to reduce nonspecific reactions such as self-priming or random-priming RT. The products of the anchored nested PCR were analyzed by Southern blotting by using DNA oligonucleotide (oligoDNA) probes 9381 and R1, which hybridized to sequences of the conventional 3′ untranslated region (UTR) and the 3′ X tail, respectively (Fig. 2B). The bands around 200 bp apparently reacted with both probes and, therefore, represented the clones extending from the conventional 3′ UTR across the poly(U) stretch to the 3′ X tail; probe 9381 was somewhat less reactive than R1 was. The lower bands of about 100 bp reacted with probe 9381 alone and probably represented clones ending with a poly(U) stretch. By sequence analysis of the longer clones, all the liver samples were found to carry the 98-nucleotide sequence downstream of the poly(U) stretch. Consensus sequences, determined for each liver sample, are aligned in Fig. 2C. The 98-nucleotide sequences of four independent liver samples were identical; on the other hand, considerable variations in the conventional 3′ UTR sequences were observed. No additional sequence extending downstream from the 98-nucleotide 3′ X-tail sequence was found in any of the liver samples. To identify the terminus of the 3′ X-tail sequence, we repeated the primer extension analysis of the four liver RNA samples; the RT was initiated from inside the 3′ X tail by primer U5, and the cDNA was tailed with dC and dT. Again, the sequencing analysis did not reveal additional sequences extending downstream of the 98-nucleotide sequence in any of the four liver RNAs. The terminal nucleotide of the 3′ X-tail sequence was identified as the 98th nucleotide, a U.

Several C, CC, or CCC residues appeared at intervals of 2 to 5 U residues at the end of the poly(U) stretch (designated the transitional region). Similar sequence motifs [(U)UUCC(C)] were found in the pY1 polypyrimidine tract of the hepatitis A virus 5′ UTR (18). The sequence pattern of the transitional region was heterogeneous among individuals (Fig. 2C) and even within the same individual, in contrast to the highly conserved 3′ X tail. The poly(U) stretch included small numbers of other nucleotides, mainly C and sometimes G or A, repre-

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A

9325(type II/1b): 9325-9344 5'-TACTCCTACTCTCTGTAGGG-3'
9348(type II/1b): 9348-9367 5'-GGCATCTACCTGCTCCCCAA-3'
9363Xh(type II/1b): 9363-9382 5'-GCGTAATCTCGAGCCCAACCGGTGAACGGGGAG-3'
9381(type II/1b): 9381-9412 5'-AGCTAAACACTCCAGGCCAATAGGCCATCCCC-3'
2a9356(type III/2a): 9356-9375 5'-GTGTCGCGTGCCCGACCCCG-3'
2a9380Xh(type III/2a): 9380-9399 5'-GTAATGTCTCGAGTTACTCCTTGGCCTACTCCT-3'
XS: 5'-TGCTGTCGACTACTCGAGCA-3'
G18XS: 5'-TGCTGTCGACTACTCGAGCAGGGGGGGGGGGGGGGGG-3'
AdpXS: 5'-TGCTGTCGACTACTCGAGCAACCTCTGGATGTCCGAAGAC-3'
Adp (RNA): 5'-CCCGUCUUCGGACAUCCAGAGGUAU-3'

UP2: (-)11'-9' 5'-TTTCCTTCTTTGGTGGCTCC-3'
U6: (-)4'-16' 5'-CTTTGGTGGCTCCATCTTAG-3'
U9: 2'-21' 5'-GTGGCTCCATCTTAGCCCTA-3'
U5: 8'-27' 5'-CCATCTTAGCCCTAGTCACG-3'
U7EXh: 13'-32' 5'-TACGAATTCTCGAG TTAGCCCTAGTCACGGCTAG-3'
R6Xh: 29'-48' 5'-CGCGATAACTCGAGCACGGACCTTTCACAGCTAG-3'
R3: 34'-53' 5'-CGGCTCACGGACCTTTCACA-3'
R5: 45'-64' 5'-CTGCAGTCATGCGGCTCACG-3'
R2: 55'-74' 5'-TCAGCACTCTCTGCAGTCAT-3'
R1: 64'-98' 5'-ACATGATCTGCAGAGAGGCCAGTATCAGCACTCTC-3'
RP2: 79'-98' 5'-ACATGATCTGCAGAGAGGCC-3'

B

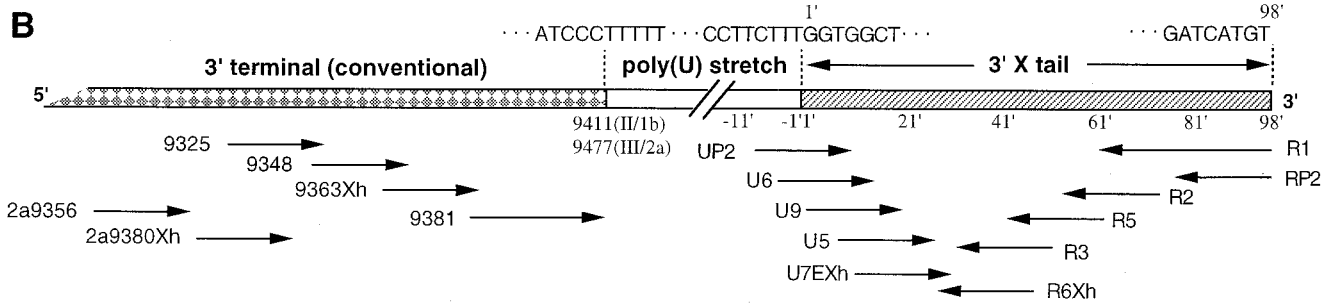

FIG. 1. Oligonucleotides used in this study. (A) Nucleotide positions and sequences of oligoDNAs and an oligoRNA; (B) location and direction of the oligoDNAs with HCV sequences. The nucleotides in the 3′ X-tail sequence are numbered 1′ to 98′ (the initial G residue of the GGUGG motif is defined as nucleotide 1′) to distinguish them from conventional HCV sequences. The nucleotides in the poly(U) stretch upstream from the 3′ X tail are shown as minus (−) numbers for convenience. Nucleotide positions of type II/1b or III/2a HCV sequences in the conventional 3′-terminal region correspond to those of HCV-JT (22) or HC-J6 (15), respectively. Some oligomers with the Xh or EXh suffix have additional tag sequences (underlined) carrying an *Xho*I site.

senting characteristic patterns for each sample. The poly(U) stretch varied in length with about 60 to 80, 50 to 80, 30 to 50, and 70 to 90 nucleotides for the clones from liver samples A, B, C, and D, respectively. The observed variability in the length of the poly(U) stretch within the same liver sample was less meaningful, because the length of the stretch may have been shortened during experimental procedures such as PCR amplification and enzymatic sequencing reactions (data not shown).

Identification of the 3′ X-tail sequence in infectious serum HCV by using an oligoRNA ligation technique. RNA was obtained from serum sample 1B-1 infected with type II/1b HCV, which has been used in our laboratory as an inoculum for the study of infection and replication of HCV in a cultured cell system (10, 11) (this serum sample is renamed in the present article). Serum sample 1B-1 should contain infectious HCV virions. 5′-end-phosphorylated oligoRNA was ligated to the 3′ end of the RNA molecules, and RT-nested PCR was performed within the 3′ X-tail sequences and the oligoRNA sequence (Fig. 3A). The PCR product gave an intense band of the expected size (about 140 bp) and a smear, or weak bands, of longer sizes (Fig. 3B). The band reacted with oligoDNA probe R1 on Southern hybridization (Fig. 3B) and was subjected to sequence analysis. In most of the analyzed clones, the oligoRNA sequence appeared just downstream of the 3′-terminal U residue of the 3′ X-tail sequence (Fig. 3C). A small number of the longer clones were detected, but the added sequence was identified as two or more tandemly repeated oligoRNAs ligated to the 3′ X tail. No extra sequence was found between the 3′ terminus of the 3′ X tail and the ligated oligoRNA.

Detection of the 3′ X-tail sequence from clinical samples. A set of primers for RT-nested PCR was prepared to detect positive-strand sequences of the 3′ X tail. The size of the product of the nested PCR was 67 bp. Uninfected liver RNA samples (N1, N2, and N3) that were negative for HCV and hepatitis B virus infections were subjected to RT-nested PCR-Southern blot analysis (Fig. 4A, left). No positive signal was observed in these uninfected RNA samples, in contrast to the situation for the positive control, HCV-infected liver sample A RNA (lane A). The liver RNA samples infected with HCV (samples A, B, C, and D) all contained positive-strand RNAs of the 3′ X tail (Fig. 4A, right). The DNA fraction from these HCV-infected livers (digested with *Eco*RI and *Hin*dIII) gave no positive signal on nested PCR-Southern blot analysis (data not shown).

Similarly, we analyzed 23 HCV-infected serum samples; 3 of the serum samples were paired with liver samples A, C, and D (sample B was not available), 1 serum sample was 1B-1, and the other 19 samples were sera that had been confirmed by a second-generation enzyme-linked immunosorbent assay (typing was not done). In this study, the RNA samples were examined without chemical modification of the 3′-terminal nucleotide (7). A representative experiment is shown in Fig. 4B. The bands showing strong positive signals by Southern blotting (serum samples E, F, I, J, and L) also were visible by ethidium bromide staining. Samples G and H were positive by Southern blotting but negative by ethidium bromide staining. Samples K and M were negative even by Southern blotting. Of a total of 23 HCV-positive serum samples, 19 were positive for the detection of positive-strand 3′ X tail as confirmed by Southern blotting. As expected, the 3′ X-tail sequence was not detected

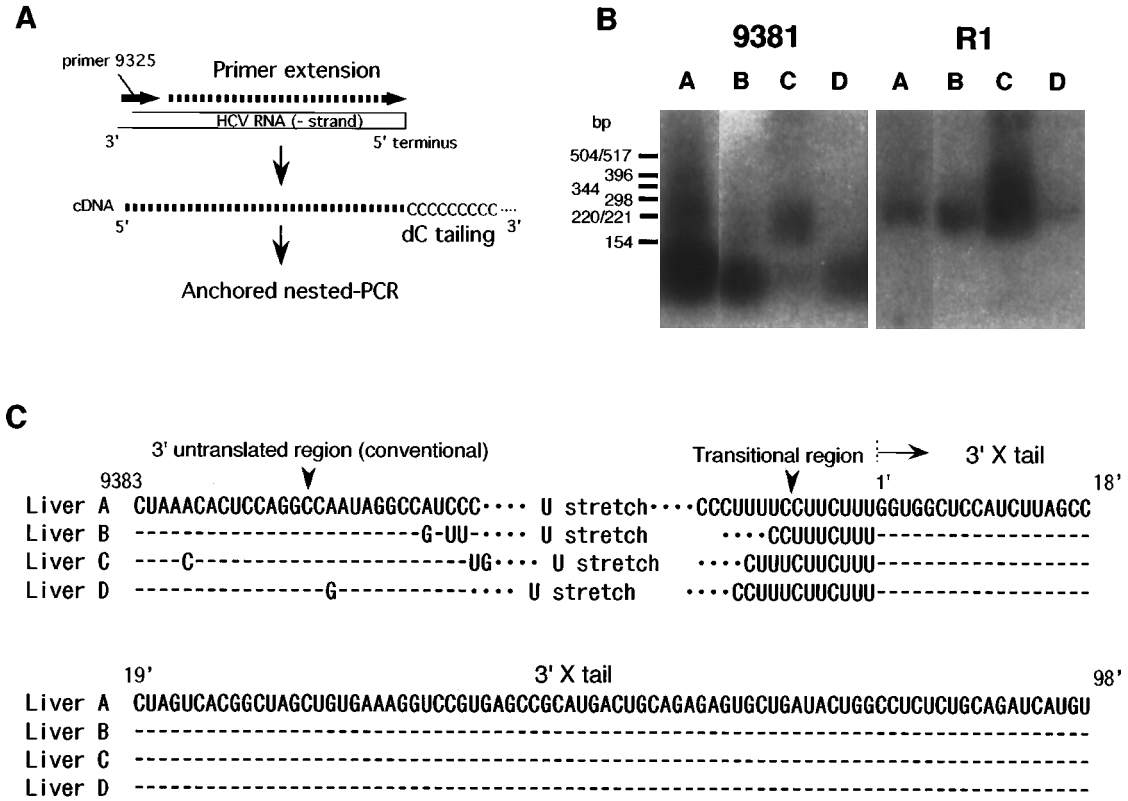

FIG. 2. 3′-terminal region of type II/1b HCV genome obtained by primer extension analysis of the negative-strand RNAs in liver. (A) Experimental strategy for the primer extension analysis. The extreme 3′ end of the HCV genome was cloned by primer extension on the 5′-end region of the antigenomic-strand RNA found in infected liver, dC tailing of the cDNA, and anchored nested PCR as described previously (21). The RT was performed with chemically modified (7) liver RNA (total of 7.5 μg in five tubes) with primer 9325. The dC-tailed cDNA was amplified by nested PCR with the following primers: first PCR, 9348 and G18XS; second PCR, 9363Xh and XS. (B) Southern blot of primer extension products. The PCR products from liver samples A, B, C, and D were analyzed by 5′-end-labeled ^{32}P-oligoDNA probes 9381 (left) and R1 (right). (C) Alignment of the 3′-terminal sequences of the HCV genomes obtained from different liver samples. Consensus sequences determined from five or more clones for each liver sample are shown. Dashes denote nucleotides identical to those in the sequence of liver sample A. Sequences in the poly(U) stretch are omitted except for those in the transitional region. The sequence of the transitional region shown here is a dominant pattern in each liver sample.

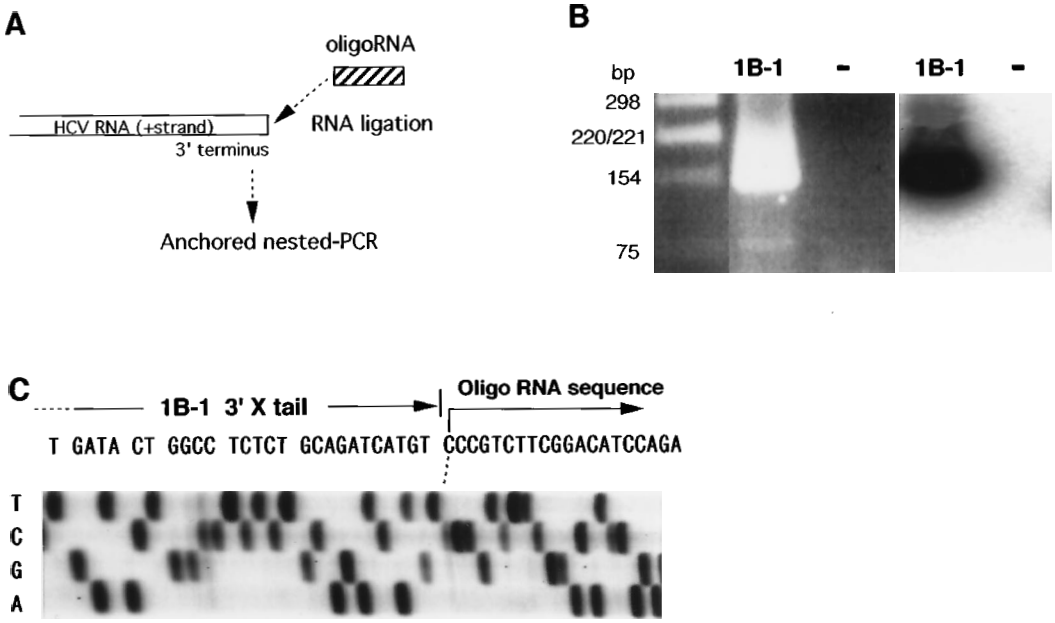

FIG. 3. Identification of the 3′ X tail from infectious serum sample 1B-1 by oligoRNA ligation. (A) Experimental strategy. An oligoRNA, Adp, was synthesized by Cruachem Kyoto Research Centre (Kyoto, Japan) and its 5′ terminus was phosphorylated with T4 polynucleotide kinase (Takara, Shuzo, Otsu, Japan) (17). The reaction mixture for RNA ligation (20 μl) contained 50 mM Tris-Cl (pH 8.1), 20 mM $MgCl_2$, 10 mM dithiothreitol, 2 μg of bovine serum albumin, 1 μl of RNasin (Promega, Madison, Wis.), 5 μM ATP, serum RNA (derived from 375 μl of 1B-1 serum), the 5′-phosphorylated oligoRNA (20 pmol), and 50 U of T4 RNA ligase (Takara Shuzo) and was incubated at 10°C for 15 h. RT-nested PCR was performed on one-half of the RNA fraction. The RT was done at 50°C for 1 h with primer AdpXS in a 20-μl reaction mixture. The RT product (1.5 μl) was amplified by nested PCR (21) with the following primers: first PCR, U6 and XS; second PCR, U7EXh and XS. (B) PCR product detected by ethidium bromide staining (left) and by Southern blotting with probe R1 (right). The "−" denotes a negative-control experiment performed without RNA. (C) Sequencing autoradiogram for the 3′-terminal region of the 3′ X tail.

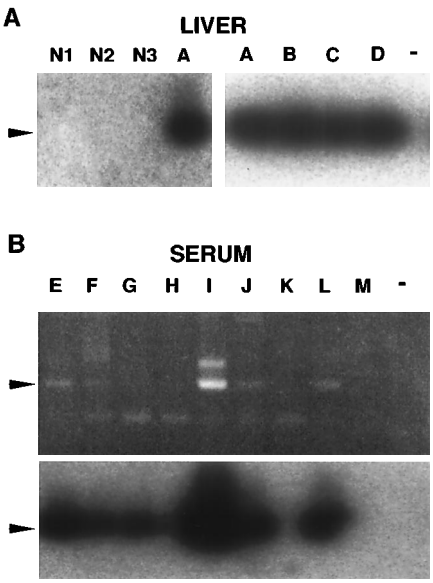

FIG. 4. Detection of the 3′ X-tail sequence in HCV-infected liver and serum samples. Liver RNA (1 μg) or serum RNA (derived from 50 to 80 μl of serum) was analyzed by RT-nested PCR and Southern blotting with probe R3 as described in the legend for Fig. 3A. The primers were as follows: RT, RP2; first PCR, UP2 and RP2; second PCR, U5 and R2. Arrowheads indicate the positions of the positive products. For the negative control (−), the same RT-nested PCR was performed without RNA. (A) Analysis of uninfected (N1, N2, and N3) and infected (A, B, C, and D) liver RNA. (B) Analysis of HCV-infected serum RNA. The products were analyzed by ethidium bromide staining (top) and Southern blotting with probe R3 (bottom). Letters E to M denote sera from different patients.

by RT-nested PCR-Southern analysis in 10 hepatitis B virus-infected serum samples (HBs antigen positive and HBe antigen or anti-HBe antibody positive) and in 10 uninfected serum samples (data not shown).

The 3′ tail of the type III/2a HCV genome. Type III/2a is the second most prevalent HCV type in Japan; however, it is the type most genetically distant from type II/1b, and more than 30% nucleotide diversity was observed between the two types. Two serum samples (X and Y) were confirmed to be infected with type III/2a HCV alone, as described by Mori et al. (12). Both samples were positive for the type II/1b HCV tail sequences by RT-nested PCR-Southern blot analysis (Fig. 5A), suggesting that the type III/2a genome had a 3′ tail sequence similar to that of the type II/1b genome. To clarify the sequence of the type III/2a 3′ tail, we performed two overlapping sets of RT-nested PCR and oligoRNA ligation experiments (Fig. 5B). The 3′ tail sequence was constructed by connecting sequences of the overlapping PCR clones (Fig. 5C). For the alignment, the 3′-terminal sequence obtained from serum sample 1B-1 is also shown in Fig. 5C. The type III/2a HCVs in sera had a 98-nucleotide 3′ X-tail sequence very similar to that of serum sample 1B-1. Nucleotide differences were seen in only four positions: nucleotides 72′, 74′, 75′, and 81′. The lengths of the poly(U) stretches were 20 to 40 and 50 to 60 nucleotides for samples X and Y, respectively. A remarkable difference between the type III/2a and II/1b HCVs was seen in a transitional region, where an additional short conserved sequence was present in the type III/2a sequences. Three A residues spaced at intervals of 4 nucleotides in the transitional region were apparent in the two type III/2a samples.

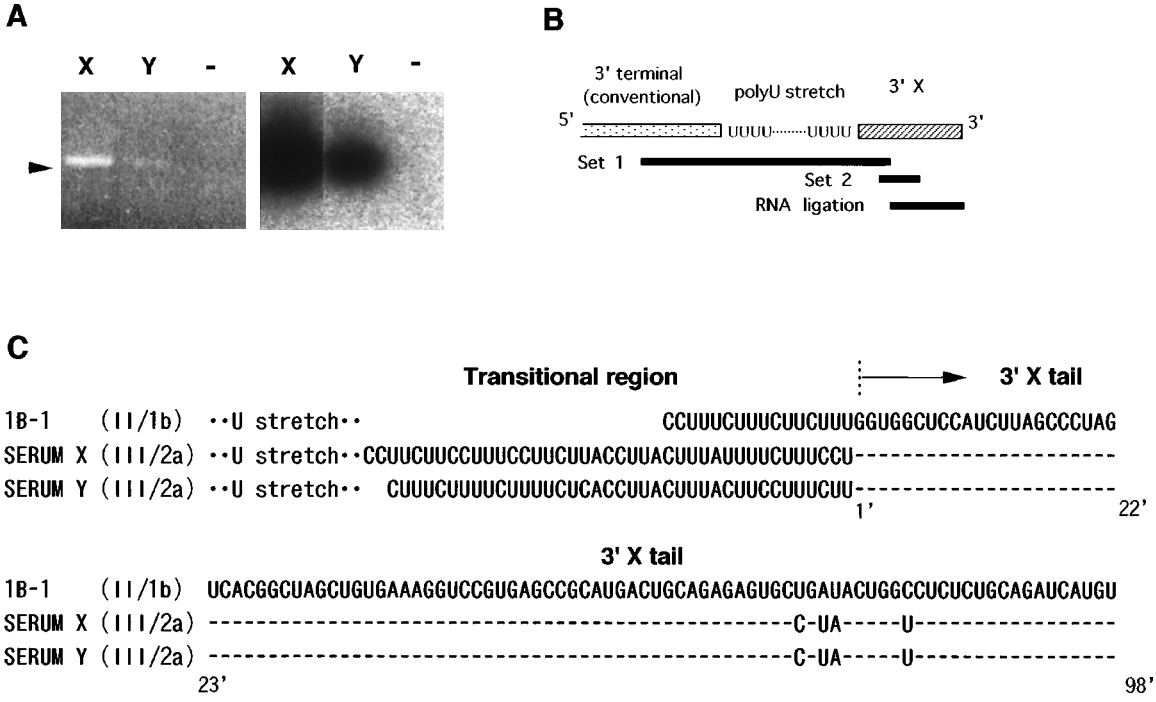

FIG. 5. 3′ X-tail sequence obtained from type III/2a HCV. (A) Detection of the 3′ X tail from sera infected with type III/2a HCV. Serum samples X and Y were analyzed by the same RT-nested PCR as described in the legend for Fig. 4 and then subjected to ethidium bromide staining (left) or Southern blotting with probe R3 (right). The "−" denotes the negative-control experiment performed without RNA. (B) Sequencing strategy for the 3′-terminal region of type III/2a HCV. The clones were obtained from two overlapping sets of RT-nested PCR and RNA ligation experiments. For set 1, the primers were as follows: RT, R2; first PCR, 2a9356 and R5; second PCR, 2a9380Xh and R6Xh. For set 2, primers were as follows: RT, RP2; first PCR, U6 and RP2; second PCR, U9 and R2. The RNA ligation experiment was performed as described in the legend for Fig. 3. Similar PCR analyses were performed for serum sample 1B-1. Primers for set 1: RT, R2; first PCR, 9348 and R5; second PCR, 9363Xh and R6Xh. Set 2 primers were the same as those used for type III/2a samples. (C) 3′-terminal sequence of type III/2a HCV. The sequences are aligned with a sequence obtained from serum sample 1B-1. Dashes denote nucleotides identical to those in the 3′ X tail of the 1B-1 sequence.

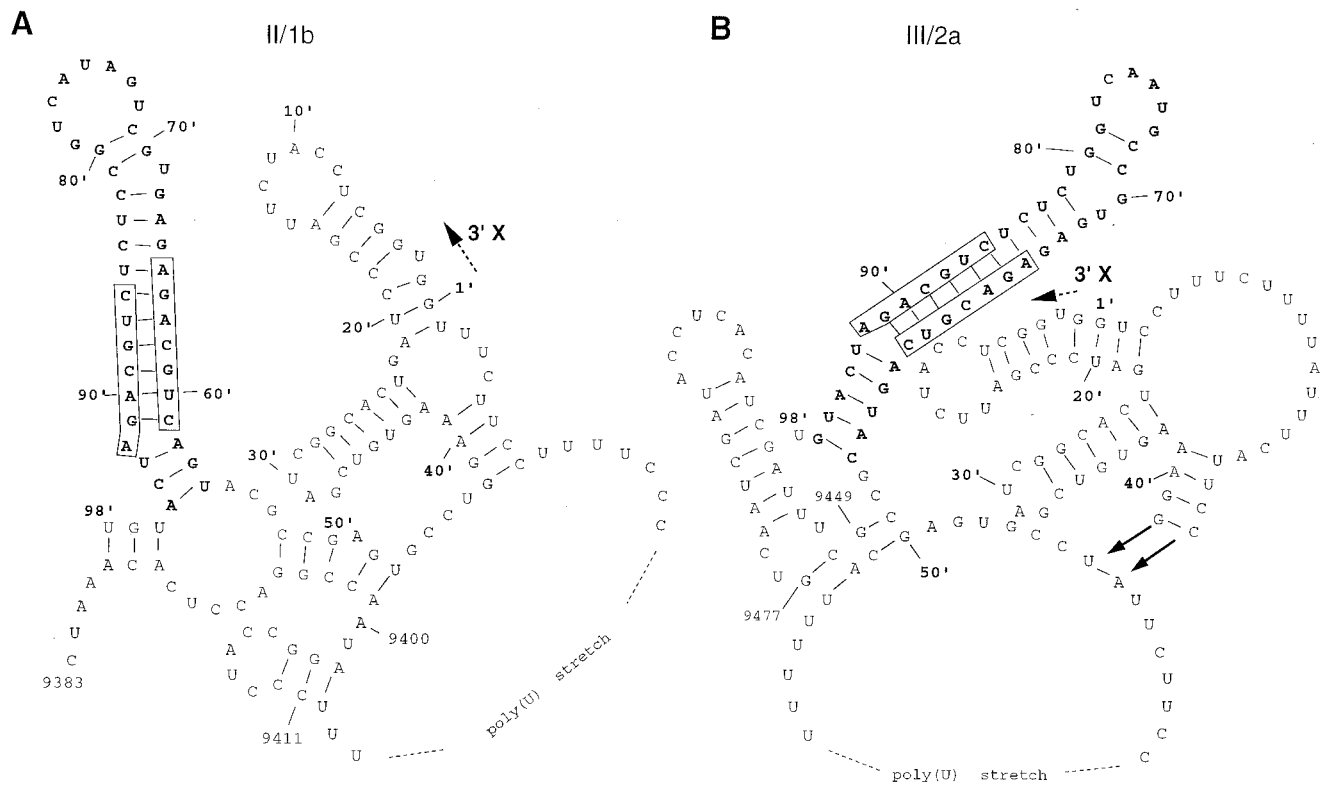

FIG. 6. Models of the secondary structure of the 3′-terminal sequence for type II/1b (A) and type III/2a (B) HCVs. Modeling was performed with the same sequence range, including a conventional 3′ UTR (29 nucleotides), the whole poly(U) stretch, and the 3′ X tail, by using the computer program GENETYX (Software Development, Tokyo, Japan). A conserved stem-and-loop structure in both models is depicted by boldface nucleotides. A 7-nucleotide direct repeat is shown boxed. In panel B, a part of the stem-and-loop structure (nucleotides (−)21′ to 42′) is moved from the original location to avoid overlaying (see arrows).

Characterization of the 3′ X-tail sequence. Figure 6 shows the possible secondary structures of the 3′ tail regions from the type II/1b and III/2a HCV genomes. The formation of the stem-and-loop structures differed in the two models, mainly because of differences in the conventional 3′ UTR sequences. However, the longest stem-and-loop structure seemed to be conserved (shown in boldface type in Fig. 6). This structure is also conserved in a model created from the 3′ X-tail sequence alone (21). A 7-nucleotide direct repeat (5′-CUGCAGA-3′) seemed to be involved in this stem structure (shown in boxes in Fig. 6). Most of the poly(U) stretch was looped out in these models. The optimal ΔG values for the structural models of the type II/1b and III/2a sequences were −69.4 and −72.6 Kcal/mol (−290.4 and −303.8 kJ/mol), respectively.

Three short open reading frames were found in the 3′ X tail, one in the positive strand (nucleotides 55′ to 96′) and two in the negative strand [nucleotides 96′ to 22′ and from nucleotide 56′ extending across the poly(U) stretch, respectively]. Sequences conserved in the 3′-terminal region of some flaviviruses (2, 16) were not found in the 3′ X tail. A sequence motif conserved in the 3′ UTR sequences of five pestiviruses, 5′-ACAGCACUUUA-3′ (6), was not found in the 3′ X tail of HCV. Significant homology or complementarity between the sequences of the 3′ X tail and the 5′ UTR of HCV was not found. A search for sequences homologous to the 3′ X-tail sequence was performed by using the computer program FASTA, provided by the DNA Research Center, National Institute of Genetics (Mishima, Japan). No sequence homologous to the whole 98-nucleotide sequence was detected in that database. However, a number of partial sequences were found to be homologous to partial sequences in the 3′ X tail. That homology was up to 85% within 25 to 40 nucleotides of the overlapping sequences. For instance, the highest degree of homology, 85.7%, was between nucleotides 22′ to 49′ of the 3′ X tail and nucleotides 3444 to 3471 of the *Mus musculus* MPS1 gene. We do not know whether this homology between two partial gene sequences is significant. The 3′ UTR of the GB virus B, a recently identified flavivirus that causes hepatitis in tamarins and probably in humans, consisted of a 27-nucleotide sequence downstream of the terminal codon, a subsequent poly(U) stretch, and a 49-nucleotide sequence at the 3′ terminus (13). The organization is similar to that of the HCV 3′ UTR, although no significant homology between the two 3′ UTR sequences was found. This structure may be one of the unique structures of the flavivirus 3′ UTR.

Implication of the 3′ X tail. The 3′ X-tail sequence was highly conserved even between the two most genetically distant HCV types, suggesting that the other types of HCV genome have very similar 3′ X-tail sequences. Both positive and negative strands of the 3′ X tail were identified in infected liver, indicating that this tail sequence is indeed implicated in the replication of the HCV genome. The tail sequence was also identified in infectious serum. These results suggest that the 3′ X tail is a conserved structure of HCV genomes in infectious virions and has an important function in HCV replication. The tail could form stable secondary structures, as reported for many positive-strand RNA viruses. Cellular proteins that bind to the 3′ stem-and-loop structure of the flavivirus genome RNA were recently reported (1). The primary, secondary, or higher structure of the 3′ X tail may be essential for interaction

with viral and cellular factors during genomic replication of HCV. The poly(U) stretch may be considered as a pyrimidine tract with some specific function yet to be elucidated. Yoo et al. (23) reported that a type I/1a HCV RNA which ended with a 15-mer of A or U bases downstream of a conventional 3′ UTR was infectious. Their result suggested that the 3′ X tail was not essential for the replication of HCV; this contradicts our concept of the 3′ X tail, although in their study the level of efficiency of genomic replication was not high. Further study is needed to resolve the question.

The 3′ X-tail sequence may be useful in the clinical study of HCV as well. Our findings suggested that the 3′ X-tail sequence is applicable as a diagnostic tool for type-independent detection of HCV, such as by PCR. Since the 3′ X tail is very likely involved in the regulation of genomic replication, it will be interesting to know whether sequence variations and quantity of the 3′ X tail are associated with clinical features of HCV infection and with infectivity of the infected serum. When an efficient experimental system for studying the replication of HCV is established, then the 3′ X tail may be used as a target for developing antiviral agents; antisense and sense nucleic acids directed against the 3′ X tail and other types of specific inhibitors may be developed.

Nucleotide sequence accession numbers. The sequences reported here have been deposited in the DDBJ, EMBL, and GenBank databases under accession numbers D63922 and D67091 to D67096.

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