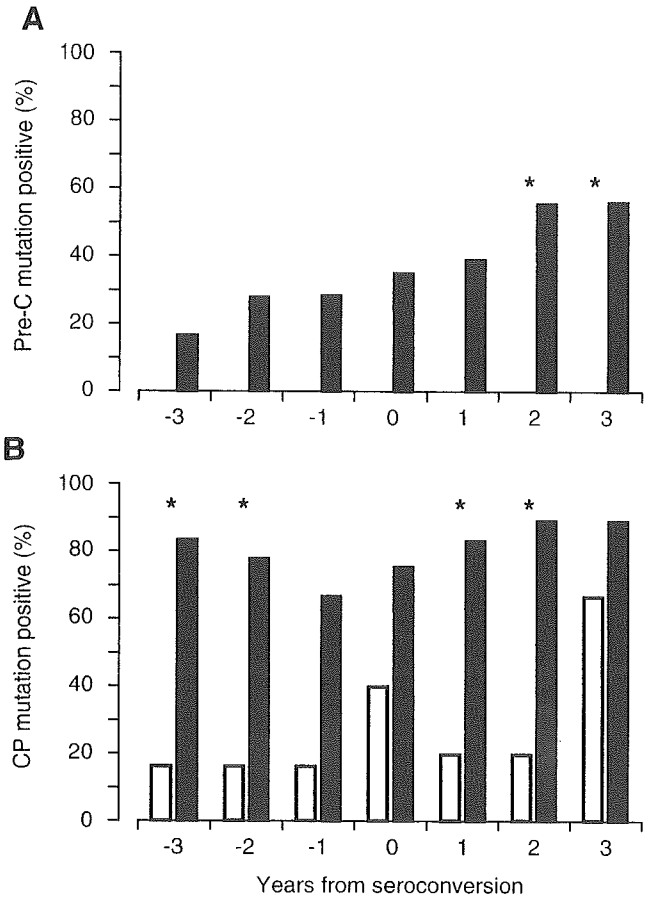

Fig. 3. Comparison of positive rates for the pre-C (**A**) and CP (**B**) mutations between the inactive and active replication groups. Open bars indicate inactive replication group and closed bars indicate active replication group. Number of patients in the inactive replication group is six at each time point except the followings: point 0 year (n = 5) in A, and points 0 year (n = 5), 1 year (n = 5), and 2 years (n = 5) in B. Number of patients in the active replication group is 18 at each time point except the followings: point 0 year (n = 17) in A and point 0 year (n = 17) in B. *$P < 0.05$ between the inactive and active replication groups.

DISCUSSION

Seroconverters were divided tentatively into two groups according to their levels of serum HBV DNA in the present study. It has been reported that older age and female gender are factors predicting occurrence of HBe antigen seroconversion in patients with chronic hepatitis B [Alward et al., 1985; Lok et al., 1987; McMahon et al., 2001]. On the other hand, in the present study, median age and gender distribution were similar between the inactive and active replication groups. A history of interferon treatment was recorded in half of the patients enrolled. The treatment history did not seem to be associated with the loss of HBV DNA after seroconversion, because the history was similarly distributed between the two groups and the duration of interferon therapy was as short as 4 weeks at most. Although the difference was not statistically significant, patients in the inactive replication group tended to show continuous normalization of ALT. Further, none of the six patients in the inactive replication group developed end stage liver diseases such as cirrhosis and hepatocellular carcinoma after the follow-up period, while 4 of the 18 patients in the active replication group developed them (data not shown). High viral load, which is usually associated with active hepatitis, has been reported to be a risk factor for development of hepatocellular carcinoma even in patients with chronic hepatitis B who achieved HBe antigen seroconversion [Ikeda et al., 2003; Ohata et al., 2004]. We could not compare long-term prognosis between patients in the inactive and active replication groups in the present study. However, patients in the active replication group tended to show active hepatitis after the seroconversion and to develop end stage liver diseases. Thus, further analysis of patients whose active viral replication continues after the seroconversion would be of clinical significance.

Analysis of the changes in HBV DNA and HBV core-related antigen revealed a clear contrast between the two. Namely, the HBV DNA level was similar between the two groups, while HBV core-related antigen was significantly lower in the active replication group than in the inactive replication group before seroconversion. The levels of both HBV DNA and HBV core-related antigen decreased remarkably around the time of seroconversion in the inactive replication group, while these levels did not change or decreased slightly in the active replication group. After seroconversion, the HBV DNA level was significantly higher in the active replication group than in the inactive replication group, while the HBV core-related antigen level was similar between the two groups. Because the discrepancy in the log ratio of HBV core-related antigen/ HBV DNA between the two groups first appeared at the time of seroconversion and continued thereafter, the difference between the HBV DNA and HBV core-related antigen changes was suggested to be closely associated with the seroconversion. The results obtained in the present study indicate that the mechanism of seroconversion was different between the two groups.

Because the serum level of HBV core-related antigen mainly reflects that of HBe antigen [Kimura et al., 2002], the low level of HBV core-related antigen seen after seroconversion in both the inactive and active replication groups might have contributed to the occurrence of seroconversion. The pre-C and CP mutations, which were associated with the seroconversion, were frequent in the active replication group and rare in the inactive replication group, at least at around the time of seroconversion. The decrease of HBV core-related antigen excretion seen after seroconversion was thought to have been caused mainly by the decrease of viral replication in the inactive replication group, because viral replication did not resume in this group. On the other hand, the decrease of HBV core-related antigen was thought to have been caused mainly by the appearance of pre-C and/or CP mutations, because active viral replication continued in this group. These results suggested that the two groups had different mechanisms of seroconversion.

It has been reported that the frequency of the pre-C and the CP mutations differs among HBV genotypes. Orito et al. reported that the CP mutation was significantly associated with genotype C [Orito et al., 2001]. Yamaura et al. [2003] reported that the CP mutation was already commonly seen several years before the seroconversion in patients with genotype C. These results are consistent with the present finding that the majority of patients in the active replication group had the CP mutation from the start of follow-up. The fact that patients in the active replication group had a lower level of HBV core-related antigen before the seroconversion may be attributable to the frequent CP mutation seen in this group.

In conclusion, the present study showed that there were different mechanisms of HBe antigen seroconversion between patients in whom HBV viraemia continued after the seroconversion and those in whom it did not. Measurement of HBV core-related antigen in addition to HBV DNA was suggested to be useful in examining specific conditions of chronic hepatitis B.

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Identification of Novel HCV Subgenome Replicating Persistently in Chronic Active Hepatitis C Patients

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In an effort to clarify the life cycle of HCV, the HCV genome in liver biopsies taken from chronic active hepatitis C patients undergoing interferon treatment was investigated. Molecular cloning by long distance reverse-transcription polymerase chain reaction (RT-PCR) revealed that the HCV genome in two patients with high viral loads in the liver had in-frame deletions of approximately 2 kb between E1 and NS2, which encode the E1–NS2 fusion protein and six other HCV proteins: core, NS3, NS4A, NS4B, NS5A, and NS5B. Among the remaining 21 chronic active hepatitis C patients, these types of deletion were found in another two patients and in two hepatocellular carcinoma patients. Out-of-frame deletions in the structural region were isolated from the other five patients, but the dominant RT-PCR products were non-truncated genomes. Retrospective analysis of a series of serum samples taken from a patient carrying the subgenome with the in-frame deletion revealed that both the subgenome and the full genome persisted through the 2-year period of investigation, with the subgenome being predominant during this period. Sequence analysis of the isolated cDNA suggested that both the subgenome and the full genome evolved independently. Western blotting analysis of HCV proteins from the HCV subgenome indicated that they were processed in the same way as those from the full genome. HCV subgenomes thus appear to be involved in the HCV life cycle. ***J. Med. Virol. 77:399–413, 2005.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: HCV; deletion; replication; biopsy

INTRODUCTION

Hepatitis C virus (HCV) is primarily transmitted via blood and blood-derived materials [Alter et al., 1989] and often causes chronic hepatic diseases that progressively worsen to chronic active hepatitis, cirrhosis, and finally to hepatocellular carcinoma (HCC) [Kiyosawa et al., 1990, 1994, 2004; Alter and Seeff, 2000]. Interferon (IFN) and interferon with ribavirin treatment are effective in eradicating HCV from patients [Iino et al., 1994; McHutchison and Fried, 2003], improving liver histological findings, and in prolonging life in patients with hepatitis C [Yoshida et al., 1999; Kasahara et al., 2004]; however, their efficacy is limited.

HCV was first identified as cDNA clones, and was characterized molecularly using cDNA isolates [Choo et al., 1989; Kuo et al., 1989]. The HCV genome is single-stranded RNA of about 9,600 nucleotides with an untranslated region (UTR) at each end, and encodes a polyprotein of about 3,010 amino acids [Choo et al., 1989; Kato et al., 1990], which is processed into 10 proteins by a host peptidase and two HCV proteases [Hijikata et al., 1991, 1993; Grakoui et al., 1993a]; Core, E1, and E2 are structural proteins for virion formation, and NS3, NS4A, NS4B, NS5A, and NS5B are components of the replication machinery for the RNA genome [Houghton et al., 1994]. However, isolation of virion particles has been difficult owing to a lack of in vitro culture systems for HCV.

HCV replication in chimpanzee following intrahepatic injection of an RNA transcript from HCV genomic cDNA proved that a molecular clone could represent a functional HCV genome [Yanagi et al., 1997]. Lohmann

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et al. [1999] established a dicistronic subgenomic RNA that replicates in a hepatoma cell line (Huh7) and consists of the HCV NS protein coding region, the 5′- and 3′-UTR of HCV and a selective marker gene. HCV subgenomic RNA replicon systems are vital to the study of the mechanisms of HCV RNA replication, but there remain problems with regard to viral replication. Information obtained from liver biopsies of hepatitis C patients thus remains important in clarifying the life cycle of HCV.

Histological grading for diagnosis [Perrillo, 1997], immunohistochemical analysis, immuno-staining [Infantolino et al., 1990; Hiramatsu et al., 1992], electron microscopic analysis [Fagan et al., 1992], in-situ hybridization and in-situ reverse transcription polymerase chain reaction (RT-PCR) [Lau et al., 1996; Dries et al., 1999], and quantitation of HCV RNA in liver biopsy specimens [Sakamoto et al., 1994; Nuovo et al., 2002] have all been used to demonstrate HCV replication in liver. However, molecularly characterized data have been limited; HCV RNA isolated from liver was found to be equivalent in size to the well-characterized RNA seen in circulating HCV [Nielsen et al., 2004], thus confirming HCV replication in liver.

In order to obtain data to elucidate the nature of HCV in liver, viral loads and the structure of the HCV genome in patient liver biopsy specimens were examined. A highly sensitive ELISA for quantitation of the HCV core antigen [Aoyagi et al., 1999; Tanaka et al., 2000] and a quantitative RT-PCR system were applied to estimate viral loads in serum and liver biopsies. For structural analysis of the whole genome in specimens, molecular clones were used. Surprisingly, it was found that novel HCV subgenomes were predominant in several patients and, in one patient, these persisted for several years. The nature of these subgenomes are described and discussed in this paper.

MATERIALS AND METHODS

Samples and Antibodies

Serum and liver biopsy specimens were taken from patients undergoing IFN-α treatment at Shinshu University Hospital. Informed consent was obtained from all patients from whom samples were taken. A 7.2 mega-unit dose of IFN-α was administered daily for 2 weeks, followed by three times per week for 22 weeks. Serum samples were collected 1 or 2 months before administration of IFN-α. A series of serum specimens was collected from one patient from before IFN treatment until 1 year after treatment. Liver biopsy specimens from two HCC patients were obtained from surgically removed cancerous liver tissues.

An anti-core monoclonal antibody (5E3) has been described previously [Kashiwakuma et al., 1996]. Anti-E1 monoclonal antibody was raised against recombinant E1 and E1/E2 proteins expressed in Sf-9 cells infected with recombinant baculoviruses (Yamaguchi unpublished). Anti-NS3 rabbit polyclonal antibody was purified from the serum of rabbits immunized with the recombinant NS3 antigen expressed in *E. coli* [Saito et al., 1992]. Anti-mouse and rabbit immunoglobulin antibody conjugated with HRP were purchased from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD) and Bio-Rad Laboratories (Tokyo, Japan), respectively. All primers were purchased from Sigma Genosys (Tokyo, Japan) and Texas Genomics Japan (Tokyo, Japan), and sequences are available on request.

Quantitation of HCV Core Antigen

Quantities of HCV core antigen were measured by EIA as described previously [Aoyagi et al., 1999; Kato et al., 2003]. The concentration of core antigen was expressed in fmol/L, and the cut-off value of the assay was set at 7.5 fmol/L. For quantitation of the core antigen in liver, extracts were diluted to 100 μg of liver protein per milliliter with negative control serum before pretreatment of the samples. Samples were heated at 56°C for 30 min with pretreatment solution containing SDS, CHAPS, and Triton X-100, and were then added to wells pre-coated with anti-HCV core antibodies, and reaction buffer was used to fill the wells. Captured core antigen after 1-hr incubation was reacted for 30 min with anti-HCV antibodies conjugated with horseradish peroxidase after stringent washing. Bound enzyme activities were measured using a Fusion plate reader (PerkinElmer, Tokyo, Japan) with a chemiluminescent reagent (SuperSinal Pico ELISA, Pierce, Rockford, IL)

Real-Time PCR Assay for HCV RNA

HCV RNA was recovered from samples by using the QIAamp viral RNA kit (QIAGEN K.K., Tokyo, Japan) according to the manufacturer's instructions. HCV RNA was reverse-transcribed and amplified using QuantiTect One-Step RT-PCR kit (QIAGEN) with primers. For quantitation of the 5′-UTR, the forward primer, chiba-s (5′-TAGTGGTCTGCGGAACCGGT-3′), and reverse primer, chiba-as (5′-TGCACGGTCTACGAGACCT-3′), yielded fragments corresponding to nucleotides 141–339 of HCV RNA. In the case of the E2 region, HC1986S (5′-TGGTTCGGCTGYACATGGATGAA-3′) and HC2199AS (5′-GGRTAGTGCCARAGCCTGTATGGGTA-3′) primers were used. Reactions were performed with a LightCycler system (Roche Diagnostics K.K., Tokyo, Japan), and fluorescence by SYBR green was monitored after each elongation reaction for real-time monitoring of DNA products during PCR. The amount of HCV RNA was calculated according to the calibration curve produced with serial dilutions of standard RNA synthesized by T7 RNA polymerase (Ambion, Inc., Austin, TX) from plasmids carrying the HCV cDNA isolate (genotype 1b). To examine the specificity of PCR, the melting point of DNA products was analyzed by melting curve analysis using LCDA software (Roche Diagnostics).

Cloning and Analysis of HCV cDNA

HCV cDNA was amplified by long distance RT-PCR (LD-RT-PCR) as described previously [Tellier et al., 1996;

TABLE I. Viral Data of Patients

Patient No.	HCV genotype	Viral loads: Serum Core antigen (fmol/L)	Viral loads: Serum HCV RNA (copies/ml)	Viral loads: Liver biopsy Core antigen (fmol/g protein)	Viral loads: Liver biopsy HCV RNA (copies/g protein)	PCR primer sets for positive results: Non-truncated genome	PCR primer sets for positive results: Truncated genome	PCR primer sets for positive results: Test primer sets
368	1b	17,108.5	1.73×10^5	5,462.4	4.71×10^8	—	j	j
207	1b	12,695.2	1.44×10^5	30,792.3	1.43×10^{10}	NT	NT	NT
204	1b	5,082.4	5.74×10^4	8,779.7	2.22×10^9	i	—	i
274	1b	1,034.4	4.24×10^3	2,651.7	3.56×10^7	a, b, c, d	e	a–e
193	1b	988.8	3.09×10^4	14,519.9	1.07×10^9	a, b, c, d, e	—	a–e
331	1b	922.2	2.03×10^3	2,387.1	2.84×10^8	a, c, d	b, e	a–e
325	1b	623.5	3.82×10^3	10,127.9	7.28×10^7	a, b, c, d, e	d	a–e
288	1b	254.5	1.00×10^1	4,037.9	9.50×10^6	a, b, c, d, e	d, e	a–e
299	1b	166.6	1.14×10^3	1,287.8	5.35×10^7	c, d	—	a–e
295	1b	1.0	5.11×10^1	261.5	2.62×10^7	a, b, c, d, e	b	a–e
171	1b	1,077.3	6.42×10^3	3,781.8	6.91×10^6	c, d	b	a–e
257	1b	12.7	1.06×10^2	568.5	2.78×10^7	d	—	a–e
372	1b	723.7	2.28×10^4	1,784.1	3.35×10^8	a, b, c, d, e	—	a–e
373	1b	597.0	8.31×10^3	33,919.0	2.65×10^9	—	a, c, d	a–e
248	2a	209.3	2.58×10^2	4,417.1	3.70×10^8	—	—	a–e
235	2a	3,616.2	3.66×10^2	7,462.1	1.55×10^9	c	—	a–e
203	2b	95.1	1.46×10^2	5,590.9	1.82×10^9	—	b, d	a–e
178	2b	34.5	8.08×10^1	609.1	4.51×10^7	—	—	a–e
297	2	3,112.7	8.35×10^3	2,883.6	1.14×10^8	—	—	a–e
298	2a	180.0	8.09×10^2	3,015.0	1.76×10^9	b	—	a–e
305	2a	173.6	1.12×10^3	1,782.8	5.96×10^7	b	—	a–e
201	2a	127.6	2.40×10^3	497.6	1.87×10^7	—	—	a–e
357	2	227.2	3.11×10^3	321.9	2.29×10^7	—	—	a–e

NT: not tested.

Yanagi et al., 1998]. HCV cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen K.K., Tokyo, Japan) with HC1b9405R primer (5′-GCCTATTGGCCTGGAGTGTTTAGCTC-3′). After RNase H (Invitrogen) treatment at 37°C, a cDNA mixture was subjected to PCR with KlenTaq DNA polymerase (BD Biosciences Clontech, Tokyo, Japan), HClong A1 primer (5′-GCCAGCCCCCTGATGGGGGCGACA-

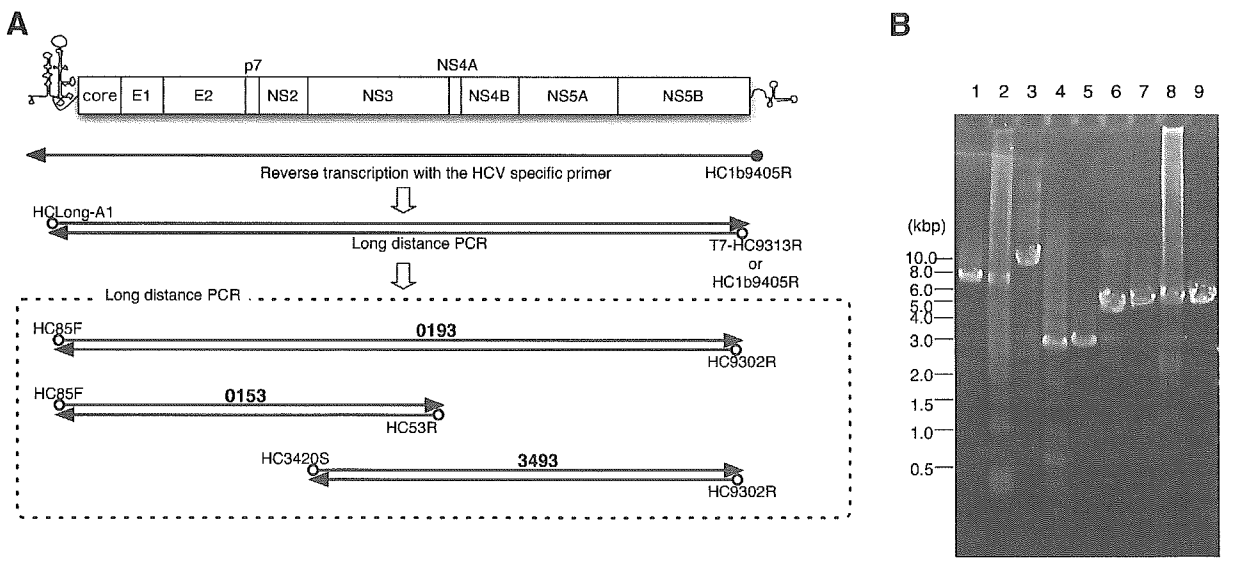


Fig. 1. Molecular cloning of HCV genome by long distance reverse-transcription PCR. **A**: Schematic view of HCV RNA is shown at the top of the figure. HCV cDNA, which was synthesized from total RNA from liver using reverse transcriptase with HCV-specific primers for the 3′-UTR, was amplified by nested PCR with HCV-specific primers. The longest LD-RT-PCR product, 0193, covered 99% (amino acids 1–2,987) of the HCV polyprotein coding sequence of genotype 1b HCV (length: 3,011 amino acids). Fragments obtained by LD-RT-PCR using HC85F and HC9302R, HC85F, and HC53R; and HC3420S and HC9302R were designated 0193, 0153, and 3493, respectively. After agarose gel electrophoresis, LD-RT-PCR products from liver biopsy samples were stained with ethidium bromide. **B**: **Lanes 1–3**, **lanes 4–6**, and **lanes 7–9** represent 0193, 0153, and 3493 fragments from Patient 207 (lanes 1, 4, and 7), 373 (lanes 2, 5, and 8), and control HCV cDNA (lanes 3, 6, and 9), respectively. The positions of markers are indicated at the left side of the image.

CTCCACC-3′) and T7-HC9313R primer (5′-TCTAGTC-GACGGCCAGTGAATTGTAATACGACTCACTCTAG-GGCGGCGGGGTCGGGCWCGNGACABGCTGTGA-3′) or HC1b9405b for 35 cycles of denaturation at 94°C for 20 sec and extension at 68°C for 9 min. Second-round PCR was carried out with the primers, HC85F (5′-ATGGCGTTAGTATGAGTGTCGTGCAGCCT-3′) and HC9302R (5′- TCGGGCACGAGACAGGCTGTGATA-TATGTCT-3′), HC85F and HC53R (5′-GCTTAAGTG-ACGACCTCCAGGTCAGCCGACAT-3′), and HC3420S (5′-GCGCCCATCACGGCCTACTCCCAACAA-3′) and HC9302R, for 20 cycles under the same conditions as first-round PCR. PCR products were purified from the gel using a QIA-quick gel kit (QIAGEN), and were then cloned into the pGEM-T easy vector (Promega K.K., Tokyo, Japan). The cDNA clones, LV207-0193-1, -3, -15, and -6, and LV373-0193-10, LV373-0153-5, and LV373-0153-6 were obtained from liver biopsy samples from Patient 207 and 373, respectively.

Nucleotide sequences of the cloned cDNA fragments were determined using a CEQ-2000 XL analysis system with a DTCS quick start kit and HCV-specific primers according to the manufacturer's instructions. Sequence data were analyzed on Macintosh computers with the

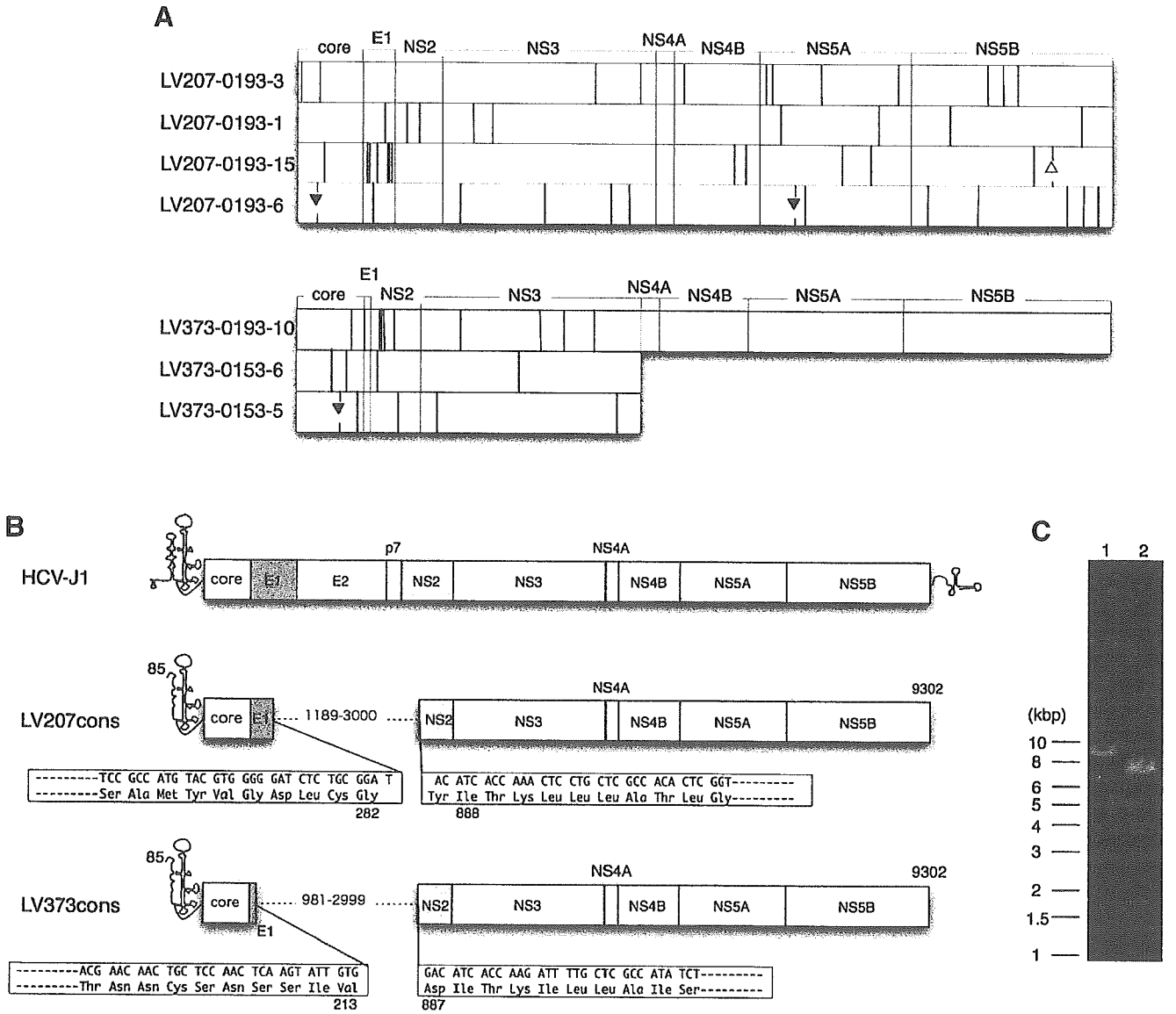


Fig. 2. Schematic presentation of HCV subgenomes from livers containing in-frame deletion. **A**: Comparisons of the deduced amino acid sequences of LD-RT-PCR fragments cloned with pGEM-T Easy are depicted. The cDNA isolates from liver biopsy are designated by the prefix LV followed by Patient numbers and fragment names described in legend of Figure 1. Bold bars indicate the positions of amino acids that differed. Closed triangles show the positions of stop codons, and open triangles indicate base deletions. **B**: Schematic HCV cDNA structures with their nucleotide sequences and deduced amino acid sequences around the boundary of the deletions are depicted. The deleted regions were represented as nucleotide positions as those corresponding to HCV-J1 by numbers between the dotted tagged boxes. Boundaries of the deletions are shown as amino acid positions corresponding to those of HCV RNA (HCV-J1) on the bottom line of the graphs. **C**: Images of agarose gel electrophoresis of LD-RT-PCR products amplified from in vitro non-truncated (**lane 1**) G14 and truncated (**lane 2**) RNA transcripts from Donor G14 and Patient 207 cDNAs, respectively.

Sequencer (Gene Code Corporation, Ann Arbor, MI), MacVector (Accelrys K.K., Tokyo, Japan), and EMBOSS [Rice et al., 2000] software packages.

Construction of HCV cDNA Expression Vectors

HCV RNA reverse transcribed with HC1b9405R from Patient 207 liver biopsy was subjected to PCR using the primers T7-HCLongH1 (5′-TCTAGTCGACGGCCAGTGAATTGTAATACGACTCACTATAGGGCGGCCAGCCCCCTGATGGGGGCGACACTCCACC-3′) and core-cla-as (5′-GCCGCATGTAAGGGTATCGATGACC-3′) in order to amplify the entire 5′-UTR, and cloned (LV207-H1-Cla). To obtain the 3′-UTR cDNA (LV373-3′UTR), cDNA was synthesized with HC8913F primer (5′-CTTGAAAAAGCCCTGGATTGTCAGAT-3′) from the minus strand RNA of Patient 373 liver sample. cDNA was amplified by PCR with HC8913F and RP2 (5′-ACATGATCTGCAGAGAGGCC-3′), and followed by PCR with HC8939F and R1 (5′-ACATGATCTGCAGAGAGGCCAGTATCAGCACTCTC-3′).

Ligation of the fragments from LV207-0193-1, -15, and -6, the 5′-UTR-core fragment and the 3′-UTR fragments gave the chimeric HCV cDNA (LV207cont). The cDNA encoding the HCV subgenome was inserted into pcDNA3.1 (Invitrogen) to construct pcD/LV207cont. A Cla I site in the core region and an Asc I site in the NS2 region of pcD/LV207cont were introduced using QuickChange II site-directed mutagenesis kits with primers. Insertion of the corresponding fragment from E1 to NS2, which was obtained from HCV-J1 cDNA [Aizaki et al., 1998] by PCR with core-cla-s (5′-GGTCATCGATACCCTTACATGCGGC-3′) and Asc-M-as (5′-CCTTCCTCGGCGCGCCGAGACRGGTAGACCCRAGATGATGTCCCCACA-3′) generated pcD/J1NLV.

In Vitro Synthesis of Truncated and Non-Truncated RNA Transcripts and Subsequent RT-PCR

Truncated cDNA, LV207cont, having the T7 promoter sequence was inserted into pBluescript II (Stratagene) to give pLV207cont. A plasmid carrying non-truncated HCV cDNA was constructed by inserting full-length cDNA derived from G14 plasma, in which only full-length HCV RNA was detected, into pBluescript II. Truncated and non-truncated RNA was synthesized using MEGAscript T7 kit (Ambion, Inc.) according to the manufacturer's instructions.

RNA transcripts (10^4 copies) were mixed with RNA extracted from uninfected liver tissue, and were reverse-transcribed and amplified by same protocol used to obtain truncated cDNAs. In addition, RNA transcripts and extracted RNA from plasma or serum were amplified by RT-PCR using primers targeting the junction site of LV207 cDNA. RNA was reverse-transcribed and amplified using QuantiTect SYBR GREEN RT-PCR Kit (QIAGEN) with sense, LV207-1S (5′-GCGTCCCCACTAAGGCAATA-3′), and antisense primers, LV207-3AS (5′-AGCAGGAGTTTGGTGATGATCCG-3′), for the

TABLE II. List of Primers Used for Detecting Truncated and Non-Truncated HCV Genomes

		Primer sets									
		a	b	c	d	e	f	g	h	i	j
Reverse transcription		HC3945R	HC3481R	HC3945R	HC3945R	HC3297R	HC3945R	HC3945R	HC3945R	HC3945R	HC3481R
1st PCR	Sense	HClongA1	HClongA1	HC813S	HC813S	HClongA1	HClongA1	HClongA1	HClongA1	HC813S	HClongA1
	Antisense	HC3945R	HC3481R	HC3945R	HC3174AS	HC3297R	HC3945R	HC3945R	HC3945R	HC3297R	HC3481R
	Sense	HC85F	HC85F	HC841S	HC841S	HC85F	HC85F	HC85F	HC841S	HC841S	HC85F
2nd PCR	Antisense	HC3297R	HC3297R	HC3759R	HC3111AS	HC3174AS	HC3297R	HC3759R	HC3759R	HC3174S	HC3297R

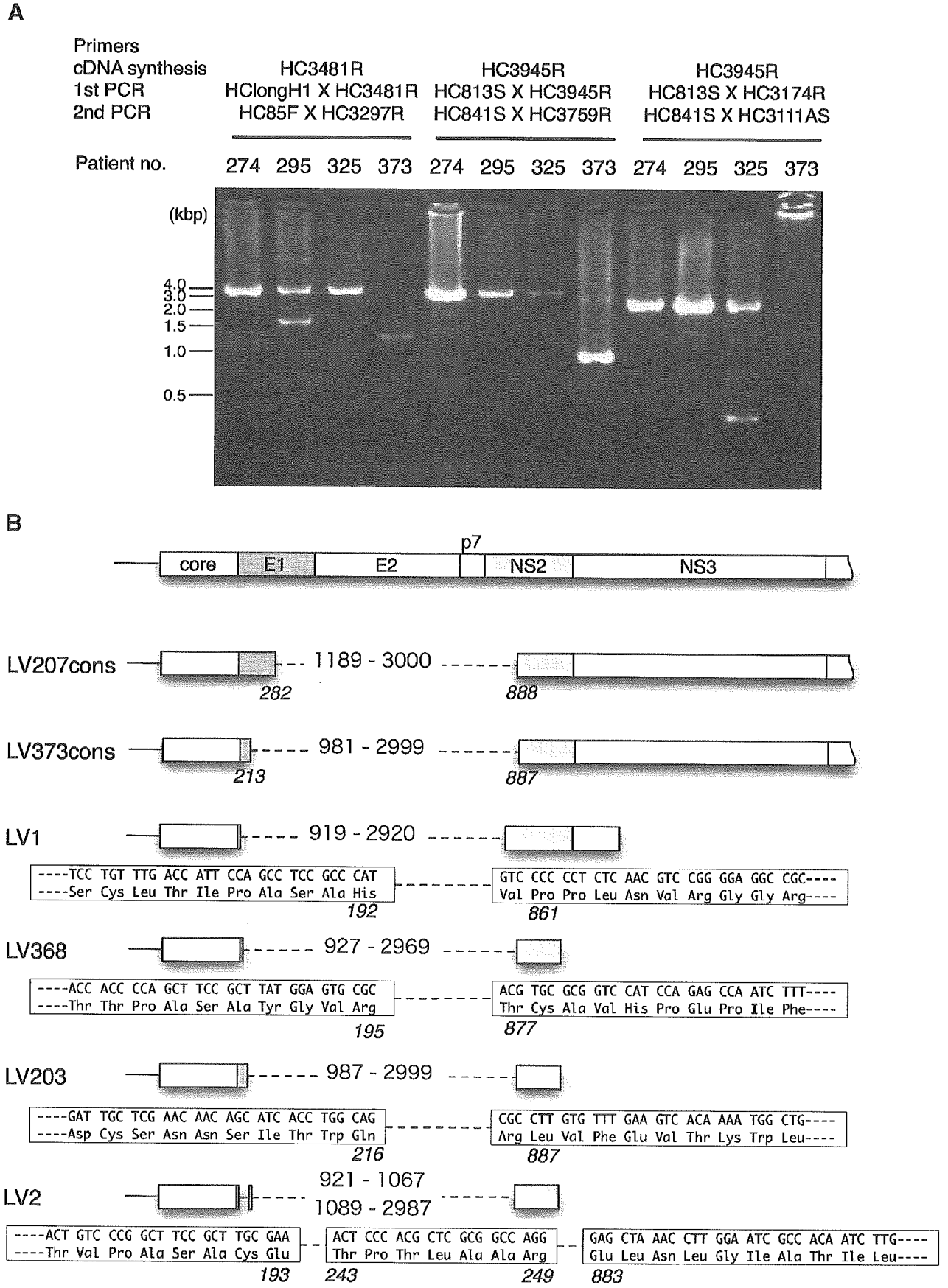

A
Primers
cDNA synthesis
1st PCR
2nd PCR
HC3481R
HClongH1 X HC3481R
HC85F X HC3297R
HC3945R
HC813S X HC3945R
HC841S X HC3759R
HC3945R
HC813S X HC3174R
HC841S X HC3111AS
Patient no.
274 295 325 373 274 295 325 373 274 295 325 373
(kbp)
4.0
3.0
2.0
1.5
1.0
0.5
B
p7
core
E1
E2
NS2
NS3
LV207cons
1189 - 3000
282
888
LV373cons
981 - 2999
213
887
LV1
919 - 2920
----TCC TGT TTG ACC ATT CCA GCC TCC GCC CAT
----Ser Cys Leu Thr Ile Pro Ala Ser Ala His
192
GTC CCC CCT CTC AAC GTC CGG GGA GGC CGC----
Val Pro Pro Leu Asn Val Arg Gly Gly Arg----
861
LV368
927 - 2969
----ACC ACC CCA GCT TCC GCT TAT GGA GTG CGC
----Thr Thr Pro Ala Ser Ala Tyr Gly Val Arg
195
ACG TGC GCG GTC CAT CCA GAG CCA ATC TTT----
Thr Cys Ala Val His Pro Glu Pro Ile Phe----
877
LV203
987 - 2999
----GAT TGC TCG AAC AAC AGC ATC ACC TGG CAG
----Asp Cys Ser Asn Asn Ser Ile Thr Trp Gln
216
CGC CTT GTG TTT GAA GTC ACA AAA TGG CTG----
Arg Leu Val Phe Glu Val Thr Lys Trp Leu----
887
LV2
921 - 1067
1089 - 2987
----ACT GTC CCG GCT TCC GCT TGC GAA
----Thr Val Pro Ala Ser Ala Cys Glu
193
ACT CCC ACG CTC GCG GCC AGG
Thr Pro Thr Leu Ala Ala Arg
243
249
GAG CTA AAC CTT GGA ATC GCC ACA ATC TTG----
Glu Leu Asn Leu Gly Ile Ala Thr Ile Leu----
883

Fig. 3.

junction site. The expected length of the PCR fragment was 112 bp.

Analysis of HCV Proteins by Western Blotting

HCV cDNA expression plasmids were transfected into HEK293 cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 24 hr, cells were recovered and lysed in RIPA buffer containing protease inhibitor cocktails. After removing debris by centrifugation, the lysate was subjected to SDS-polyacrylamide gel electrophoresis (10–20% gradient gel, Daiichi Chemical, Tokyo, Japan), and proteins were transferred to a PVDF membrane (Millipore, Tokyo, Japan) under semi-dry conditions. The membrane was probed with the relevant antibodies. Bound antibodies were detected with anti-mouse or rabbit antibodies conjugated with HRP, and visualized by detecting the chemiluminescent signals developed using SuperSignal West Pico reagent (Pierce) with the LAS-1000 image analysis system (Fujifilm K.K., Tokyo, Japan) or exposure to Kodak Bio Max film. The membrane was rinsed in Restore reagent (Pierce) in order to remove bound antibodies, and was then probed with other antibodies.

RESULTS

Quantitation of HCV in Clinical Specimens

Table I summarizes viral parameters in 23 chronic active hepatitis C patients subsequently treated with interferon. All patients responded to IFN administration, exhibiting reduced virus titers and alanine transaminase (ALT) levels in serum; however, IFN efficacy varied (not shown). Serum and liver specimens were taken before IFN administration, and HCV viral loads were then determined by quantitation of HCV RNA by real-time RT-PCR, and by measuring HCV core antigen levels using core antigen ELISA (Table I). Correlations among viral loads were analyzed. The amount of HCV core antigen and RNA in serum and liver were well correlated ($R = 0.968$ and $R = 0.728$, respectively). In contrast, the correlation between HCV titers in serum and liver was significant but poor ($R = 0.575$ stand for core antigen and $R = 0.373$ for RNA). No obvious correlation was observed between efficacy and viral load in liver biopsy specimens.

Truncated Subgenomic HCV RNA in the Liver

In order to investigate the structure of the HCV genome in patient liver, HCV cDNAs were cloned from two cases with high viral load (207 and 373). Figure 1A shows the strategy of LD-RT-PCR for cloning of the HCV genome. The length of LD-RT-PCR products varied between the patients. Comparison with the control cDNA, which was previously cloned from a blood donor (G14) plasma sample, indicated a 2-kb deletion occurred in the 5′-half of the HCV RNA from these patients (Fig. 1B).

The nucleotide sequences of cDNA clones from Patient 207 liver (LV207-0193-1, -3, -6 and -15) were analyzed and the deduced amino acid sequences were compared (Fig. 2A). All clones had almost identical sequences. While the open reading frames (ORFs) of two isolates were discontinued (by two stop codons in LV207-0193-6 and a one-base deletion in LV207-0193-15), LV207-0193-1 and -3 encoded a continuous 2,383 amino acid polypeptide. In the case of Patient 373, LV373-0193-10 encoded a 2,314 amino acid polypeptide. Two cDNA clones, LV373-0153-6 and -5, overlapped with LV373-0193-10, and identity in the nucleotide sequence of the overlapped region was 98%. With the exception of LV373-0153-5, which contained an in-frame stop codon, each cDNA had a contiguous ORF. The consensus HCV cDNA sequence for each patient was generated from these cDNA isolates (Fig. 2B).

Sequence comparison with authentic genotype 1b HCV (J1) [Aizaki et al., 1998] is illustrated in Figure 2B. Both consensus sequences from patient livers lacked sequences between E1 and NS2; the regions corresponded to the nucleotides 1189–3000 (amino acids 283–887), and 981–2999 (amino acids 214–886) of HCV-J1.

To confirm that the deletion was not produced during LD-RT-PCR (due to effects such as strong RNA secondary structure), non-truncated RNA transcripts were reverse-transcribed and amplified by the same protocol to give the truncated genome in Patient 207. While a deleted DNA fragment of about 7 kbp was amplified from truncated RNA transcripts of Patient 207, a full-length DNA fragment of about 9 kbp was amplified from non-truncated RNA transcripts of Donor G14, (Fig. 2C).

Deletions Found in Livers of Other Patients

In order to examine the incidence of HCV subgenomes with E1–E2 deletions, HCV genomes in other liver specimens were examined by RT-PCR with the primers listed in Table II. Two surgical samples (Patient 1 and 2) from hepatic cirrhosis patients with hepatocellular carcinoma were also subjected to this analysis. While the patterns of PCR fragments depended on the primer sets (Fig. 3A, Table II), they were classified into four

Fig. 3. Demonstration of deletions in the structural protein coding sequences of HCV genomes from patient livers. **A**: RNA from patient biopsies was analyzed by RT-PCR with primers for 5′-UTR to NS2 or core to NS2. Primer sets and Patient numbers are shown above the agarose electrophoresis image, and positions of the DNA markers are indicated on the left side of the image. **B**: Illustration shows schematic views of in-frame deletions and sequences of PCR fragments obtained by RT-PCR with the 5′-UTR and NS2 primers. cDNA isolates were designated as described in legend of Figure 2. The clones (LV1, LV368, LV203, and LV2) of Patient 1, 368, 203, and 2 were obtained by RT-PCR using primer sets g, j, b, and f (Table II), respectively. Only truncated fragments were amplified in these cases after RT-PCR for 5′-UTR to NS2 (Table I). Structures of the truncated HCV genomes, LV207cons and LV373cons, are shown for comparison. Numbers between dot-tagged graphs represent deleted regions as they correspond to HCV-J1. Bottom lines show the corresponding amino acid positions of the deletion boundaries.

types (Table I): no amplified fragments (five cases); fragments corresponding to the non-truncated HCV genome (eight cases); fragments corresponding to both the truncated and non-truncated genomes (six cases); and fragments corresponding only to the truncated genome (four cases, including Patient 207 and 373).

RT-PCR of HCV RNA in Patient 1, 368, and 203 yielded only fragments corresponding to the truncated HCV genome. The isolated molecular clones contained in-frame deletions of amino acids extending from E1 to NS2, similarly to those isolated from Patient 207 and 373. Although cDNA from Patient 2 contained two in-frame deletions in E1 and E1–NS2, it shared the characteristics of the truncated genome; entire core, partial E1, and NS2 (Fig. 3B).

In cases with both truncated and non-truncated HCV cDNA, the predominant PCR product was non-truncated (Table I, Fig. 3A). Figure 4 shows the structures of the cDNA isolates. Deletions similar to those described above were found only in Patient 325. Patient 295, 288, 274, and 331 had out-of-frame deletions, and except for a clone from Patient 331 [LV331-(i)], sequence comparison between truncated and non-truncated HCV cDNA indicated sequence identity (3–8 nucleotides in length) in these out-of-frame deletions between the deletion donor and acceptor regions.

In Patient 207, Both Truncated and Non-Truncated HCV RNA Co-Existed for Years

E2 primers for PCR were designed to avoid PCR bias under competitive PCR conditions in order to confirm the presence of the non-truncated HCV genome in Patient 207 [Alvarez et al., 2000]. Two overlapping HCV cDNA sequences (LV0922 and LV2030) were isolated from a liver biopsy specimen from Patient 207 (Fig. 5A). The overlapping regions of the consensus sequences (LV0922cons and LV2030cons in Fig. 5B) were identical; however, the amino acid sequence identity to the truncated HCV genome (LV207cons: consensus sequence determined with isolates) was 92.1% in the E1 region, and 82.1% in the NS2 region (Fig. 5B).

Both the truncated and non-truncated genomes were detected by RT-PCR in serum from Patient 207 (Fig. 5A). The sequence of an isolate (S831) showed 99.4% nucleotide and amino acid sequence identity with LV207cons and the same in-frame deletions (Fig. 5A). The cDNA for the non-truncated genome (S2531) was also isolated from the serum by PCR with primers for E2 sequences. This cDNA was nearly identical (99.8% in nucleotide sequence) to that from the liver (LV2030cons), but differed from the truncated HCV genome identified in the liver (LV207cons) and serum (S831) (Fig. 5B).

From the RNA extracted from Patient 207 serum, a PCR fragment of the expected length was amplified using anti-sense primer for junction site at the deleted position between E1 and NS2 of the truncated genome of Patient 207. However, the PCR fragment was not amplified from the RNA of Donor G14 plasma, in which only the full-length HCV genome was detected. Similarly, the PCR fragment was amplified from truncated RNA transcripts, but not amplified from non-truncated RNA transcripts (Fig. 5C).

In order to examine the persistence of both HCV genomes, a series of serum specimens consisting of samples obtained from Patient 207 were examined. Only the truncated RNA was detected by RT-PCR with primers for the core and NS2 in all samples. The sequences of PCR fragment clones were conserved at both the nucleotide and amino acid level (92–99% identity, Fig. 6A). All clones had the same deletions as observed in biopsy samples (Fig. 6A) at the E1–NS2 junction. Novel quasispecies with amino acid deletions at 13 amino acids upstream of the junction were found in March 1998 (designated as 9803). Another quasispecies was identified in March 1999. HCV E2 cDNAs of the non-truncated genome were isolated by RT-PCR from all serum samples, and were found to have conserved sequences (Fig. 6B).

Significant Difference in the Ratio of Truncated to Non-Truncated Genome Between Serum and Liver

HCV RNA from Patient 207 was measured by real-time RT-PCR for the 5′-UTR and E2 sequences. The quantity of 5′-UTR is indicative of the entire HCV genome, while that of E2 is only indicative of the non-truncated genome. The ratio of HCV E2 RNA to 5′-UTR RNA in serum was nearly constant throughout the 15-months monitoring period (Table III). Significant differences in the ratio of truncated to non-truncated genome were observed between liver and serum RNA samples; the ratio of HCV 5′-UTR to E2 RNA in liver specimens was about a hundred times of those in serum.

Expression of Truncated HCV cDNA In Vitro

HCV proteins processed from subgenomic HCV cDNA were investigated in a transient cDNA expression experiment. Chimeric cDNA for the truncated genome, LV207cont, consisted of cDNA fragments selected from 4 cDNA isolates from Patient 207 and the 3′-end of NS5B to the 3′-UTR sequence isolated from another patient (Fig. 7A). For expression of full-length HCV cDNA, chimeric cDNAs having the core–NS2 sequence of LV207cont instead of the corresponding region of J1 HCV cDNA (J1NLV) were used.

Figure 7B shows the Western blots of lysates from cells transfected with cDNA expression vectors. Anti-core monoclonal antibody detected a 19-kDa protein, thus indicating that the truncated polyprotein was correctly processed at the core-E1 junction. Each 35-kDa protein expressed from HCV cDNA, which was reactive to anti-E1 monoclonal antibody, was susceptible to endoglycosidase (Endo H), and converted to different molecular masses by enzyme treatment. The migration of deglycosylated E1 reactive peptide from the truncated HCV cDNA corresponded to the predicted molecular mass of the E1–NS2 fusion protein, 24 kDa, which differed from that of the non truncated E1 (19 kDa). The

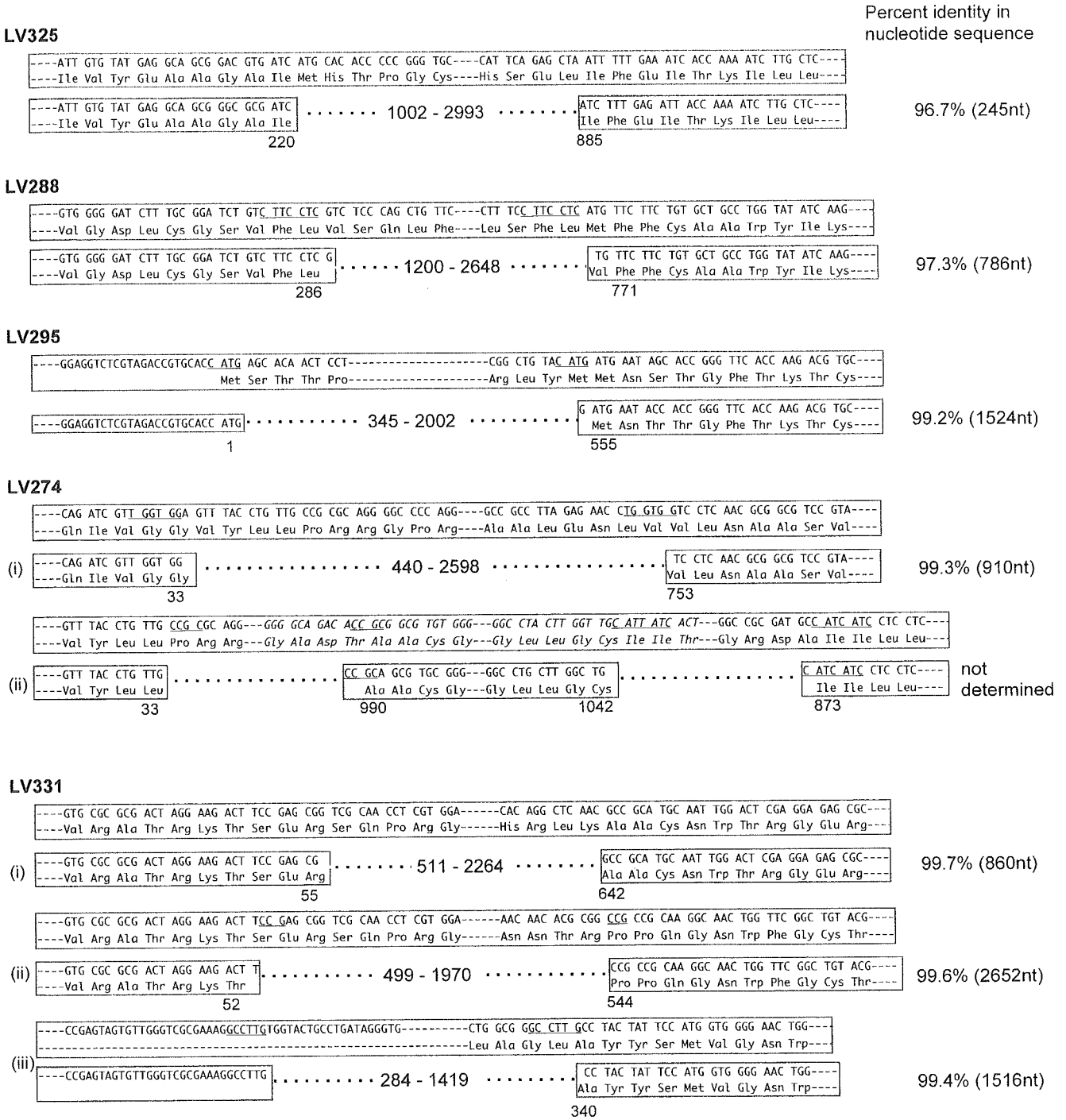
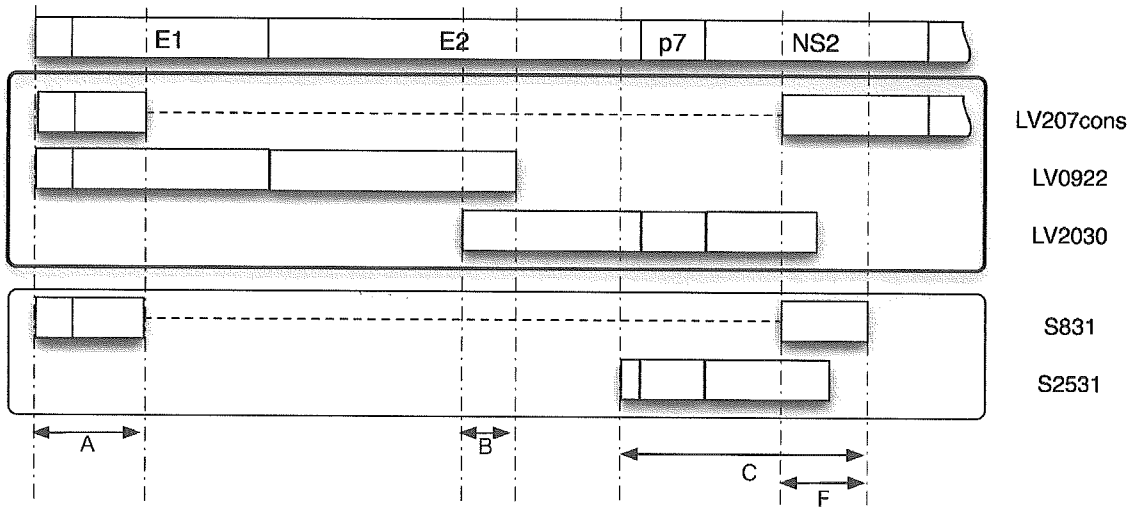


Fig. 4. Sequence comparisons between HCV genomes with and without deletions isolated from the same patient. Partial sequences of RT-PCR fragments isolated from liver biopsies are shown. cDNA fragments are labeled as described for previous figures. Nucleotide and amino acid positions are represented as the corresponding positions in HCV-J1. In these cases, both non-truncated and truncated HCV RNA were amplified from each biopsy specimen (Table I). The non-truncated and truncated RT-PCR fragments from Patient 325, 288, 295, 274, and 331 were obtained with primer sets d and d, d and d, b and b, b and e, and a and b (Table II), respectively. Numbers between tagged graphs represent deleted regions as they correspond to HCV-J1. Bottom lines show the corresponding amino acid positions of the boundaries. The right side of the sequences shows the identities of overlapping nucleotide sequences in the truncated and non-truncated genome, and the length of the overlap is given in parentheses. The LV274-(ii) had a translocation of the NS3 sequence inserted between the core and NS2 sequences. Because we did not clone the NS3 sequence from this case, the corresponding region of HCV-J1 is italicized in the figure and the sequence identity was not determined. Sequences that coincided between the boundaries of the deletions are underlined.

A



B

			Serum cDNA		
			S831		S2531
Liver cDNA	LV207cons	Region	A	F	F
		Nucleotide	99.7% (315)	98.9% (180)	91.8% (98)
		Amino acid	100% (105)	98.3% (60)	81.8% (33)
	LV0922cons	Region	A		
		Nucleotide	95.0% (303)		
		Amino acid	92.1% (101)		
	LV2030cons	Region		F	C
		Nucleotide		90.2% (82)	99.8% (513)
		Amino acid		81.4% (27)	99.4% (171)

			Liver cDNA	
			LV0922cons	LV2030cons
Liver cDNA	LV207cons	Region	A	F
		Nucleotide	95.0% (303)	91.6% (98)
		Amino acid	92.1% (101)	82.1% (28)
	LV2030cons	Region	B	
		Nucleotide	100% (76)	–
		Amino acid	100% (25)	

Number in parentheses expresses
The length of the overlapped sequences

C

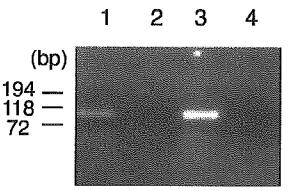


Fig. 5. Cloning of the non-truncated HCV RNA from patient 207. **A**: Schematic views of RT-PCR fragments for non-truncated HCV RNA in liver (LV0922 and LV2030) and in serum (S2531) from Patient 207, and for the truncated sequence in serum (S831). LV0922 fragment was obtained by RT-PCR with HC1b9405R for cDNA synthesis, HC841S and HC2199AS for 1st PCR, and HC948S and HC2199AS for 2nd PCR. LV2030 fragment was obtained by RT-PCR with XR58R for cDNA synthesis, HC2048SLV and LVC1392AS for 1st PCR, and HC2069S and LVC1280AS for 2nd PCR. S2531 fragment was obtained by RT-PCR with HC3174AS for cDNA synthesis, HC2430S and HC3174AS for 1st PCR, and HC2546S and HC3111AS for 2nd PCR. Arrows indicate regions being compared in the Tables (**B**), which show sequence identities between the non-truncated and truncated sequences in serum (**upper**), and those between sequences isolated from liver and liver (**lower**). **C**: Images of agarose gel electrophoresis of RT-PCR products amplified using junction site primer, from extracted RNA from Patient 207 serum (**lane 1**) and Donor G14 plasma (**lane 2**), truncated in vitro RNA transcripts of Patient 207 (**lane 3**) and non-truncated in vitro RNA transcripts of Donor G14 (**lane 4**).

protein at the 70-kDa position, which was the predicted mass of NS3, was detected by anti-NS3 polyclonal antibody. These data suggested that processing of the truncated HCV polyprotein is same as that of the full-length HCV polyprotein at the core–E1 and NS2–NS3 junctions.

DISCUSSION

Characteristics of HCV Subgenome With In-Frame Deletion

Novel truncated HCV genomes with in-frame deletions from E1 to NS2 were identified in the livers of two

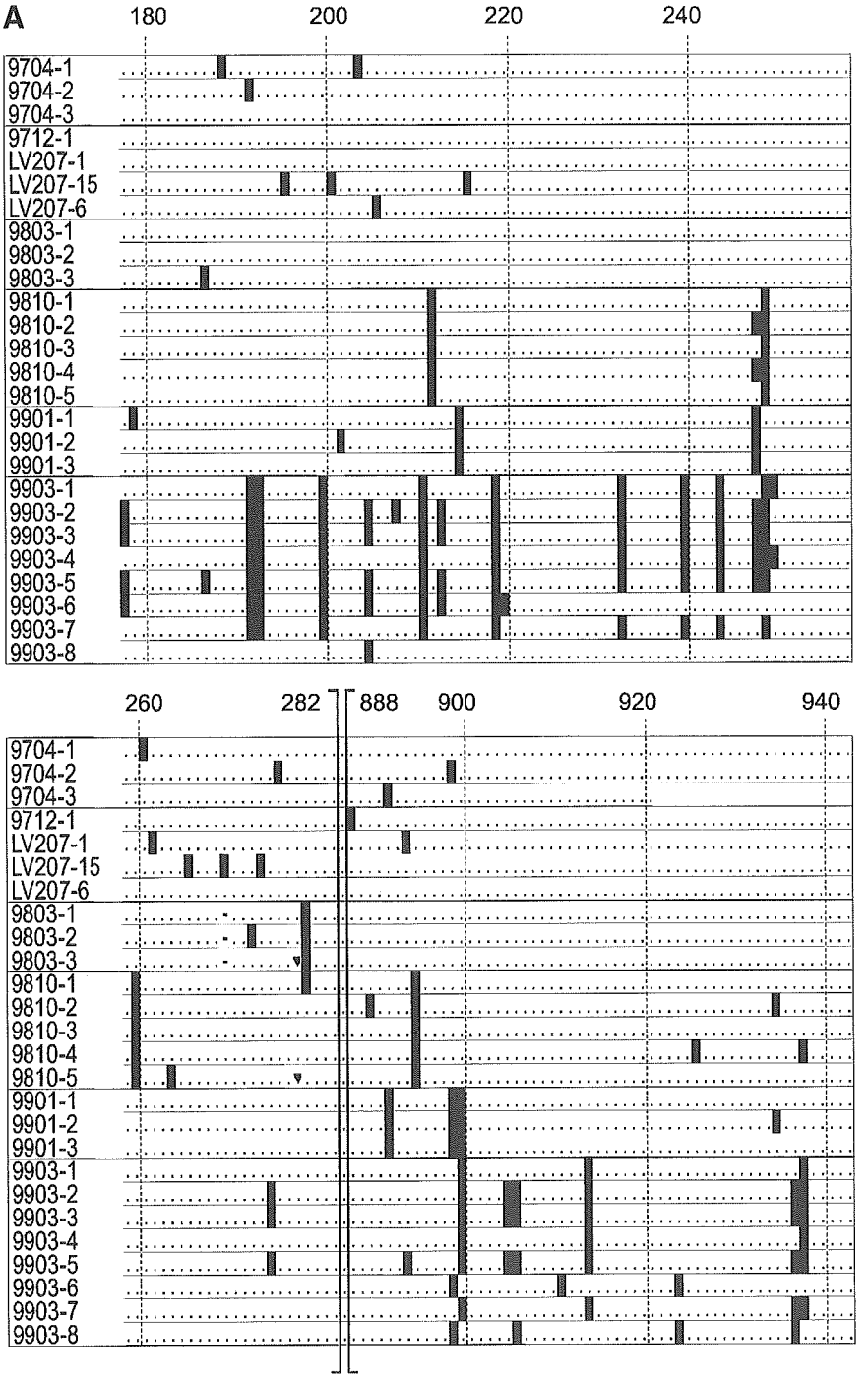


Fig. 6. Alignment of amino acid sequences of cDNA isolates from a series of serum samples from Patient 207. Comparison of nucleotide sequences of truncated genome (**A**) and non-truncated genome (**B**) was carried out using Clustal W algorithm. Numbers on top of the alignment show the corresponding amino acid positions of HCV J1. Names of the sequences indicate the date the serum was taken; 9712, for example, refers to December 1997. Bold bar represents amino acid position that differs from consensus sequence. Coinciding amino acid positions are represented by dots. All cDNA isolates from March 1998 (9803) had an amino acid deletion at the position marked by the horizontal bar. Bold vertical bars in A show the boundaries of the deletion. Inverted triangles indicate positions of nucleotide deletions in cDNA isolates. Deduced amino acid sequences with nucleotide deletions were obtained by inserting a nucleotide at the position. Fragments of the truncated genome (A) were obtained by RT-PCR with HC3297R for cDNA synthesis, HC813S and HC3297R for 1st PCR, and HC841S and HC3174AS for 2nd PCR. Fragments of the non-truncated genome (B) were obtained by RT-PCR with HC2378R for cDNA synthesis, HC1979S and HC2378R for 1st PCR, and HC1979S and HC2300R for 2nd PCR.

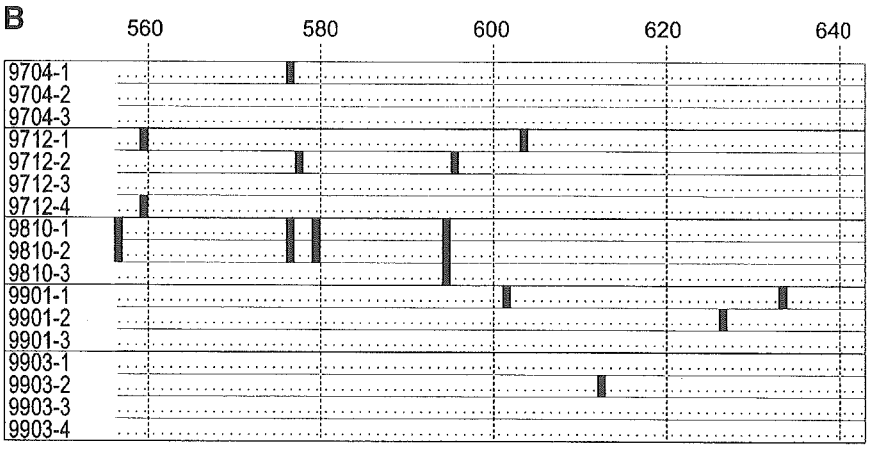


Fig. 6. (*Continued*)

chronic active hepatitis C patients. These HCV subgenomes encoded a single polyprotein for the entire core, five NS proteins (from NS3 to NS5B), and E1–NS2 fusion protein. This type of HCV subgenome was found in 4 of 23 chronic active hepatitis patients (16 cases with positive results by RT-PCR with primer sets tested), and in 2 hepatocellular carcinoma patients. These data suggested that HCV subgenomic RNA is generated in a certain number of chronic active hepatitis C patients.

HCV polypeptide expressed in cells with the subgenomic cDNA was processed in the same way as the authentic HCV polyprotein. The E1–NS2 fusion protein from subgenomic HCV cDNA was glycosylated and was susceptible to Endo H treatment, thus suggesting that it was located in the ER. NS2 is a membrane protein located in the ER region [Santolini et al., 1995; Kim et al., 1999]. On the other hand, localization and modification of E1 protein was governed by E2 [Cocquerel et al., 1998; Duvet et al., 1998]. These data indicate that ER retention in the E1–NS2 fusion protein is determined by NS2.

TABLE III. Quantitation of HCV RNA in a Series of Serum Samples Taken From Patient 207

Date	5′-UTR (copies/ml)	Percentage of E2/UTR
December 8, 1997	7.75×10^4	19.5%
	(5.75×10^3	0.2%)
	Numbers in parenthesis represents HCV quantity in liver biopsy specimen	
March 6, 1998	2.93×10^3	36.2%
March 9, 1998	1.19×10^4	22.1%
	IFN treatment From March 9, 1998 to September 2, 1998	
September 29, 1998	4.17×10^4	23.8%
October 28, 1998	5.25×10^4	21.8%
December 8, 1998	2.24×10^4	26.1%
January 12, 1999	5.81×10^4	28.5%
February 10, 1999	2.57×10^4	20.3%
March 31, 1999	5.30×10^4	23.1%

Do HCV Subgenomes Replicate Autonomously in Patients?

Viral subgenomes have been isolated from viruses closely related to HCV, such as flavivirus (Murray Valley encephalitis virus) [Lancaster et al., 1998], pestivirus (classical swine fever virus; SFV [Aoki et al., 2001], and bovine viral diarrhea virus; BVDV [Tautz et al., 1994; Kupfermann et al., 1996]. The HCV subgenomes shared common structural features with these subgenomic RNAs.

The HCV subgenomes fulfilled the minimal requirements for autonomous RNA replication; the 5′-UTR, nonstructural proteins (NS3–NS5B), and the 3′-UTR, as demonstrated using artificial HCV subgenomic replicons [Lohmann et al., 1999; Blight et al., 2000]. In addition, defective genomes of DI autonomously replicate their RNA [Behrens et al., 1998]. Furthermore, sequence comparisons of the truncated and non-truncated HCV genome sequences, which were isolated from a serum series obtained from a single patient, suggested that both genomes have been replicating independently for years. These data suggest that HCV subgenomes with in-frame deletions in structural proteins replicate themselves. However, it is possible that the full-length genome is required for the replication of the subgenome, as both genomes were present for years despite the dominance of the subgenome.

The dominance of the subgenome over the full-length genome (approximately 500-fold in the liver) was indicated by real-time RT-PCR analysis for the HCV 5′-UTR and E2. The dominance of HCV subgenome suggests an advantage in RNA replication. The length of the genome is probably a key factor in viral replication. If processing velocities in translation and transcription are equal over the HCV genome, the HCV subgenome would be replicated about 20% faster than the non-truncated genome. However, other mechanisms affecting efficiency are likely present.

Heterogeneous molecular clones with out-of-frame deletions, which shared sequences with the full-length

genome, indicated that the HCV subgenome frequently arises from its full-length genome by such mechanisms found in other RNA viruses [Nagy and Simon, 1997]. However, the fact only one type of subgenome with an in-frame deletion persisted suggested that a competent subgenome for replication is selected. The NS2 in all HCV subgenomes, which preserved their NS2–NS3 protease domains [Grakoui et al., 1993a; Hijikata et al., 1993], indicated protease activity is involved in the persistence of the HCV subgenome. Furthermore, we believe that core protein is required for virus replication in vivo, because the core sequence in the HCV subgenomes was preserved among the dominant HCV subgenomes.

Comparison With HCV Subgenomes or Recombinants Described Previously

Quadri and Negro [2001] identified recently a positive-strand subgenomic RNA starting from the 5′-UTR without the 3′-UTR, and a negative-strand subgenomic RNA with the 3′-UTR lacking the 5′-UTR. Although we did not clone the 3′-UTR from the Patient 207 sample, an HCV subgenome with same deletion was isolated from cDNA with a primer corresponding to the X-region in the 3′-UTR(data not shown), and we isolated the 3′-UTR from Patient 373 liver RNA. Based on these observations, it is considered that the subgenomic HCV RNA contains the entire 3′-UTR, rather than their proposed RNA populations.

Intergenotypic recombination has been described between genotype 2k and genotype 1b HCV at between nucleotides 3175 and 3176, about 200 nucleotides from the recombination region of the HCV subgenomes [Kalinina et al., 2002]. We did not examine the possibility of this type of recombination because the number of HCV cDNAs covering this region was too few in the present study.

HCV Subgenome and Pathogenesis

The question whether the HCV subgenome involved in the mechanism of viral persistence and pathogenesis in a similar manner as the DI particles of other viruses

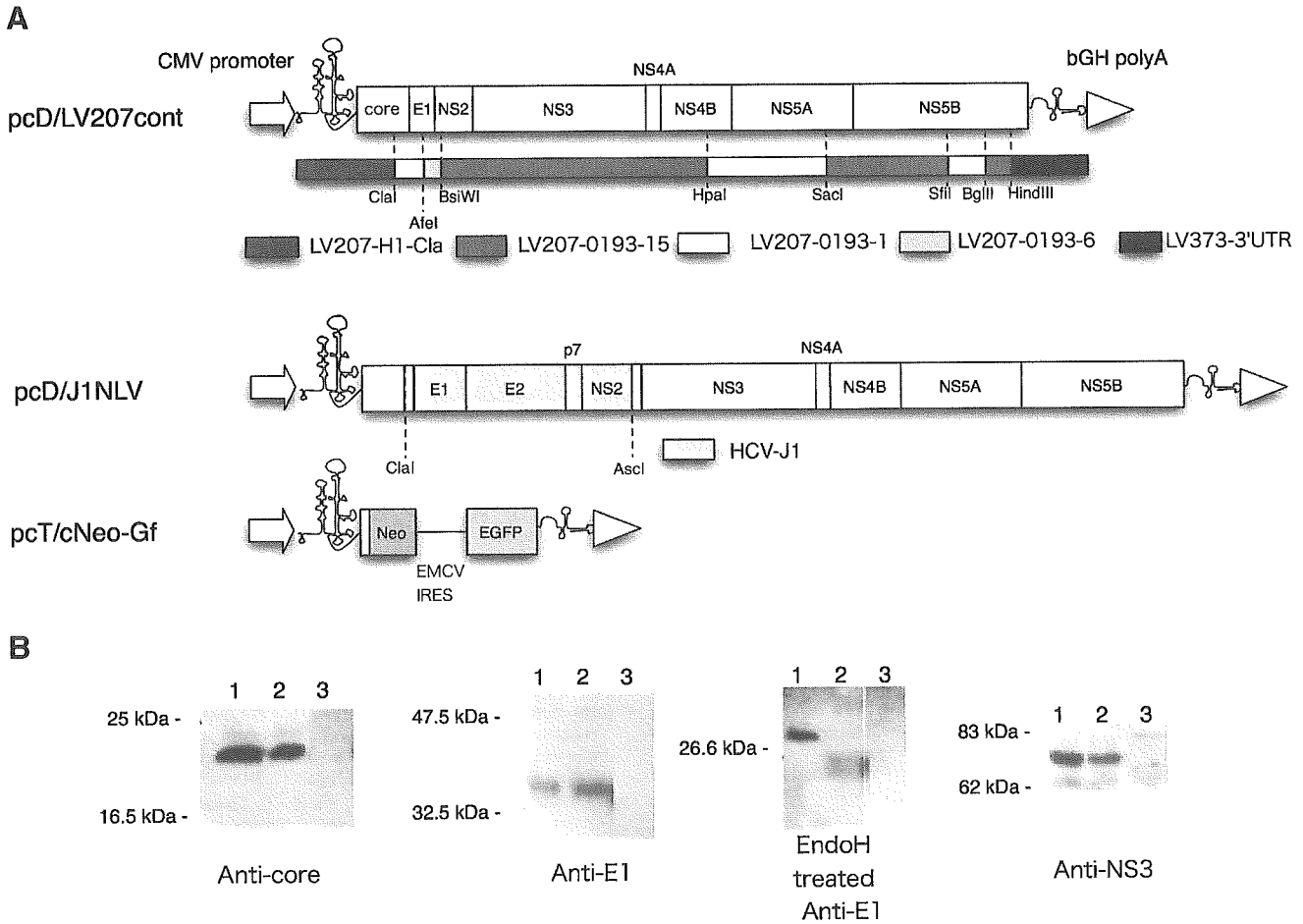


Fig. 7. Expression of HCV proteins from truncated and non-truncated HCV cDNA in mammalian cells. **A**: Chimeric HCV subgenomic cDNA, LV207cont, was composed of fragments from four cDNAs from patient liver (LV207-H1-Cla, 0193-15, 0193-1, and 0193-6) and a fragment from Patient 373 (LV373-3′-UTR) by using restriction sites depicted. Other chimeric cDNAs consisted of LV207cont and a fragment of HCV-J1 (J1NLV), as indicated. Dicistronic DNA constructs of HCV core-Neo resistant fusion and EGFP genes, cNeo-EGFP, was used as a negative control for HCV protein expression. All DNA constructs were transiently expressed in HEK293 cells under the control of the CMV promoter in pcDNA3.1. **B**: Western blotting analysis results for core, E1 and NS3 in transfected cells are shown. The positions of pre-stained molecular weight markers are indicated on the left side of the images.

[Tautz et al., 1994; Kupfermann et al., 1996] is remained. The amounts of core protein in patients with the HCV subgenome were larger than in patients without the subgenome (not statically significant). Transgenic mice expressing core protein in liver developed steatosis and later cancer, indicating that the core protein is a potent carcinogen in mice [Moriya et al., 1997, 1998]. It was found that two HCC patients had this subgenome. These data suggested that the involvement of the truncated genome in pathogenesis; however, we must examine more cases in order to elucidate any correlations between HCV subgenome and disease, particularly for progression of the disease to HCC.

The heterogeneous nature of the HCV genomes in patients may contribute to the persistence of HCV in escaping the host defense system. Particularly, the deletion of E1/E2 proteins may have a great impact on host immune response to the virus; E1/E2 is believed to be a target molecule for neutralizing antibodies, which block the binding of virions to virus receptor [Beyene et al., 2002]. The function of the truncated HCV genome in the life cycle of HCV is uncertain, but we believe the presence of this subgenomic RNA in both the liver and serum is important for illustrating that much about the nature of HCV remains unknown.

Added in Proof

Wakita et al. recently reported that a man-made HCV subgenomic RNA lacking E1/E2 replicated in vitro. [Wakita et al., 2005, NatureMedicine, published online 12 June]

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Age-specific antibody to hepatitis E virus has remained constant during the past 20 years in Japan

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SUMMARY. We investigated the presence of antibodies to hepatitis E virus (anti-HEV) and hepatitis A virus (anti-HAV) by enzyme immunoassays in sera from 1015 individuals collected in 1974, 1984 and 1994. Age-specific profiles of anti-HEV remained unchanged with a peak at 40–49 years, while those of anti-HAV started to increase in individuals aged 20–29 years in 1974, 30–39 years in 1984 and 40–49 years in 1994. These results suggest that a silent HEV infection has been taking place in the last 20 years or so in Japan, while HAV infection has been terminated at least since 1974.

Keywords: hepatitis A virus, hepatitis A virus antibody, hepatitis E virus, hepatitis E virus antibody, seroepidemiology.

INTRODUCTION

Hepatitis E virus (HEV) is transmitted mainly by the faecal–oral route, and causes waterborne outbreaks and sporadic cases of acute hepatitis in developing countries with poor sanitary conditions [1]. Outbreaks of HEV have been primarily noted in developing countries, whereas cases in developed countries were considered to have been exposed in foreign countries. However, cases of acute hepatitis because of indigenous HEV strains were reported in patients in the United States, Europe and Japan who had never travelled abroad [2–4]. Recently, HEV strains have been isolated from pigs in developed countries, which are closely related to local human HEV strains, suggesting zoonotic infection [5–7]. Because of these lines of evidence, HEV has attracted increasing attention even in developed countries where HEV is not endemic. For example, in Japan, more than 20% of acute, sporadic hepatitis cases are non-A, non-B, non-C [8].

The impact of HEV infection in developed countries, however, has not been fully explored. Past exposure to HEV can be examined by enzyme-linked immunosorbent assay (ELISA) by detecting antibody to HEV (anti-HEV). In the present study, we surveyed the extent and changes of HEV infection in Japan by testing sex- and age-specific prevalence of anti-HEV in serum samples collected in 1974, 1984 and 1994. Using the same samples, we also tested for antibodies to hepatitis A virus (anti-HAV) for comparison.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; HAV, hepatitis A virus; HEV, hepatitis E virus; VLP, virus-like particles.

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MATERIALS AND METHODS

Serum samples

A total of 1015 samples were selected at random from the Serum Reference Bank of the National Institute of Infectious Diseases, Tokyo. They were obtained from healthy volunteers aged from 0 to 89 years (median 35.6 years) living in seven prefectures in the central part of Japan. Of them, 349 were collected in 1974, 324 in 1984 and 342 in 1994. The present study, was reviewed by the ethical committee of the National Institute of Infectious Diseases.

Hepatitis viral markers

Anti-HAV (total antibody) was determined by radioimmunoassay using a commercial kit (HAV-AB RIA kit; Dainabot Co., Ltd, Tokyo, Japan). Positive and negative results were judged according to the manufacturer's instructions with intermediate results recorded as negative. Anti-HEV was determined by ELISA using the method of Li *et al.* [9]. Briefly, wells of microtitre plates were coated with purified virus-like particles (VLP) of HEV expressed by a recombinant baculovirus. One hundred microlitres of test serum was then added to each well and the plate was incubated at 37 °C for 1 h. The plate was washed six times with 10 mM phosphate

buffered saline containing 0.05% Tween 20, and then 100 μL of buffer containing goat anti-human IgG conjugated with horseradish peroxidase was delivered to each well. The plate was incubated for a further 1-h period at 37 °C, washed six times, and thereafter, each well received 100 μL of buffer containing orthophenylenediamine. The plate was incubated at room temperature for 30 min, and then 50 μL of 4 N H_2SO_4 are added to each well. The absorbance at 492 nm was recorded and positive and negative results were scored as described by Li *et al.* [9].

Statistical analyses

Statistical analyses were performed using the chi-square test, and $P < 0.05$ was considered significant.

RESULTS

Age-specific prevalence of anti-HEV and anti-HAV

Basic patterns of age-specific prevalence of anti-HEV were similar in the three examination years (Fig. 1). The prevalence of anti-HEV increased gradually until 40–49 years old, and then decreased slowly with age. In contrast, the prevalence of anti-HAV was almost nil in people younger than 20 years, increased steeply at a certain age range, and then reached 80–90% in older people in all of the three years of examination. The age range at which the prevalence of anti-HAV started to increase sharply was 20–29 years in 1974, 30–39 years in 1984 and 40–49 years in 1994. Thus, it shifted by 10 years at each examination year. Anti-HAV was significantly more prevalent than anti-HEV in all age ranges over 30 years in 1974 ($P < 0.001$ in all). Similarly, anti-HAV was significantly more prevalent in all age ranges over 40 years in 1984 ($P < 0.001$ in all), and in all age ranges over 50 years in 1994 ($P < 0.001$ in all).

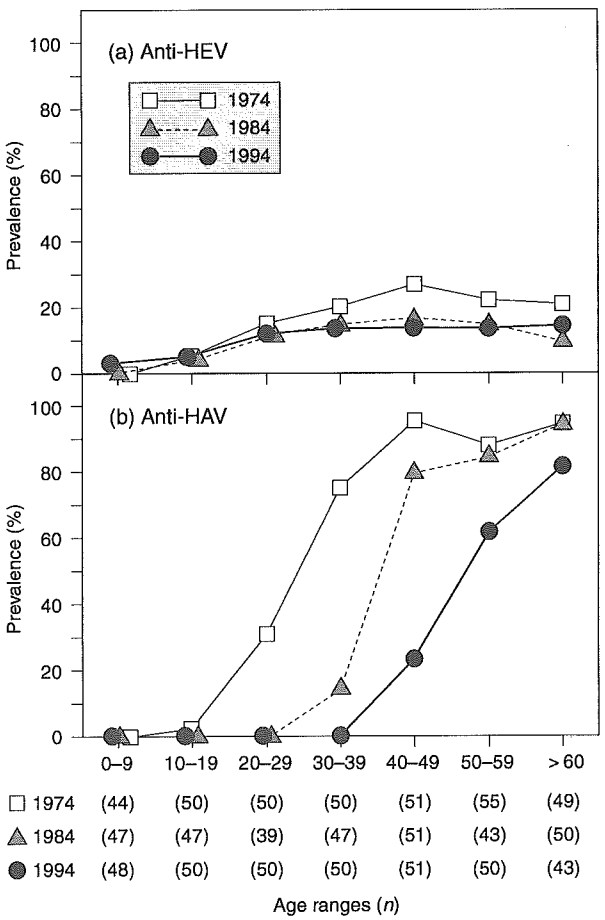

Fig. 1 Age-specific prevalence of anti-HEV and anti-HAV in Japan at three different times. Number of individuals tested in each age group and year is indicated below in parentheses.

Sex- and age-specific prevalence of anti-HEV

Figure 2 illustrates the prevalence of anti-HEV in serum samples from different age groups of healthy Japanese volunteers collected in 1974, 1984 and 1994 stratified by

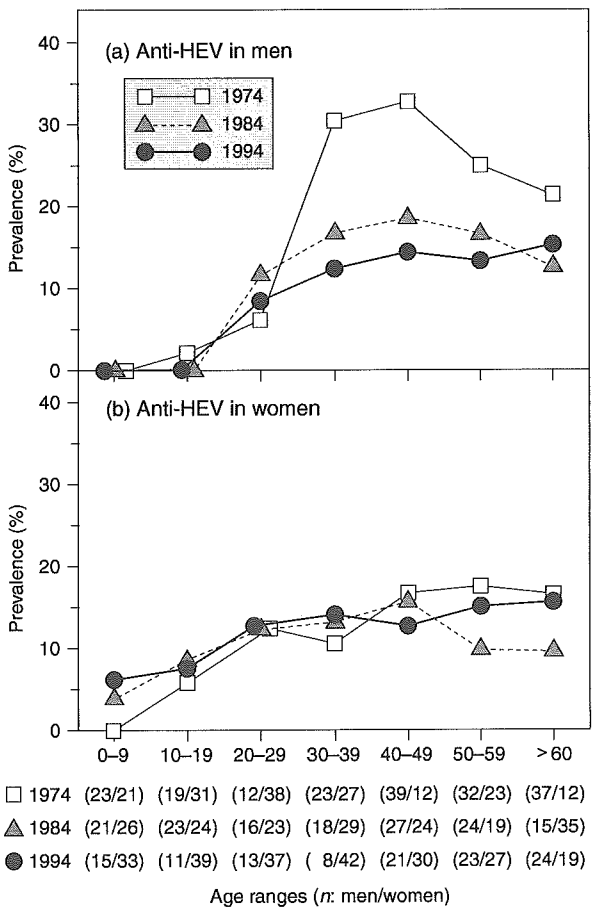

Fig. 2 Sex and age-specific prevalence of anti-HEV in Japan at three different times. Numbers of men/women tested in each age group and year are indicated below in parentheses.

sex and age. Although basic profiles of the prevalence of anti-HEV did not differ between men and women, anti-HEV in men was significantly more frequent in 1974 (21.6%) than in 1984 (11.1%, $P = 0.012$) and 1994 (10.4%, $P = 0.013$); the difference is attributed to a high frequency of anti-HEV in age groups older than 30 years in 1974. In sharp contrast, the age-specific prevalence of anti-HEV in women stayed unchanged in the three different years examined (11.0% in 1974, 10.6% in 1984 and 11.9% in 1994). Differences in the prevalence of anti-HEV between men and women were significant only in the year 1974 ($P = 0.008$). Age-specific prevalence of anti-HAV was quite similar between males and females in each year of examination (data not shown).

DISCUSSION

Many immunological methods have been developed for the determination of anti-HEV utilizing natural and recombinant viral proteins as antigens. They are, however, disappointingly nonspecific and have been shown to yield discrepant results using the same panel of sera with or without anti-HEV [10]. Lack of reliable serological assays for the detection of anti-HEV has hampered the accurate examination of exposure to HEV in various epidemiological and clinical settings.

Recently, Li *et al.* [11] succeeded in developing VLP using a recombinant baculovirus containing the coding sequences for capsid protein of the virus. The VLP appear to have similar antigenicity to the authentic HEV particles [12]. Using these VLP, a novel ELISA for anti-HEV has been developed that is sensitive and specific in seroepidemiological surveys for HEV infection. A considerable proportion of Japanese adults (around 10–20%) appear to have had previous exposure to HEV, although Japan is not endemic for hepatitis E [9,13].

The clinical features of HAV infection are similar to those of HEV infection, in that they both are transmitted by the faecal–oral route and cause acute hepatitis without chronic sequelae. In the present study, serological markers of HAV and HEV infection were determined and compared among healthy Japanese volunteers at three different time points (1974, 1984 and 1994). Age-specific prevalence of anti-HAV increased steeply and reached 90% at a certain age range dependent on the year of examination. The age range at which anti-HAV increased shifted by 10 years in subsequent time points, indicating that HAV infection was endemic several decades ago in Japan and has been contained thereafter. This would be mainly because of an improvement of sanitary conditions in Japan since the 1950s.

Age-specific profiles distinct between anti-HAV and anti-HEV during the last 20 years in Japan would be not only of epidemiological but also of clinical relevance. The prevalence of anti-HAV increasing with age involving by far the most aged individuals signifies a life-long immunoprotection against HAV. By sharp contrast, the prevalence of anti-HEV did not increase linearly with age, and peaked in individuals aged 40–49 years. Furthermore, unlike anti-HAV that has become increasingly absent in younger age groups, anti-HEV was detected in younger individuals aged 20–29 years in both men and women, and among women aged <20 years, in the three examination years. Similar age-specific profiles of anti-HEV have been reported in India [14]. Prevalence of anti-HEV in Japanese individuals younger than 30 years old was somewhat higher in the present study, than the almost zero prevalence reported by Li *et al.* [9]. Although there was some difference in percentages, the basic pattern of age-specific prevalence of anti-HEV was almost similar between the two studies.

Based on the age-specific distribution of anti-HEV, the exposure to HEV has not decreased during the past 20 years in Japan, unlike that to HAV. This implies that the principal transmission route of HEV may be different from that of HAV and would not be prevented only by improved sanitary conditions, despite the lower infectivity and transmissibility of HEV than HAV [14]. Zoonotic transmission of HEV through domestic and wild animals may account for the perpetuation of HEV infection, and deserves to be examined in future studies. Recently, transmission of HEV from pigs and deer to human beings has been reported in Japan [15,16].

In conclusion, exposure to HEV has stayed unchanged during the last 20 years in Japan, contrasting with HAV, which has diminished over the same period. These results warrant closer attention to infection with HEV, especially because it can induce fulminant hepatitis not only in pregnant women in developing countries [14], but also in sporadic cases in developed countries [15].

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