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Heterogeneity of Hepatitis B Virus Genotype D in Japan

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Key Words
Hepatitis B virus · Genotype D · Hepatitis B surface antigen

Abstract
Objective: Hepatitis B virus (HBV) genotypes B and C are predominant in Japan. Previously, we reported that approximately 9% of HBV carriers in the Ehime area of western Japan were infected with genotype D (HBV/D) and their sequences closely related. Recently, serum samples from 3 patients with chronic HBV/D infections living in Tokyo and the surrounding area became available for testing. The purpose of this study was to determine whether the HBV/D isolates from these different areas of Japan are closely related. ***Methods:*** Of the 3 Tokyo area patients infected with HBV/D, 2 had chronic hepatitis, and 1 had hemophilia with a history of frequent coagulation factor injections. The complete HBV/D genome sequences of each were determined, and compared with those of subjects from the Ehime area. ***Results:*** All 3 HBV/D sequences had a genomic length of 3,182 bases, and the hepatitis B surface antigen subtype was ayw3. Phylogenetic analysis revealed that the 1 of the HBV/D isolates was closely related to the isolates from Ehime Prefecture, while 1 was similar and 1 was clearly distinct. ***Conclusion:*** Our results indicate that HBV/D infections in Japan are heterogeneous.

Introduction

Hepatitis B virus (HBV) is an incomplete double-stranded DNA virus with approximately 3,200 bases in genomic length. More than 350 million people throughout the world are chronically infected with HBV, which had been classified into several serotypes and genotypes according to the antigenicity of the hepatitis B surface antigen (HBsAg) and the entire nucleotide sequence, respectively [1–4]. HBV genotypes are classified as A to H [4–7], and have distinct geographic distributions [8–11], as genotypes B (HBV/B) and C (HBV/C) are found mainly in East Asia, while genotypes A (HBV/A) is mainly distributed in Europe, North America and central Africa, with genotype D (HBV/D) is distributed widely. A number of studies regarding the significance and clinical relevance of HBV genotypes have been reported [12–18].

In Japan, HBV/B and HBV/C are the most prevalent. Although the frequency of HBV/D was reported to comprise only 0.4% of HBV carriers in Japan [8], approximately 9% of the HBV carriers in a small geographic locale in western Japan (Ehime Prefecture) were found to be infected with HBV/D [19]. It was reported previously that Gianotti-Crosti syndrome caused by HBV subtype *ayw* was endemic in the 1970s in the Ehime area and an HBV isolate with subtype *ayw* from a patient with that

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Table 1. Clinical and virological data of the 3 patients

Patient	Sex	Age	Diagnosis	Year of sampling	ALT IU/l	HBeAg	Anti-HBe	HBV-DNA LGE/ml (TMA)*	Isolate	Accession No.	Length bp	Deduced HBsAg subtype
1	M	12	CH, hemophilia A	1990	53	–	+	<3.7	Toshima 1	AB210819	3182	ayw3
2	M	27	ASC	2001	12	–	+	<3.7	Tokyo 1	AB210820	3182	ayw3
3	M	8	CH	2001	501	+	–	8	Kokusai-Iryo 2-OS	AB210822	3182	ayw3

CH = Chronic hepatitis; ASC = asymptomatic carrier (inactive HBsAg carrier state).
* Log genome equivalents/ml, transcription-mediated assay (Chugai Diagnostics Science Co. Ltd., Tokyo, Japan).

syndrome was determined to be HBV/D [20–22]. In our previous report, we showed that HBV/D strains from Ehime Prefecture formed a definite cluster in a phylogenetic tree, and that several isolates from Russia and north Europe were closely related to them [23]. Further, evolutionary analyses revealed that the root of the HBV/D phylogenetic tree in Ehime was approximately 1900. Combined with the history of Japan, we speculated that the origin of HBV/D in Ehime is Russia.

HBV/D is rare in other areas of Japan, however, serum samples from 3 patients in Tokyo and the surrounding area (greater Tokyo area) infected with HBV/D have become available for study. In the present study, we analyzed the genomic sequences of the HBV/D isolates from the Tokyo area and compared them to those from the Ehime area to elucidate whether the strains from these areas are closely related.

Patients and Methods

Patients

Three Japanese patients living in the Tokyo area and confirmed to be infected with HBV/D were included in this study. Patient 1 was a 12-year-old male, with chronic hepatitis and hemophilia, who had a history of frequent injections of coagulation factor VIII, which was considered to be the route of infection. Patient 2 was a 27-year-old male, asymptomatic HBV carrier, with an unknown infectious route, and patient 3 was an 8-year-old male, with chronic hepatitis, and an unknown infectious route. None of the 3 patients had ever lived in the Ehime area, and were not related. Serum samples were stored at –80° prior to genotyping and sequencing.

The purpose and the details of the study were explained, and written informed consent was obtained from the subjects or their parents.

HBV Genotyping

The HBV genotype was determined using the genotype-specific epitopes of the preS2 region of HBV with a commercially available enzyme immunoassay kit (HBV Genotype EIA Kit, Institute of Immunology, Tokyo, Japan) according to the manufacturer's instructions, as reported previously [24].

Complete Genome Sequencing

The complete genome sequences were determined by direct sequencing of the PCR- products, as explained in detail in our previous report [25]. Briefly, DNA was extracted from the serum sample, then to obtain the full-length HBV-DNA sequence, 2 amplicons were obtained by PCR, with 1 fragment shown to be 2,936 bases in length (nt 1994 to nt 1747), and the other 1,080 bases in length (nt 1399 to nt 2478). Sequencing was performed by direct sequencing using a commercially available kit (BigDye Terminator Cycle Sequencing FS Ready Reaction Kit, Applied Biosystems, Alameda, Calif., USA) with suitable sequencing primers. The accuracy of the sequences was ensured by identification of the sequence data of the complete genome obtained by the sense and anti-sense sequencing primers.

Phylogenetic Tree

The 3 complete HBV genomes in the present study were compared with those of HBV/D isolates in Ehime as well as other isolates available in databases. The nucleotide sequences were multiple-aligned using software (GENETYX for Windows version 7.0, Genetyx, Tokyo, Japan). Genetic distances were calculated using the Kimura two-parameter method and phylogenetic tree was constructed by the neighbor-joining method [26]. Phylogenetic and molecular evolutionary analyses were performed using MEGA version 2.1 [27].

Results

Complete Genome

Complete genome sequencing revealed that all 3 isolates were 3,182 bases in length, and the deduced HBs antigen subtype was ayw3 in each (table 1). The homol-

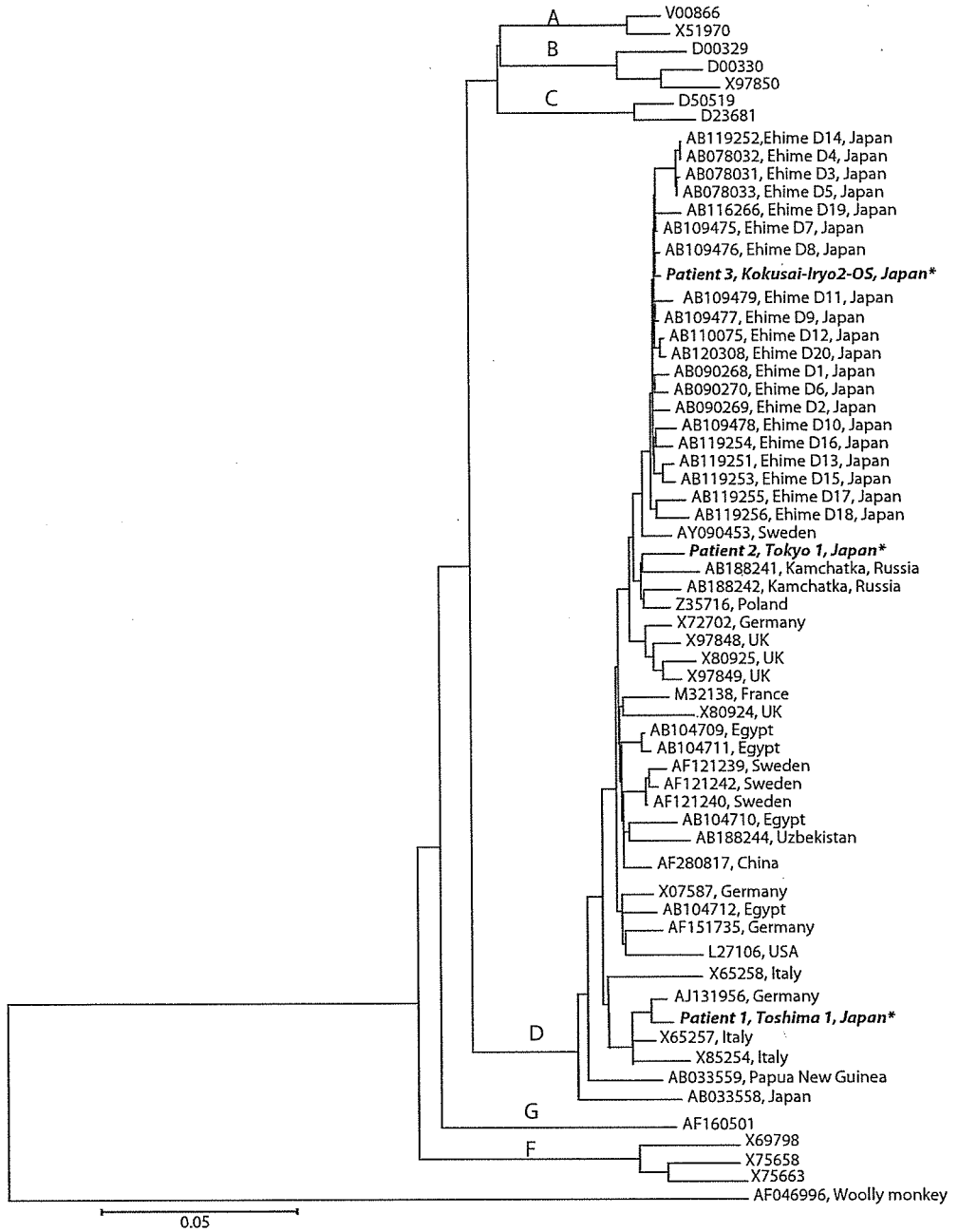

Fig. 1. Phylogenetic tree of HBV isolates. The accession numbers and information regarding the country or area where the isolate was obtained are included. Those from Japanese subjects are shown in italic bold letters and marked with an asterisk.

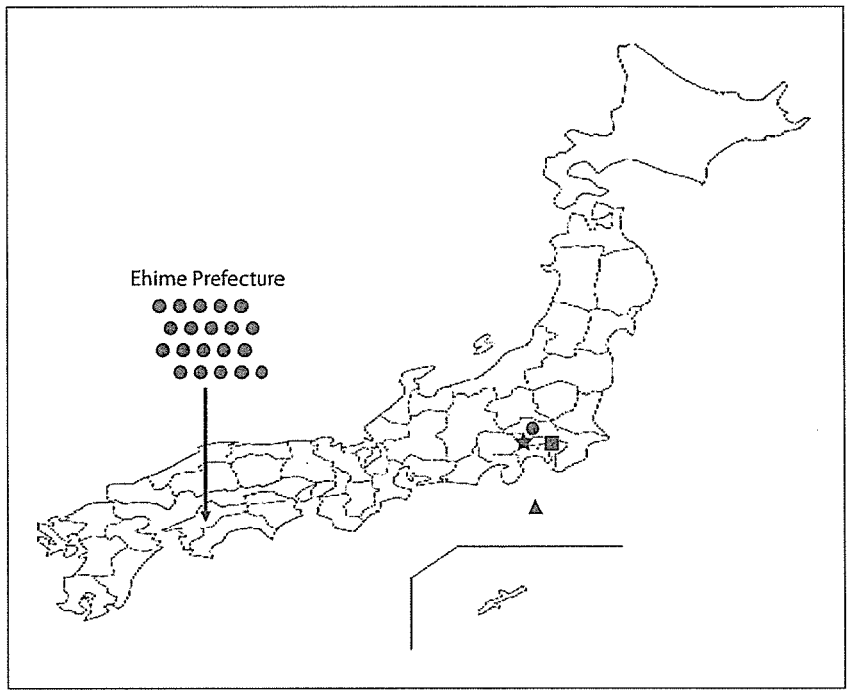

Fig. 2. HBV/D isolates in Japan and residence of subjects who harbored the isolates. ● = Isolates from Ehime [23] and the isolate from patient 3 in the present study that formed the cluster in the phylogenetic tree shown in figure 1. ■ = The isolate from patient 2 that has a close relation with several isolates from Kamchatka in Russia. ★ = The isolate from patient 1 that is distinct from the isolates from Ehime. ▲ = The isolate reported by Okamoto et al. [4] that is distinct from isolates from the Ehime, though residence information for that person is not available.

ogy of the nucleotide sequences (3,182 bases) among the 3 isolates was 96.39% between AB210819 (patient 1) and AB210820 (patient 2), 96.64% between AB210819 (patient 1) and AB210822 (patient 3), and 97.77% between AB210820 (patient 2) and AB210822 (patient 3).

Phylogenetic Analysis

Figure 1 shows a phylogenetic tree constructed from the complete nucleotide sequence of HBV. In addition to the 3 isolates in the present study, the complete genome sequences of 21 isolates of HBV/D isolated from Japanese subjects have been reported (fig. 1), which include 20 isolates from the Ehime area [23], and 1 (AB033558) from a Japanese blood donor reported by Okamoto et al. [4]. The isolate from the present patient 3 (AB210822) was very closely related to the isolates from the Ehime area, while that from patient 2 (AB210820) was closely related to several isolates from Kamchatka in Russia and relatively close to those from Ehime in the tree. In contrast, the isolate from patient 1 (AB210819), who had hemophilia, was clearly distinct from the isolates described above. Based on the present phylogenetic tree, we suspect that 1 of the isolates (AB210822) have the same origin as those in Ehime Prefecture, while the isolate from patient 1 was clearly distinct from the other 2 isolates and the isolates from Ehime area. The isolate reported by Okamoto et al. [4] was also shown to be distinct from the 3 Tokyo area isolates and all of those from Ehime. Therefore, we concluded that HBV/D in Japan is heterogeneous (fig. 2).

The nucleotide homologies between the 3 HBV/D isolates in the present study and the 20 from Ehime shown in figure 2 were calculated. The nucleotide homology between AB210819 (patient 1) and the 20 isolates from Ehime ranged from 95.91 to 96.64%, while that between AB210820 from patient 2 and the 20 isolates ranged from 97.01 to 97.86%, and that between AB210822 from patient 3 and the 20 isolates ranged from 98.68 to 99.81%.

Discussion

In the present study, we found that 1 HBV/D isolates from a patient living in Tokyo area was closely related to the isolates from Ehime, while 1 was close to several isolates from Kamchatka in Russia, and 1 was distinct from all of the isolates from Ehime. In our previous study, we speculated that HBV/D infection in the Ehime area had originated from Russia, and that Japanese-Russo war may relate with the import of HBV/D as approximately 6,000 Russian military personnel were interned in a pris-

on camp in Ehime during the Japanese-Russo war [23]. In addition, during and after that war, approximately 70,000 Russian individuals were interned in several prison camps throughout Japan, including the Tokyo area. Therefore, the 2 isolates from patients 2 and 3 are also suspected to have originated from Russia. From our results, we speculated that HBV/D strains originating from Russia may be the dominant HBV/D strains in Japan.

One of the present isolate from a patient in the Tokyo area (AB210819) was clearly distinct from the other 2 isolates from Tokyo and the 20 isolates from Ehime. That patient has a history of repeated injections with a coagulation factor produced in the 1970s and 1980s, during which time the screening and elimination of contaminated viruses in blood products was not sufficient. The ingredients used in coagulation factor products during those years were imported into Japan mainly from the United States, therefore, the origin of the HBV/D isolate in that patient may be related to imported coagulation factor product ingredients. Thus, imported coagulation factor products is suspected to be another route of HBV/D infection in Japan.

A few studies have reported subgrouping of HBV genotypes [28–30]. Norder et al. [11] classified HBV/D into 4 subgenotypes (D1-D4). According to that classification, the 20 isolates from Ehime area [23] and the isolate from the present patient 3 (AB210822) are D2, whereas the isolate from patient 1 (AB210819) and the isolate reported by Okamoto et al (AB033558) [4] are distinct from D2. These findings support the notion that HBV/D in Japan is heterogeneous.

HBV/B and HBV/C are indigenous to Japan, though it has been reported that HBV/A is increasing not only in Japanese patients with acute hepatitis, but also in chronic carriers [31–33], while other genotypes, such as HBV/E, F, G, H, have been identified from Japanese patients [34–37]. These HBV genotypes, as well as HBV/D, are suspected to have been transmitted horizontally, as they are not indigenous to Japan. A nationwide prophylactic policy for preventing mother to child transmission of HBV has been introduced in Japan, however, horizontal transmission, especially sexual transmission of HBV, remains uncontrolled [38], because a universal vaccination of HBV has not introduced. To prevent the transmission of HBV, especially HBV with non-indigenous genotypes, additional efforts are needed to prevent horizontal transmission routes.

In conclusion, our results show that HBV/D in Japan is heterogeneous. Further, we speculated that the various HBV/D strains in Japan originated from different areas of the world.

Acknowledgement

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Clinical Studies

Measurement of hepatitis B virus core-related antigen is valuable for identifying patients who are at low risk of lamivudine resistance

Tanaka E, Matsumoto A, Suzuki F, Kobayashi M, Mizokami M, Tanaka Y, Okanoue T, Minami M, Chayama K, Imamura M, Yatsuhashi H, Nagaoka S, Yotsuyanagi H, Kawata S, Kimura T, Maki N, Iino S, Kiyosawa K, HBV Core-Related Antigen Study Group. Measurement of hepatitis B virus core-related antigen is valuable for identifying patients who are at low risk of lamivudine resistance.
Liver International 2006: 26: 90–96. © Blackwell Munksgaard 2005

Abstract: *Objective:* The clinical usefulness of hepatitis B virus core-related antigen (HBVcrAg) assay was compared with that of HBV DNA assay in predicting the occurrence of lamivudine resistance in patients with chronic hepatitis B. *Patients:* Of a total of 81 patients who were treated with lamivudine, 25 (31%) developed lamivudine resistance during a median follow-up period of 19.3 months. *Results:* The pretreatment positive rate of HBe antigen, or pretreatment levels of HBVcrAg or HBV DNA did not differ between patients with and without lamivudine resistance. Levels of both HBVcrAg and HBV DNA decreased after the initiation of lamivudine administration; however, the level of HBVcrAg decreased significantly more slowly than that of HBV DNA. The occurrence of lamivudine resistance was significantly less frequent in the 56 patients whose HBV DNA level was less than 2.6 log copy/ml at 6 months of treatment than in the remaining 25 patients. The cumulative rate of lamivudine resistance was as high as 70% within 2 years in the latter group, while it was only 28% in the former group. Lamivudine resistance did not occur during the follow-up period in the 19 patients whose HBVcrAg level was less than 4.6 log U/ml at 6 months of treatment, while it did occur in 50% of the remaining patients within 2 years. *Conclusion:* These results suggest that measurement of HBV DNA is valuable for identifying patients who are at high risk of developing lamivudine resistance, and that, conversely, measurement of HBVcrAg is valuable for identifying those who are at low risk of lamivudine resistance.

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Key words: chronic hepatitis B – HBV core-related antigen – HBV DNA – lamivudine resistance

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Lamivudine, a nucleoside analogue that inhibits reverse transcriptases, was first developed as an anti-viral agent against human immunodeficiency virus (HIV). It was later also found to be effective against hepatitis B virus (HBV) because HBV is a member of the Hepadnaviridae family of viruses, which use reverse transcriptases in their replication process (1, 2). Lamivudine was found to inhibit the replication of HBV, reduce hepatitis, and improve histological findings of the liver in long-term treatment (3–5). Furthermore, it has been shown that lamivudine treatment improves the long-term outcome of patients with chronic hepatitis B (6, 7). However, there are a number of problems with lamivudine therapy, such as relapse of hepatitis because of the appearance of YMDD mutant viruses and the reactivation of hepatitis after discontinuation of the treatment (8–11).

The concentration of HBV DNA in serum decreases and usually becomes undetectable during lamivudine administration, but it rapidly increases when HBV becomes resistant to lamivudine. Thus, the measurement of HBV DNA is useful for monitoring the anti-viral effects of lamivudine. However, a negative result of HBV DNA in serum does not necessarily indicate a good outcome of lamivudine therapy, because lamivudine resistance may occur even if HBV DNA levels remain undetectable during therapy (11–13). Recently, a chemiluminescence enzyme immunoassay (CLEIA) was developed in our laboratory for the detection of hepatitis B virus core-related antigen (HBVcrAg) (14, 15). The assay reflects the viral load of HBV in a similar manner to that used in assays, which detect HBV DNA. HBVcrAg consists of HBV core and e antigens; both proteins are transcribed from the precore/core gene and their first 149 amino acids are identical (16–18). The HBVcrAg CLEIA simultaneously measures the serum levels of hepatitis B core (HBc) and e (HBe) antigens, using monoclonal antibodies, which recognize common epitopes of these two denatured antigens. In the present study, we analyzed the clinical significance of the HBVcrAg assay in monitoring the anti-viral effects of lamivudine treatment.

Patients and methods

Patients

A total of 81 patients with chronic hepatitis B, who received lamivudine therapy, were enrolled in the present study. These were 58 men and 23 women with a median age of 49 years (range 24–79 years). The 81 patients were selected retrospectively from six medical institutions in Japan (Shinshu University Hospital, Toranomon Hospital, Nagoya City University Hospital, Kyoto Prefectural University Hospital, Hiroshima University Hospital, National Nagasaki Medical Center). Eight to 25 patients who met the following three criteria were selected consecutively in each institution: the first, a daily dose of 100 mg lamivudine was administered for at least 6 months in a period from 1999 to 2004; the second, histologically confirmed for chronic hepatitis without liver cirrhosis; and the third, serum samples at several time points available for testing. All patients were naive for lamivudine therapy. Chronic hepatitis B was defined as positive hepatitis B surface (HBs) antigen for more than 6 months with elevated levels of serum transaminases. The HBV genotype was A in two patients, B in three and C in 76. Serum HBV DNA was detectable in all patients, and HBe antigen was positive in 51 (63%) of the 81 patients just before lamivudine administration. The median follow-up period was 19 months with a range from 6 to 50 months. Follow-up of patients ended when lamivudine administration was discontinued. Written informed consent was obtained from each patient.

The occurrence of lamivudine resistance was defined as a rapid increase in serum HBV DNA levels with the appearance of the YMDD mutations during lamivudine administration. Using this criteria, resistance appeared in 27 (33%) of the 81 patients. The median period from the start of lamivudine administration to the occurrence of resistance was 12 months with a range from 4 to 37 months.

Serological markers for HBV

HBs antigen, HBe antigen and anti-HBe antibody were tested using commercially available enzyme immunoassay kits (Abbott Japan Co., Ltd., Tokyo, Japan). Six major genotypes (A–F) of HBV can be detected using the method reported by Mizokami et al. (19), in which the surface gene sequence amplified by polymerase chain reaction (PCR) is analyzed by restriction fragment length polymorphism. The YMDD motif, that is, lamivudine resistant mutations in the active site of HBV polymerase, was detected with an enzyme-linked mini-sequence assay kit (HBV YMDD Mutation Detection Kit, Genome Science Laboratories Co., Ltd., Tokyo, Japan) (20).

Serum concentration of HBV DNA was determined using Amplicor HBV monitor kit (Roche, Tokyo, Japan), which had quantitative range from 2.6 to 7.6 log copy/ml. Sera containing

over 7.0 log copy/ml HBV DNA were diluted 10- or 100-fold with normal human serum and re-tested to obtain the end titer.

Serum concentrations of HBVcrAg were measured using the CLEIA method reported previously (10, 11). Briefly, 100 μL serum was mixed with 50 μL pretreatment solution containing 15% sodium dodecylsulfate and 2% Tween 60. After incubation at 70 °C for 30 min, 50 μL pretreated serum was added to a well coated with monoclonal antibodies against denatured HBc and HBe antigens (HB44, HB61 and HB114) and filled with 100 μL assay buffer. The mixture was incubated for 2 h at room temperature and the wells were then washed with buffer. Alkaline phosphatase-labeled monoclonal antibodies against denatured HBc and HBe antigens (HB91 and HB110) were added to the well, and the mixture was incubated for 1 h at room temperature. After washing, CDP-Star with Emerald II (Applied Biosystems, Bedford, MA) was added and the plate was incubated for 20 min at room temperature. The relative chemiluminescence intensity was measured, and the HBVcrAg concentration was determined by comparison with a standard curve generated using recombinant pro-HBe antigen (amino acids, 10–183 of the precore/core gene product). The HBVcrAg concentration was expressed as units/ml (U/ml) and the immunoreactivity of recombinant pro-HBe antigen at 10 fg/ml was defined as 1 U/ml. In the present study, the cutoff value was tentatively set at 3.0 log U/ml. Sera containing over 7.0 log U/ml HBVcrAg were diluted 10- or 100-fold in normal human serum and re-tested to obtain the end titer.

Statistical analysis

The Mann–Whitney *U*-test and Wilcoxon signed-ranks test were utilized to analyze quantitative data, and Fisher's exact test was used for qualitative data. A log-rank test was used to compare the occurrence of lamivudine resistance. Statistical analyses were performed using the SPSS 5.0 statistical software package (SPSS, Inc., Chicago, IL). A *P*-value of less than 0.05 was considered to be statistically significant.

Results

Table 1 shows a comparison of the clinical and virological backgrounds of the 27 patients who showed lamivudine resistance and the 54 patients who did not. Median age, gender distribution and median follow-up period did not differ between the two groups, and the positive rate of HBe antigen was similar. Both HBV DNA and HBVcrAg levels at the beginning of lamivudine administration were similar between the two groups; however, both HBV DNA and HBVcrAg levels at 6 months after the start of lamivudine administration were significantly lower in the lamivudine resistance negative group than in the positive group. ALT level was normal at the beginning in eight (15%) of the 54 patients without lamivudine resistance and in two (7%) of the 27 patients with it ($P > 0.2$).

Table 1. Comparison of the clinical and virological backgrounds of patients who showed lamivudine resistance and those who did not

Characteristics	Appearance of lamivudine resistance: Negative ($n = 54$)	Positive ($n = 27$)	P
Age (years)*	47.0 (24–79)	50.6 (34–67)	0.140†
Gender (male %)	74%	67%	>0.2‡
Follow-up period (months)*	16 (6–50)	21 (9–43)	>0.2†
HBV genotype (A/B/C)	2/2/50	0/1/26	>0.2‡
HBe antigen (positive %)	59%	70%	>0.2‡
ALT (IU/ml)*			
Initial	85 (22–713)	95 (20–1140)	>0.2†
At 6 months	27 (11–115)	30 (15–92)	>0.2†
HBV DNA (log copy/ml)*			
Initial	7.0 (3.5–9.1)	7.3 (4.2–9.2)	>0.2†
At 6 months	<2.6 (<2.6–4.8)	3.3 (<2.6–6.6)	<0.001†
HBVcrAg (log U/ml)*			
Initial	6.2 (<3.0–8.8)	7.3 (4.4–9.1)	0.073†
At 6 months	5.2 (<3.0–6.7)	5.8 (4.7–8.4)	<0.001†

HBe antigen, hepatitis B e antigen; HBV, hepatitis B virus; ALT, alanine aminotransferase; HBVcrAg, HBV core-related antigen. *Data are expressed as median (range). †Mann–Whitney *U* test. ‡χ^2-test.

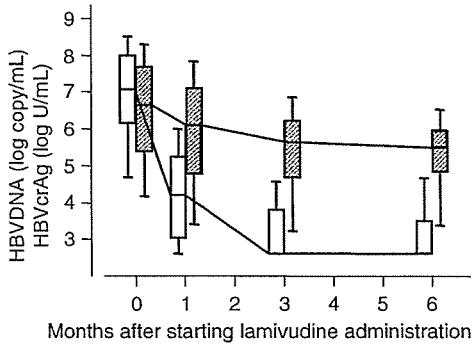

Fig. 1. Changes in the median levels of hepatitis B virus core-related antigen (HBVcrAg) and hepatitis B virus (HBV) DNA during lamivudine administration. The box plots show the 10th, 25th, 50th, 75th and 90th percentiles, with the open boxes indicating HBV DNA and shaded boxes indicating HBVcrAg. The median amount of decrease from the baseline in HBVcrAg levels was significantly smaller (Wilcoxon signed-ranks test) than that in HBV DNA level at 1 (2.80 log copy/ml vs. 0.27 log U/ml, $P < 0.001$), 3 (3.60 log copy/ml vs. 0.83 log U/ml, $P < 0.001$) and 6 months (3.90 log copy/ml vs. 1.15 log U/ml, $P < 0.001$) after the initiation of lamivudine administration.

Figure 1 shows changes in HBV DNA and HBVcrAg levels during lamivudine treatment in all patients. The level of HBV DNA decreased rapidly and became undetectable at 3 months after treatment was initiated. On the other hand, although HBVcrAg levels decreased continuously, the median amount of decrease from the base-line was significantly lower than that in HBV DNA levels at 1, 3 and 6 months after starting lamivudine administration (Wilcoxon signed-ranks test, $P<0.001$ at all analyzed points in time).

Changes in HBV DNA and HBVcrAg levels during lamivudine administration are compared in Fig. 2 between the 27 patients who showed lamivudine resistance and the 54 patients who did not. Serum HBV DNA levels were found to decrease rapidly and become undetectable within 6 months in 45 (83%) of the 54 patients without lamivudine resistance. On the other hand, only 11 (41%) of the 27 patients with lamivudine resistance showed a similar rapid decrease, and the HBV DNA levels of the remaining patients stayed above the detection limit during the follow-up period. HBVcrAg levels decreased but did not reach levels lower than 4.7 log U/ml (5000 U/ml) in the 27 patients with lamivudine resistance. In 19 (35%) of the 54 patients without lamivudine resistance, on the other hand, the levels decreased to levels below 4.7 log U/ml within 6 months after the start of lamivudine administration. The level of HBVcrAg increased rapidly as did the level of HBV DNA when lamivudine resistance occurred.

The occurrence of lamivudine resistance was significantly less frequent in the 56 patients whose HBV DNA level was less than 2.6 log copy/ml at 6 months after the initiation of treatment than in the remaining 25 patients (Fig. 3). The cumulative occurrence of lamivudine resistance was as high as 70% within 2 years in the latter group, while it was only 28% in the former group. There was no occurrence of lamivudine resistance during the follow-up period in the 19 patients whose HBVcrAg levels were less than 4.6 log U/ml at 6 months after the initiation of lamivudine therapy (Fig. 3). On the other hand, lamivudine resistance occurred in 50% of the remaining patients within 2 years.

Discussion

The HBVcrAg assay is a unique assay, which measures the amounts of e and core antigens

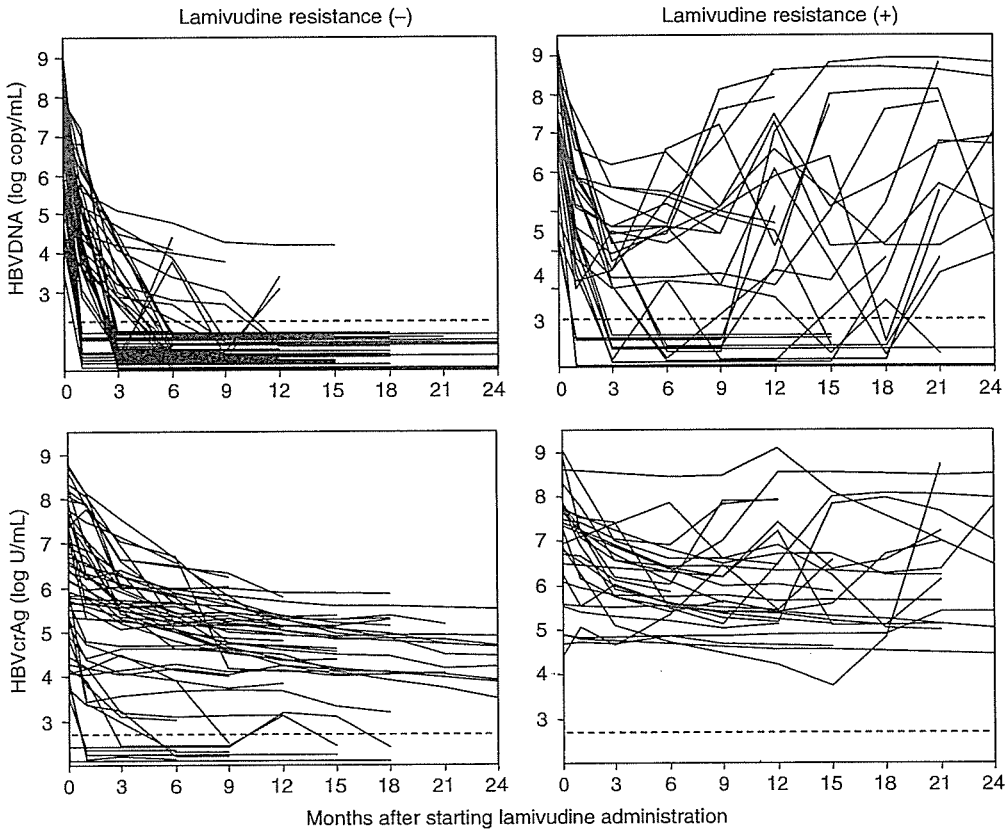

Fig. 2. Comparison of changes in serum hepatitis B virus (HBV) DNA and serum HBV core-related antigen (HBVcrAg) levels between patients who showed lamivudine resistance and those who did not. The broken lines indicate the detection limit of each assay.

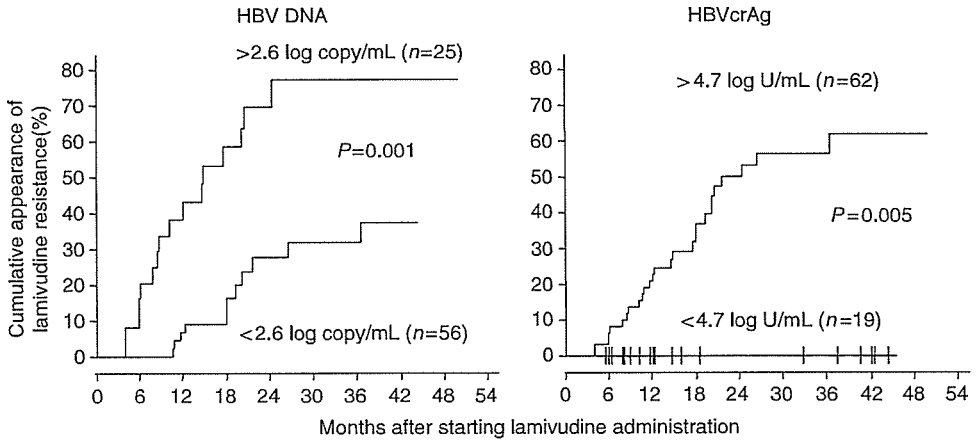

Fig. 3. Comparison of the cumulative occurrence of lamivudine resistance between patients who showed hepatitis B virus (HBV) DNA levels of less than the detection limit (2.6 log copy/ml) at 6 months after starting lamivudine administration and those who did not (left figure), and similarly between patients who showed HBV core-related antigen (HBVcrAg) levels of less than 4.7 log U/ml and those who did not (right figure).

coded by the core gene of the HBV genome with high sensitivity and a wide quantitative range. Serum HBVcrAg levels reflect the viral load in the natural course because these levels correlate linearly with those of HBV DNA (14, 15). On the other hand, the character of HBVcrAg is somewhat different from that of HBV DNA in patients undergoing anti-viral therapies such as lamivudine. That is, HBVcrAg levels decrease significantly more slowly than those of HBV DNA after the initiation of lamivudine administration.

HBV is an enveloped DNA virus containing a relaxed circular DNA genome, which is converted into a covalently closed circular DNA (cccDNA) episome in the nucleus of infected cells (18, 21–23). The cccDNA molecules serve as the transcriptional template for the production of viral RNAs that encode viral structural and non-structural proteins. Reverse transcription of the viral pregenomic RNA and second-strand DNA synthesis occur in the cytoplasm within viral capsids formed by the HBV core protein. Because lamivudine, a nucleoside analogue, inhibits reverse transcription of the pregenomic RNA, it directly suppresses the production of HBV virion. Thus, serum HBV DNA levels decrease rapidly after the initiation of lamivudine administration. On the other hand, the production of viral proteins is not suppressed by lamivudine because the production process does not include reverse transcription. Furthermore, it has been reported that the amount of cccDNA, which serves as a template for mRNA, decreases quite slowly after starting the administration of nucleoside analogues (24–26). Thus, it is reasonable that serum HBVcrAg levels decrease much more slowly than HBV DNA levels after the initiation of lamivudine therapy.

Significant markers that can predict the presence or absence of lamivudine resistance are clinically valuable because the emergence of this resistance and the subsequent recurrence of hepatitis are fundamental problems in lamivudine therapy. Serum markers that reflect the activity of HBV replication have been reported to be associated with the occurrence of lamivudine resistance (11, 12, 27, 28). However, neither the pretreatment existence of HBe antigen nor pretreatment levels of HBV DNA or HBVcrAg were found to be significant markers in the present study. These results may reflect a weak association between the pretreatment activity of HBV replication and the occurrence of lamivudine resistance (13, 29). Changes in HBV DNA and HBVcrAg levels after starting lamivudine administration clearly differed between patients with and without lamivudine resistance. Thus, HBV DNA and HBVcrAg levels at 6 months after starting lamivudine administration were analyzed to determine whether these levels might serve as predictive markers; both were found to be significantly lower in patients without lamivudine resistance at the tested point in time. Furthermore, patients who showed higher levels of HBV DNA and HBVcrAg at 6 months after the initiation of treatment were significantly more likely to develop lamivudine resistance than those who showed lower levels.

We believe that the measurement of HBV DNA levels is useful to identify patients who are at high risk for lamivudine resistance because as many as 70% of patients who were positive for HBV DNA at 6 months after starting lamivudine

administration developed lamivudine resistance within 2 years. However, a negative result of HBV DNA at 6 months does not necessarily guarantee the absence of lamivudine resistance because nearly 30% of such patients developed resistance within 2 years. On the other hand, HBVcrAg levels of less than 4.7 log U/ml at 6 months are a useful indicator of patients who are unlikely to develop lamivudine resistance, because no such patients developed resistance during the follow-up period in the present study. Lower serum HBVcrAg levels may reflect lower levels of cccDNA in hepatocytes because the mRNAs of HBVcrAg are transcribed from the cccDNA (18, 22, 23). This possibility may explain our finding that patients whose HBVcrAg levels decreased sufficiently were unlikely to develop lamivudine resistance, because cccDNA provides the templates for viral and pregenomic messenger RNA (18, 22, 23), which may be a source of lamivudine-resistant strains.

In conclusion, our results suggest that measurement not only of HBV DNA but also of HBVcrAg is useful for predicting the occurrence of lamivudine resistance. HBV DNA measurement is valuable for identifying patients who are at high risk of developing this resistance and HBcrAg measurement is valuable for identifying those who are at low risk.

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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-

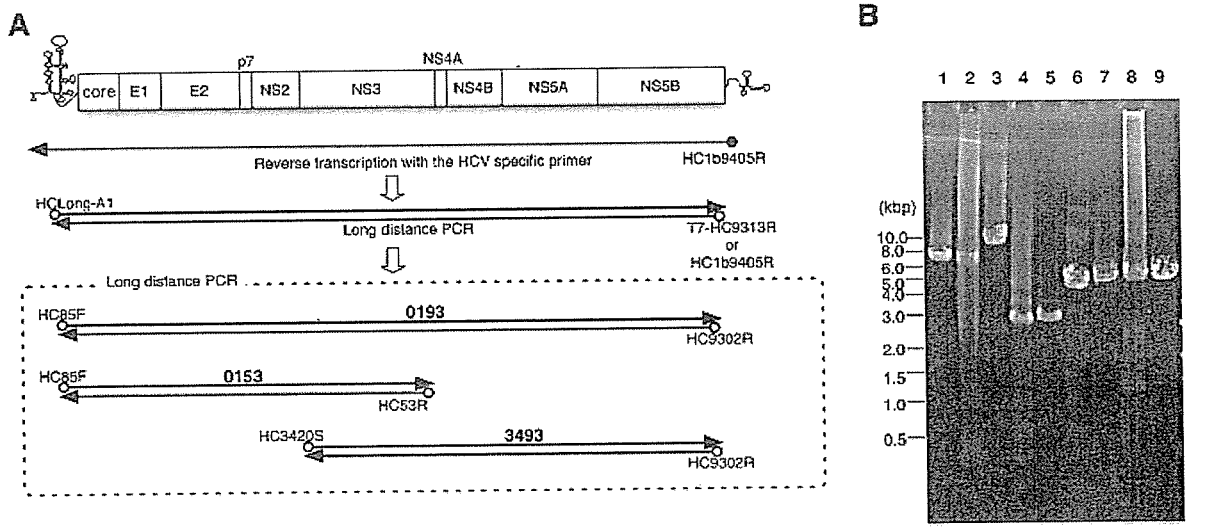

Fig. 1. Molecular cloning of HCV genome by long distance reverse-transcription PCR. A: Schematic view of HCV RNA is shown at the top of the figure. HCV cDNA, which was synthesized from total RNA from liver using reverse transcriptase with HCV-specific primers for the 3′-UTR, was amplified by nested PCR 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

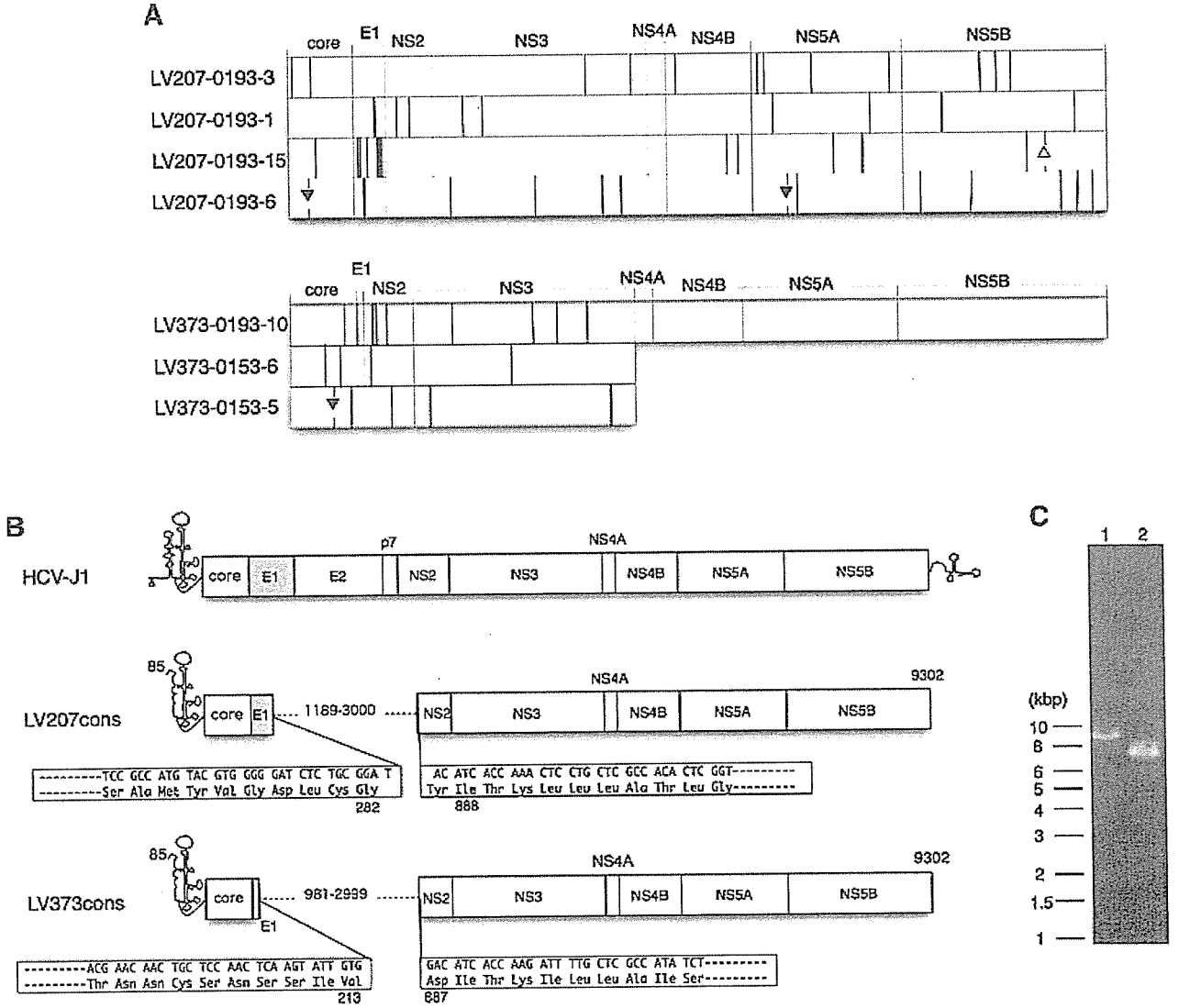

Fig. 2. Schematic presentation of HCV subgenomes from livers containing in-frame deletion. **A**: Comparisons of the deduced amino acid sequences of LD-RT-PCR fragments cloned with pGEM-T Easy are depicted. The cDNA isolates from liver biopsy are designated by the prefix LV followed by Patient numbers 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′-TCTAGTCGACGGCCAG-TGAATTGTAATACGACTCACTATAGGGCGGCCAG-CCCCCTGATGGGGGCGACACTCCACC-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′-ACATGATCTGCAGA-GAGGCCAGTATCAGCACTCTC-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 Quick-Change 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′-GGTCATCGA-TACCCTTACATGCGGC-3′) and Asc-M-as (5′-CCTTC-CTCGGCGCGCCGAGACRGGTAGACCCRAGATGAT-GTCCCCACA-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′-GCGTCCCC-ACTAAGGCAATA-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	Sense	HC3945R	HC3481R	HC3945R	HC3945R	HC3297R	HC3945R	HC3945R	HC3945R	HC3945R	HC3481R
	Antisense	HClongA1	HClongA1	HC813S	HC813S	HClongA1	HClongA1	HClongA1	HClongA1	HC813S	HClongA1
1st PCR	Sense	HC3945R	HC3481R	HC3945R	HC3174AS	HC3297R	HC3945R	HC3945R	HC3945R	HC3297R	HC3481R
	Antisense	HC85F	HC85F	HC841S	HC841S	HC85F	HC85F	HC85F	HC841S	HC841S	HC85F
2nd PCR	Sense	HC3297R	HC3297R	HC3759R	HC3111AS	HC3174AS	HC3297R	HC3759R	HC3759R	HC3174S	HC3297R

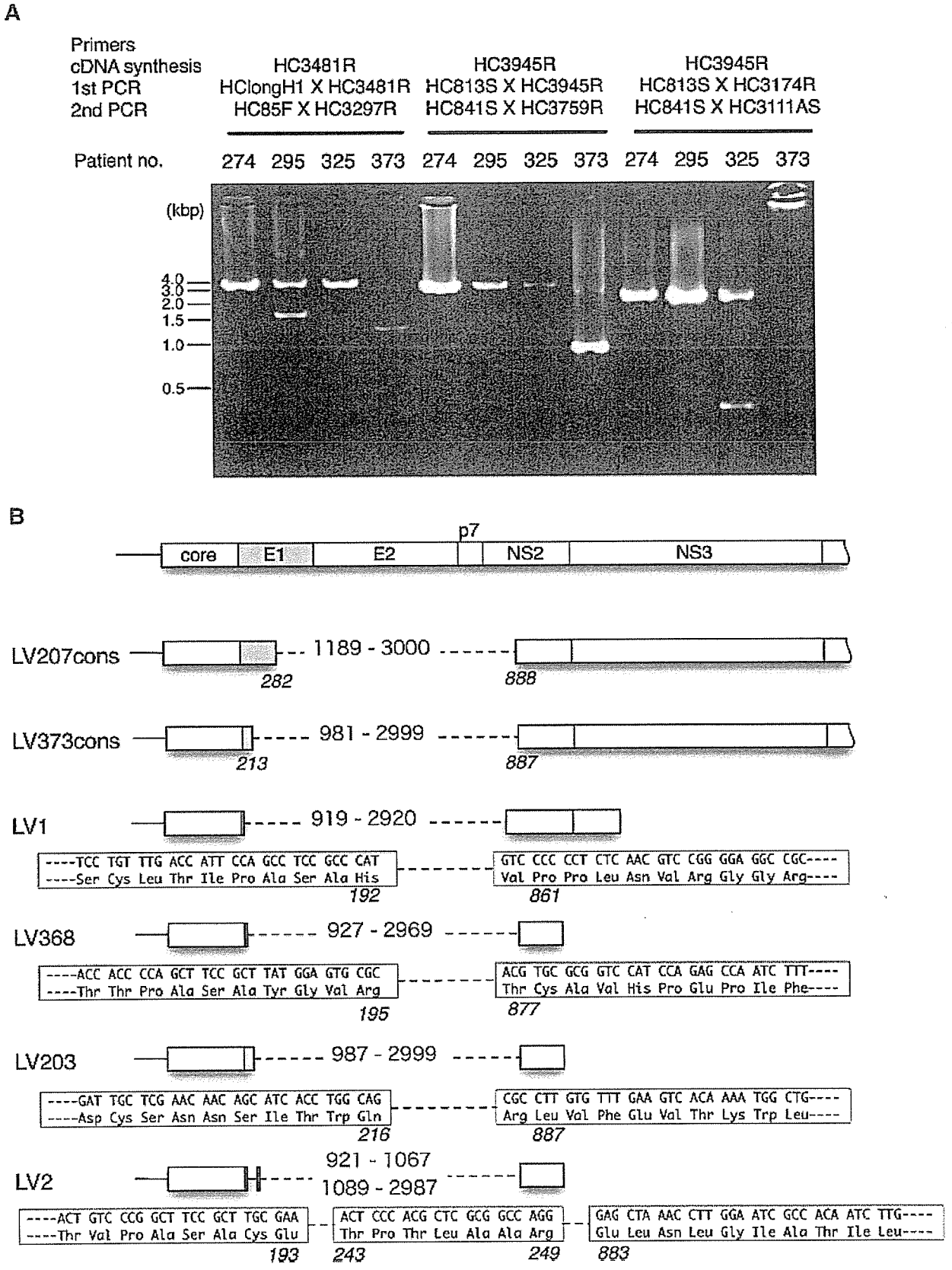
A
Primers
cDNA synthesis
1st PCR
2nd PCR
HC3481R
HClongH1 X HC3481R
HC85F X HC3297R
HC3945R
HC813S X HC3945R
HC841S X HC3759R
HC3945R
HC813S X HC3174R
HC841S X HC3111AS
Patient no.
274 295 325 373 274 295 325 373 274 295 325 373
(kbp)
4.0
3.0
2.0
1.5
1.0
0.5
B
p7
core
E1
E2
NS2
NS3
LV207cons
1189 - 3000
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.