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## Abstract

The negative strand RNA of hepatitis C virus, supposed to be a replicative intermediate of the virus appears to indicate viral replication. In this study, we detected the negative strand RNA by using reverse transcription-polymerase chain reaction with RNase A digestion to degrade the remaining positive strand genomic sequence of the virus after complementary DNA (cDNA) synthesis. In vitro transcribed positive-stranded mutant RNA was not detected by this method. Sample sera and liver tissues of 16 patients with chronic hepatitis C virus infection (liver fibrosis, 1; chronic hepatitis, 13; liver cirrhosis, 2) were analysed for negative strand RNA of hepatitis C virus. The negative strand RNA sequence was detected in 15 (93%) of 16 liver tissues and in 11 (78%) of 14 sera. The study demonstrated that negative strand RNA of hepatitis C virus in serum and liver tissue could be specifically detected.

**KEYWORDS:** hepatitis C virus, replication, replicative intermediate, polymerase chain reaction, chronic liver disease

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## Detection of Negative Strand RNA of Hepatitis C Virus in Infected Liver and Serum

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The negative strand RNA of hepatitis C virus, supposed to be a replicative intermediate of the virus appears to indicate viral replication. In this study, we detected the negative strand RNA by using reverse transcription-polymerase chain reaction with RNase A digestion to degrade the remaining positive strand genomic sequence of the virus after complementary DNA (cDNA) synthesis. *In vitro* transcribed positive-stranded mutant RNA was not detected by this method. Sample sera and liver tissues of 16 patients with chronic hepatitis C virus infection (liver fibrosis, 1; chronic hepatitis, 13; liver cirrhosis, 2) were analysed for negative strand RNA of hepatitis C virus. The negative strand RNA sequence was detected in 15 (93 %) of 16 liver tissues and in 11 (78 %) of 14 sera. The study demonstrated that negative strand RNA of hepatitis C virus in serum and liver tissue could be specifically detected.

**Key words :** hepatitis C virus, replication, replicative intermediate, polymerase chain reaction, chronic liver disease

Hepatitis C virus (HCV), carrying a single positive-stranded RNA genome, has been identified as a major causative agent for blood-borne non-A, non-B hepatitis (1). Isolation of its cDNA fragments from infected human (2) and chimpanzee plasma (1) permitted the assay of circulating antibodies to HCV (anti-HCV) using recombinant proteins derived from the viral genome (3, 4). These assays for HCV infection have shown its high seroprevalence in patients with acute and chronic non-A, non-B liver diseases including hepatitis, cirrhosis, and hepatocellular carcinoma (3-5). Several attempts at molecular cloning of HCV genome succeeded in characterization of its whole sequence spanning ~10,000 bases and revealed the diversity among HCV strains (5). HCV has been thought to be related to flaviviridae and pestiviridae families based on the similarity of their genomic sequences (5). As in the flaviviridae (6, 7), the HCV genome is supposed to replicate in the host cell using its complementary sequence as a template, which is called a replicative intermediate.

Although clinical materials have been assumed to contain too little HCV genome to be detected by conventional hybridization techniques, reverse transcription-polymerase chain reaction (RT-PCR) permits extremely sensitive assays for HCV-RNA to provide direct evidence of viral existence in tested samples (8-10). Recently, the competitive RT-PCR method has made it possible to quantify the amount of HCV-RNA (11-13). Using this strategy, we have found a close relationship between the amount of HCV-RNA in serum or liver tissue and histological severity of chronic hepatitis (14), which may be reflected by intrahepatic viral replication. To evaluate the evidence of the viral replication, we modified the RT-PCR assay to detect the negative strand RNA of HCV, the complementary sequence to the HCV genome. We detected it in liver tissue and serum from patients with chronic HCV infection and discussed its significance in the study of the pathophysiology of chronic hepatitis C.

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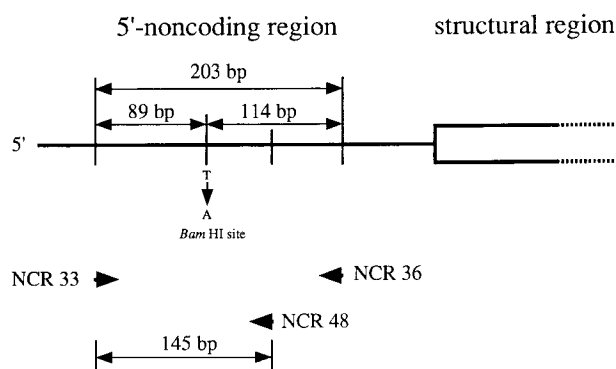
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## Subjects and Methods

**Patients.** We studied 16 patients (9 men + 7 women; mean age, 55 years) with chronic HCV infection. All of them were observed for at least six months to evaluate chronicity of their diseases by routine clinical and laboratory studies at the First Department of Internal Medicine of Okayama University. Serum was screened with the commercially available first-generation (c100-3) and second-generation (c200/c22-3) anti-HCV ELISA systems (Ortho HCV ELISA, Ortho Diagnostics, Tokyo). All patients were seropositive for anti-HCV antibodies and seronegative for HBs antigen. One man with hepatocellular carcinoma associated with cirrhosis underwent hepatic resection. The others underwent laparoscopy with guided biopsy to evaluate liver histology before interferon treatment. Liver histology demonstrated chronic persistent hepatitis in 5, chronic active hepatitis in 8, and cirrhosis in 2. One specimen showed portal fibrosis without significant hepatic necroinflammation and nodule formation. Informed consent was obtained from all patients. A part of the liver biopsy specimen was immediately frozen with dry ice and stored at  $-70^{\circ}\text{C}$  until used. Serum samples obtained on the same day were stored at  $-20^{\circ}\text{C}$ .

**RNA preparation.** All chemicals used in the present study were reagent-grade and purchased from Wako Pure Chemical Ind., Ltd., Osaka, Japan unless otherwise indicated. All manipulations in RNA preparation were performed under RNase-free condition using equipment stringently restricted to RNA experiments. Total RNA was isolated from liver tissue and serum by the method of Chomczynski and Sacchi (15) with several modifications (16). The frozen tissue (9–86 mg) was minced on ice with scissors and homogenized with 500  $\mu\text{l}$  of denaturing solution consisting of 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5 % sarcosyl, 0.1 M 2-mercaptoethanol. The homogenate was mixed with 50  $\mu\text{l}$  of 2 M sodium acetate (pH 4), 500  $\mu\text{l}$  of water-saturated phenol, and 100  $\mu\text{l}$  of chloroform-isoamylalcohol mixture (49:1), and then cooled on ice for 15 min. After centrifugation (14,000 g, 4 $^{\circ}\text{C}$ , 20 min), the aqueous phase was re-extracted once with phenol: chloroform (1:1), once with chloroform, and precipitated with isopropanol. The RNA pellet was dissolved in 150  $\mu\text{l}$  of the denaturing solution, reprecipitated with ethanol, washed with 70 % ethanol, and redissolved in water treated with 0.1 % diethyl pyrocarbonate (DEPC). The yield of RNA from liver tissue was determined by spectrophotometry (17). Serum samples were incubated with 4 % polyethylene glycol on ice for an hour and pelleted by centrifugation (14,000 g, 4 $^{\circ}\text{C}$ , 10 min). RNA was extracted from the pellet by the method described above with two additional extractions, and precipitation with isopropanol in the presence of 20  $\mu\text{g}/\text{ml}$  of glycogen. All liver and serum samples were treated in parallel with negative control throughout RNA preparation.

**Oligonucleotide primers.** Oligonucleotide primers used in the present study were generated on a DNA synthesizer (Applied Biosystems Japan, Tokyo, Japan). The highly conserved 5'-



**Fig. 1** Locations and directions of the oligonucleotide primers deduced from the 5'-noncoding region of hepatitis C virus (HCV) genome (19). A primer NCR 36 is used in cDNA synthesis for detection of positive strand HCV-RNA, while a primer NCR 33 is used for detection of negative strand HCV-RNA. A cDNA fragment of 203 base pairs (bp) is expected to be amplified by the first round polymerase chain reaction (PCR) with primers NCR 33 and NCR 36, and so is 145 bp of that by the nested one with primers NCR 33 and NCR 48. A novel *Bam* HI site created by site-directed mutagenesis is also represented.

noncoding region of the HCV genome (18, 19) was used to prepare oligonucleotide primers. The nucleic acid sequences of the primers were as follows: NCR 33: 5'-TTCACGCAGAAAGC-GTCTAG-3'; NCR 48: 5'-GTTGATCCAAGAAAGGACCC-3'; NCR 36: 5'-AACACTACTCGGCTAGCAGT-3'. Locations and directions of these primers were shown in Fig. 1.

A pair of primers for  $\beta$ -actin mRNA was designed to span an intron to distinguish genomic DNA from cDNA based on size. 3' primer, 5'-CAGCACTGTGTTGGCGTACA-3' (ACT4) is complementary to nucleotides 919–938 of the human  $\beta$ -actin cDNA (20, 21). 5' primer, 5'-CCAGCCATGTACGTTGCTAT-3' (ACT1), corresponds to nucleotides 429–448 of its cDNA sequence.

**cDNA synthesis and RNase A treatment.** To determine the presence of both positive and negative strand HCV-RNA, RT-PCR was performed as described previously (22). To detect negative strand HCV-RNA, the cDNA material was subjected to RNase A treatment prior to PCR. The first-strand cDNA was synthesized in 10  $\mu\text{l}$  of reaction mixture containing RNA derived from 100  $\mu\text{l}$  of serum or 1.5  $\mu\text{g}$  of total RNA from liver tissue, 1  $\times$  PCR buffer (50 mM KCl/10 mM Tris-HCl, pH 9.0/0.1 % Triton X-100) (Promega Biotec, Wisconsin, USA), 3.0 mM  $\text{MgCl}_2$ , 0.1 mM dNTPs (Perkin-Elmer Cetus, Connecticut, USA), 1 mM dithiothreitol, 4 units of a ribonuclease inhibitor, RNasin (Promega), 1 mM antisense primer, and 2.5 units of Moloney leukemia virus reverse transcriptase (New England Biolabs, Massachusetts, USA). NCR 36 and NCR 33 were used as antisense primers for the positive and negative strand HCV sequence, respectively. The mixture was incubated at 25 $^{\circ}\text{C}$  for 10

min, and then at 42°C for 30 min, and heated to 95°C for 5 min. Sequentially, it was treated or untreated with 1 mg/ml RNase A at 37°C for 1 h.

**PCR.** The cDNA products were supplemented with 10 µl of PCR mixture containing 1 × PCR buffer, 0.1 mM dNTPs, 1 mM sense primer, and 0.5 units of Taq DNA polymerase (Promega). NCR 33 and NCR 36 were used as sense primers for the positive and negative strand HCV sequence, respectively. The amplification was performed for 35 cycles with denaturation at 94°C for 1 min, primer annealing at 45°C for 1 min, and polymerization at 72°C for 1 min in a DNA thermal cycler (Perkin-Elmer Cetus). After the first round of amplification, 2 µl of the product was reamplified using an inner primer pair, NCR 48 and NCR 33 for 20 cycles under the same conditions except for primer annealing at 55°C. The PCR products were electrophoresed on 3% agarose gels (2.25% NuSieve, 0.75% SeaKem LE) (FMC Bio-products, Maryland, USA), stained with ethidium bromide, and visualized by UV fluorescence. Contamination of RT-PCR reactions were monitored using multiple negative controls and prevented by following several recommendations (24).

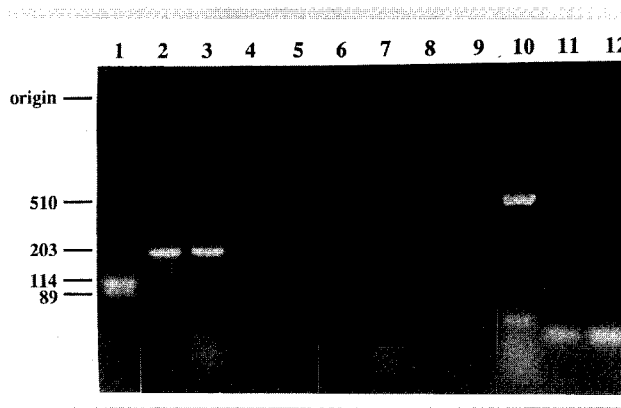
To determine if the liver tissue RNA was intact, RT-PCR was performed with primers for β-actin as an internal control. β-actin cDNA was amplified under the same conditions used for the qualitative assay of HCV-RNA except for 30 cycles of amplification in the first round of PCR.

The amount of HCV-RNA was determined by competitive RT-PCR targeting 5' noncoding region of HCV genome as described elsewhere (13, 14).

**Mutant RNA.** A mutant positive-stranded fragment of HCV-RNA was generated to provide a control template for a competitive RT-PCR assay for measuring the amount of HCV-RNA as described elsewhere (13, 14). Briefly, site-directed mutagenesis was carried out to substitute a T for an A at nucleotide 138 of the 5'-noncoding region which creates a novel Bam HI site on a recombinant plasmid (pHSN24) carrying a 239 base pairs cDNA fragment corresponding to nucleotide 13 to 251 of the 5'-noncoding region of the HCV genome (18). *In vitro* transcription was performed to synthesize the mutant RNA, which was treated with DNase I (Takara Biochemicals, Kyoto, Japan) to remove the DNA template. Following purification by phenol/chloroform extraction, the mutant RNA was dissolved in DEPC-water, measured spectrophotometrically, and serially diluted to a series of known concentrations.

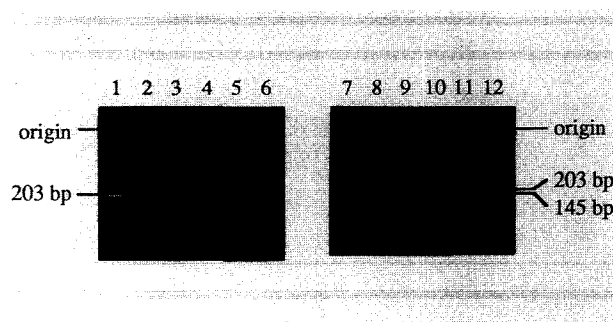
## Results

**Strand specific detection of the negative strand HCV-RNA in the liver tissue.** To confirm the strand-specific amplification in our assay, we examined a liver tissue RNA sample which was estimated to contain 10<sup>6</sup> copies of HCV sequence by the competitive RT-PCR



**Fig. 2** Strand specific detection of the negative strand RNA of hepatitis C virus (HCV). Reverse transcription-polymerase chain reaction (RT-PCR) was carried out for the positive strand HCV sequence (lanes 1-5), the negative strand one (lanes 6-9), and β-actin mRNA (lanes 10-12). An RNA sample prepared from a patient's liver tissue was examined (lanes 2-4, 6, 8, 10-11). To make sure the strand-specificity of this method, the positive-stranded mutant HCV RNA (10<sup>7</sup> copies) was reverse transcribed and amplified with (lanes 6-9) and without (lane 1) the RNA sample. Lanes 5, 9, 12 represent negative controls using water instead of the RNA sample. Each reaction was treated with 1 mg/ml RNase A at 37°C for 1 h before (lanes 4, 8, 11) or after (lanes 2, 6) cDNA synthesis. After 55 cycles of amplification, the products on the lanes 1 and 6-9 were digested with 5 units of Bam HI at 37°C for 2 h. All of the PCR products were analysed by 3% agarose gel electrophoresis. In this experiment, only the first round amplification was carried out for 55 cycles instead of 35 cycles. The second PCR for 20 cycles was not performed.

assay. To confirm the specific detection of the negative strand HCV-RNA, 10<sup>7</sup> copies of the mutant RNA was added into the RNA sample as a control template of the positive strand HCV-RNA prior to reverse transcription. As shown in Fig. 2, the disappearance of PCR product by RNase A treatment before cDNA synthesis (lanes 4, 8, 11) indicated both the efficiency of this enzyme to degrade templates in samples, and the absence of template DNA in the RNA preparations. When RNase A treatment of the cDNA product was carried out, RT-PCR for negative strand HCV-RNA resulted in generation of 203 base pairs of PCR product (lane 6), which has the identical size with that obtained by RT-PCR for positive strand HCV-RNA (lane 2). Bam HI digestion of this PCR product did not generate two fragments (lane 6) corresponding to 114 and 89 base pairs derived from the positive-stranded mutant RNA (lane 1). This result suggested that negative strand HCV-RNA could be specifically detected by this method even in the presence of excessive amounts of positive strand HCV-RNA.



**Fig. 3** Detection of the positive and negative strand HCV-RNA in the serum. RT-PCR for the each strand HCV-RNA (positive strand: lanes 1, 3, 5, 7, 9, 11; negative strand: lanes 2, 4, 6, 8, 10, 12) was carried out on the different day avoiding contamination by carry-over of the PCR products, although they were analysed on a 3% agarose gel at the same time. Lanes 1-6 represent products by the first round amplification for 35 cycles. Lanes 7-12 represent those by the nested PCR. Positive strand HCV-RNA was detected in an RNA sample from patient 11 (Table 1) (lanes 1, 7) and another sample from patient 3 (Table 1) (lanes 3, 9), but not in negative controls using water in place of RNA samples (lanes 5, 11). Negative strand HCV-RNA was detected in patient 11 (lanes 2, 8), but not in patient 3 (lanes 4, 10) and negative controls (lanes 6, 12). HCV, PT-PCR: See Fig. 2.

There was no difference in the intensity of fluorescence of the PCR product between the cDNA treated and untreated with RNase A after reverse transcription (lane 2 and 3, 6 and 7) indicating that additional RNase A did not inhibit the PCR reaction.

$\beta$ -actin cDNA was amplified in all RNA samples from liver tissues suggesting that no significant RNA degradation occurred.

*Negative strand HCV-RNA in chronic liver diseases.* All patients examined in this study were positive for circulating and intrahepatic positive strand HCV sequence (HCV genome) by nested RT-PCR. The results of qualitative detection of the negative strand HCV-RNA in patient's sera and liver tissues were presented with their clinical backgrounds in Table 1. The intrahepatic negative strand HCV-RNA was detected in 15 patients out of 16. The negative strand HCV sequence was detected also in the serum in 11 patients out of 14 examined. Fig. 3 shows representative results in two patients (Table 1, patients 3 and 11). Patients with negative test results for the negative strand HCV sequence had normal serum alanine aminotransferase levels (Table 1, patients 1-3).

**Table 1** Clinicopathological profile and the negative strand hepatitis C virus (HCV) RNA in patients with chronic HCV infection.

Patients	Age/Sex	ALT <sup>a</sup> (IU/l)	Histology <sup>b</sup>	Negative strand HCV-RNA <sup>c</sup>	
				Liver	Serum
1	43/F	16	CPH	-	-
2	54/F	23	LF	+	-
3	68/M	25	CAH	+	-
4	64/M	33	CPH	+	+
5	59/F	69	CPH	+	+
6	42/M	135	CPH	+	+
7	39/F	486	CPH	+	+
8	63/F	38	CAH	+	+
9	54/F	42	CAH	+	+
10	60/M	62	CAH	+	NT
11	56/F	81	CAH	+	+
12	61/M	81	CAH	+	+
13	59/M	110	CAH	+	+
14	57/M	150	CAH	+	+
15	58/M	91	LC	+	+
16	61/M	58	LC <sup>d</sup>	+	NT

a: Values of serum alanine aminotransferase at the time of liver biopsy; normal value  $\leq 37$  IU/l

b: LF, liver fibrosis; CPH, chronic persistent hepatitis; CAH, chronic aggressive hepatitis; LC, liver cirrhosis

c: +, positive; -, negative; NT, not tested

d: This patient was accompanied with hepatocellular carcinoma.

## Discussion

We determined the presence of negative strand HCV-RNA in the liver tissue and serum using the RT-PCR method. The presence of the negative strand HCV-RNA has been determined using RT-PCR (25, 26). According to our previous study, the amount of HCV-RNA ranged from  $10^8$  to  $10^{11}$  copies/g liver tissue and from  $2 \times 10^3$  to  $2 \times 10^8$  copies/ml serum in patients with hepatitis C (13, 14). The amount of HCV-RNA in such clinical materials was too little to allow detection by conventional Northern or spot-blot hybridization. We used the RT-PCR method to address the following problems; a) blocking the activity of reverse transcriptase before the sense primer is added into the reaction for PCR when cDNA synthesis was completed, b) Taq DNA polymerase in itself has been reported to behave like reverse transcriptase (23), c) evaluation of strand specificity of the PCR product. Several authors who detected the negative strand HCV-RNA using RT-PCR inactivated the reverse transcriptase by heating at 95-100°C for more than 20 min (25, 26), but we used RNase A to degrade RNA as a potential template of reverse transcription during PCR cycles. We devised a method to check the false positive result using

a mutant RNA of the positive strand HCV-RNA, whose PCR product can be distinguished from that of endogenous HCV sequence by a created novel restriction enzyme site. In our system for detecting the negative strand HCV-RNA, the mutant RNA was not detected suggesting that the negative strand HCV sequence was exclusively amplified.

We found negative strand HCV-RNA in liver tissue and in serum. The presence of the intrahepatic negative strand HCV-RNA is consistent with the notion that HCV replicates in the hepatocytes and generates a replicative intermediate as in the flaviviridae (6, 7). Our data indicates that serum is not always available as a negative control for the presence of the negative strand HCV-RNA in the liver tissue. However, we previously observed that 1 g of the infected liver tissue contained  $10^2$  to  $10^4$  times as many copies of HCV-RNA as did 1 ml of the serum (14) supporting the intrahepatic viral replication. The significance of a circulating form of the negative strand HCV sequence remains unclear. Fong TL *et al.* suggest the replicative intermediate might be associated with a virion or bound to membrane keeping RNA free from the activity of endogenous RNase in the serum (25). The absence of the circulating replicative intermediate has also been reported (26). The difference in efficiency of reverse transcription or PCR reaction may cause such a discrepancy.

The replicative intermediate is supposed to play an important role in viral replication, through which viral components are newly synthesized and assembled into virions. The presence of its circulating form only in patients with elevated serum alanine aminotransferase levels may suggest that the severity of hepatocellular injury is related to the amount of replicative intermediate in the liver. This idea is not contrary to a hypothesis that HCV-induced hepatocellular injury is not simply mediated by a direct cytotoxicity of the virus but modified by other factors including host immune responses (27), because the decrease in the activity of the viral replication may lead to a reduced degree of the HCV-related antigen presentation. We have observed that the amount of HCV-RNA in the serum or liver tissue correlated with histological severity of chronic hepatitis but not with serum aminotransferase activities (14). The loss of a close relationship between the viral amount and serum alanine aminotransferase levels in chronic hepatitis C does not support the direct cytotoxicity of HCV although the mechanism of hepatocellular injury in HCV infection

remains unclear. In the present study, we did not measure the amount of the negative strand HCV-RNA. Our findings also suggest that the replicative intermediate may be available, if measured quantitatively, as a novel marker of the viral replication.

In conclusion, we determined the presence of negative strand HCV-RNA using RT-PCR with several modifications which permitted strand-specific amplification. We believe that detection of the intrahepatic negative strand HCV sequence, the replicative intermediate, will help us to understand better the pathophysiology of chronic hepatitis C.

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