

Sequence Analysis of the Hepatitis C Virus Genome Recovered from Serum, Liver, and Peripheral Blood Mononuclear Cells of Infected Chimpanzees

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Of 13 different strains of hepatitis C virus (HCV) in the inoculum used, only 1 persisted in human lymphocyte cell lines infected in vitro (N. Nakajima, M. Hijikata, H. Yoshikura, and Y. K. Shimizu, *J. Virol.* 70:3325–3329, 1996). To determine whether that particular strain (designated H1-2) has a tropism for lymphocytes in vivo, we sequenced hypervariable region 1 (HVR1) of the genome of HCV recovered from the sera, livers, and peripheral blood mononuclear cells (PBMC) of chimpanzees infected with plasma H77, the same inoculum used for the in vitro studies. In the PBMC collected from two chimpanzees during the early phase of infection, H1-2 was detected as the only or predominant HVR1 sequence. H1-2 was also detected in PBMC obtained during persistent infection from a chimpanzee that had been treated with immunosuppressants. From the livers of these chimpanzees, two to six different strains were recovered but H1-2 was not detected. Thus, H1-2 appeared to have an affinity for lymphocytes not only in vitro but also in vivo. In samples collected from a chimpanzee after 6 years of infection, however, such tissue compartmentalization of the HCV genome was not observed; a single strain became predominant in the serum, liver, and PBMC. An HCV strain capable of replicating in both the liver and PBMC probably emerged during in vivo replication and persisted.

Hepatitis C virus (HCV) persists in experimentally infected chimpanzees, as well as in humans, for many years. The HCV virion contains a single-stranded, positive-sense RNA genome which is taxonomically related to those of flaviviruses and pestiviruses. By analogy with these viruses, the HCV genome is presumed to replicate by using the negative strand as a template.

Negative-strand HCV RNA has been detected by reverse transcription (RT)-PCR not only in the liver but also in the peripheral blood mononuclear cells (PBMC) of infected individuals (2, 9, 11, 17). Recently, we and others reported that HCV can replicate in cultured lymphocytes (1, 10, 12–14, 16). In such cells, a few particular HCV strains among the various strains in the inoculum continued to replicate (10, 12). This may suggest the existence of an HCV strain which has an affinity for lymphocytes. In the present study, we sequenced the genome of HCV recovered from the sera, livers, and PBMC of chimpanzees infected with the same inoculum used for the in vitro studies to investigate whether that particular HCV strain also has a tropism for PBMC in vivo.

MATERIALS AND METHODS

Specimens. Chimpanzees 1313 and 1304 were inoculated on 30 January 1989 with 0.5 ml of undiluted plasma H77 containing 10^{6.5} 50% chimpanzee-infectious doses of HCV per ml (4). Details of the outcome of infection in these animals were reported elsewhere (15). Serum, liver biopsy specimens, and PBMC collected from chimpanzee 1313 on 8 February 1989 (day 9) and 8 March 1989 (day 37) and from chimpanzee 1304 on 9 February 1989 (day 10) were used in the present studies. An additional set of samples was collected from chimpanzee

1304 on 27 April 1995 (year 6). PBMC specimens collected daily during the first 10 days of infection were also examined. Chimpanzee 177 has been a chronic carrier of HCV since it was inoculated with plasma H77 on 11 December 1984. During 1988 and 1989, the animal had been used in experiments which required immunosuppression. Serum, liver, and PBMC samples were collected from this chimpanzee on 24 March 1989 (year 5) and 7 March 1995 (year 11). Liver biopsy specimens taken from this chimpanzee on 19 January 1988 (year 3) and 1 August 1988 (year 3.5) were also included in the present study. Preparation of PBMC was done as follows. Ten milliliters of whole blood was mixed with an equal volume of phosphate-buffered saline, underlaid with 15 ml of Ficoll-Paque (Pharmacia, Uppsala, Sweden), and centrifuged in a TOMY RL-100 centrifuge at 2,500 rpm for 30 min. A white layer of PBMC was collected, washed three times, resuspended with RPMI 1640 containing 10% dimethyl sulfoxide (Sigma, St. Louis, Mo.), and frozen at –80°C until use.

RT-PCR. For detection of the HCV genome, total nucleic acids were extracted from a 100- μ l portion of each serum sample, from an approximately 1-mm³ portion of each liver biopsy specimen, or from a pellet of approximately 10⁶ PBMC. Extraction of nucleic acids, RT, and a two-step PCR with nested primers were carried out as described previously (12). The primers were synthesized on the basis of the published sequence of strain H (7) to detect the E1/E2 region of the HCV genome. We used primer sets 5'-GGGGAGTCCTGGCGGCATA-3' (nucleotide positions 1399 to 1418) and 5'-CGTCGGCAGCTGGCAACC T-3' (nucleotide positions 1723 to 1704) as an external pair and 5'-GGGAATTCCCATGGTGGGGAAGCTGGGCG-3' (nucleotide positions 1429 to 1448) and 5'-GGAAGCTTGCCGTGCTATTGATGTGCCA-3' (nucleotide positions 1618 to 1599) as an internal pair (italicized nucleotides represent restriction enzyme sites which were attached to HCV sequences). For detection of the 5' noncoding region of the HCV genome, we used nested primers 5'-TGGGGGCGACACTCCACCAT-3' (nucleotide positions 14 to 33) and 5'-GGTGCACGGTCTACGAGACC-3' (nucleotide positions 341 to 332) as an external pair and 5'-GGGAATTCTTACGCGAGAAGCGTCTAG-3' (nucleotide positions 63 to 82) and 5'-GGAAGCTTCCCTATCAGGCAGTACCACA-3' (nucleotide positions 301 to 288) as an internal pair. In the RT step, the external antisense primer was used to prime cDNA synthesis from positive-strand HCV RNA and the external sense primer was used to prime cDNA synthesis from negative-strand HCV RNA.

Cloning and sequencing. RT-PCR products from the serum, liver, and PBMC samples were purified with Wizard PCR-prep (Promega, Madison, Wis.), digested by *Eco*RI and *Hind*III, and subcloned into cloning vector Bluescript SK+ (Stratagene) by the standard procedure. Twelve independent colonies were picked up, and the cDNA clones were sequenced in both directions with an ABI 373A sequencer and a *Taq* dye terminator cycle sequencing kit (Perkin Elmer).

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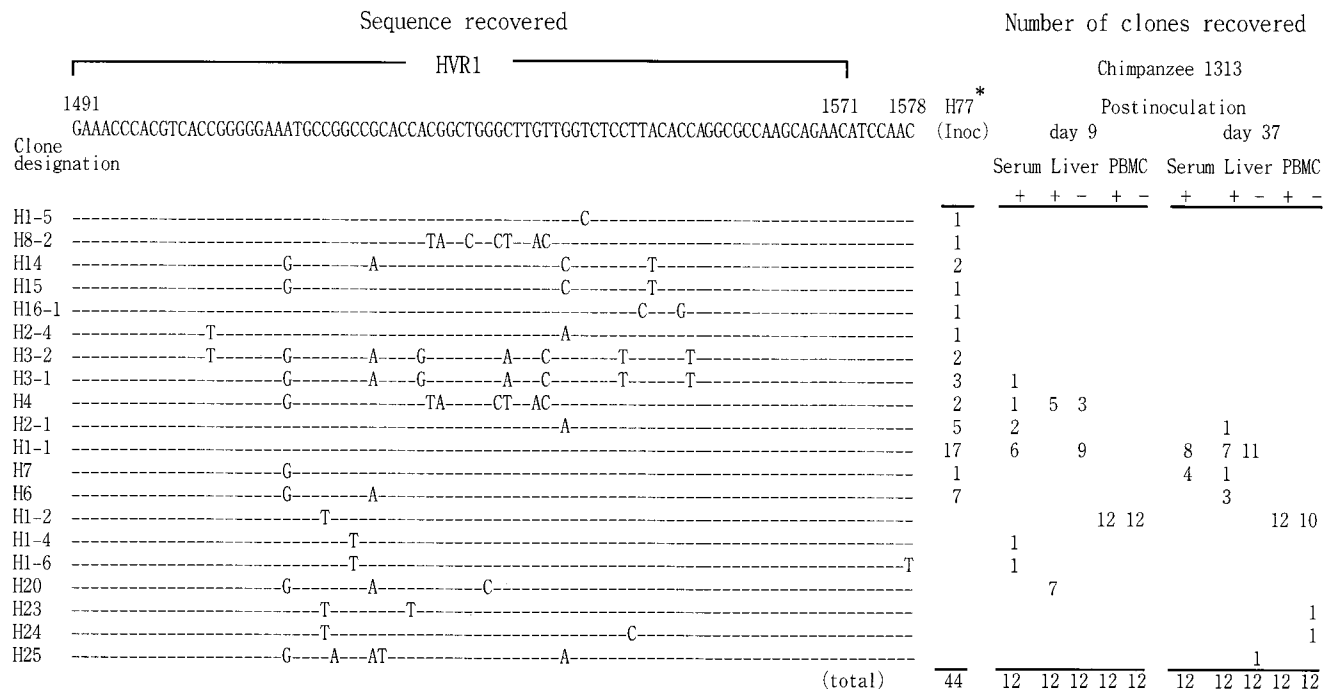


FIG. 1. HVR1 nucleotide sequences of HCV and numbers of clones recovered from the serum, liver, and PBMC of chimpanzee 1313 on days 9 and 37 after inoculation with plasma H77. Horizontal bars indicate nucleotides identical to those of the published sequence of strain H (7). *, data from previous work (12); Inoc, inoculum; +, positive-strand HCV RNA; -, negative-strand HCV RNA.

RESULTS

Sets of serum, liver, and PBMC samples collected on the same day from the same chimpanzee were tested by RT-PCR for hypervariable region 1 (HVR1) of the HCV genome, and 12 cDNA clones from each product were sequenced. For the liver and PBMC samples, both the positive and negative strands of HCV RNA were examined.

Figure 1 shows the results for chimpanzee 1313. From the PBMC collected on day 9, only one sequence, designated H1-2, was recovered as the positive and negative strands; no other sequences were detected. H1-2 was the sequence which persisted in HPBMA10-2 cells (a human T-cell line) and Daudi cells (a human B-cell line) (12). In the liver, sequences different from H1-2 were recovered, H4 and H20 as the positive strand and H4 and H1-1 as the negative strand. There were one to eight nucleotide differences between H1-2 and the sequences detected in the liver. This may indicate that HCVs in the PBMC and those in the liver were different. In the serum, sequences H3-1, H4, H2-1, H1-4, H1-6, and H1-1 (the major population) were detected. On day 37, H1-2 remained in the PBMC as the major sequence, while sequence H1-1 was predominant in the serum and liver. Judging from the HVR1 sequence, HCV strains in the serum became more homogeneous at 37 days.

In chimpanzee 1304, H1-2 was again detected as the major sequence from the PBMC collected on day 10 (Fig. 2). In the liver, H4 and H1-1 were abundant as the negative strand and H4 was the major population of the positive strand. In serum, the detected sequences were H3-1, H4, H2-1, H1-1, and H10. The distribution of the sequences appearing in the serum, liver, and PBMC collected during the early stage of infection was grosso modo similar to that in chimpanzee 1313.

In addition, we examined HVR1 sequences recovered from the PBMC during the first 10 days of infection of these chim-

panzees (Table 1). The signal of HCV RNA was first detected on day 4 as the negative strand in chimpanzee 1313 and as the positive strand in chimpanzee 1304. All of the sequences determined by direct sequencing of the RT-PCR products from the PBMC samples collected during the first 10 days of infection were H1-2. It is improbable that the sequence detected was derived from the inoculum simply phagocytosed by the PBMC, because (i) there was a cryptic period before the first detection and (ii) the sequence detected was H1-2, which was a minor part of the inoculum used.

In the samples obtained 6 years later from chimpanzee 1304, the major population was H40 in the serum, liver, and PBMC (Fig. 2); no tissue compartmentalization was observed.

Figure 3 shows the results for chimpanzee 177. Sequence H1-2 was found to be the major negative strand in the PBMC at both 5 and 11 years. For the positive strand, H32 was predominant at 5 years and H30 was predominant at 11 years (as both H32 and H30 were present in the serum, the possibility that these sequences were derived from the serum could not be excluded). In the liver, H1-1 was dominant at 3 and 3.5 years and H30 was dominant at 5 and 11 years. Sequences H1-1 and H30 were the same except for one nucleotide. H32, which was relatively abundant in the liver at 5 years, differed from H30 by only one nucleotide and from H1-1 by two nucleotides. On the whole, the pattern of sequence distribution in this animal stayed relatively unchanged for 11 years. This might be related to the fact that chimpanzee 177 had been treated with immunosuppressants during 1988 and 1989. Immunoprecipitation studies indicated that most of the virions in the serum collected at 11 years from this chimpanzee were free from anti-HCV envelope antibodies; most of the HCV was not precipitated by anti-human immunoglobulins. The immune status of this animal merits further investigation.

A similar sequence analysis of the 5' noncoding region (nu-

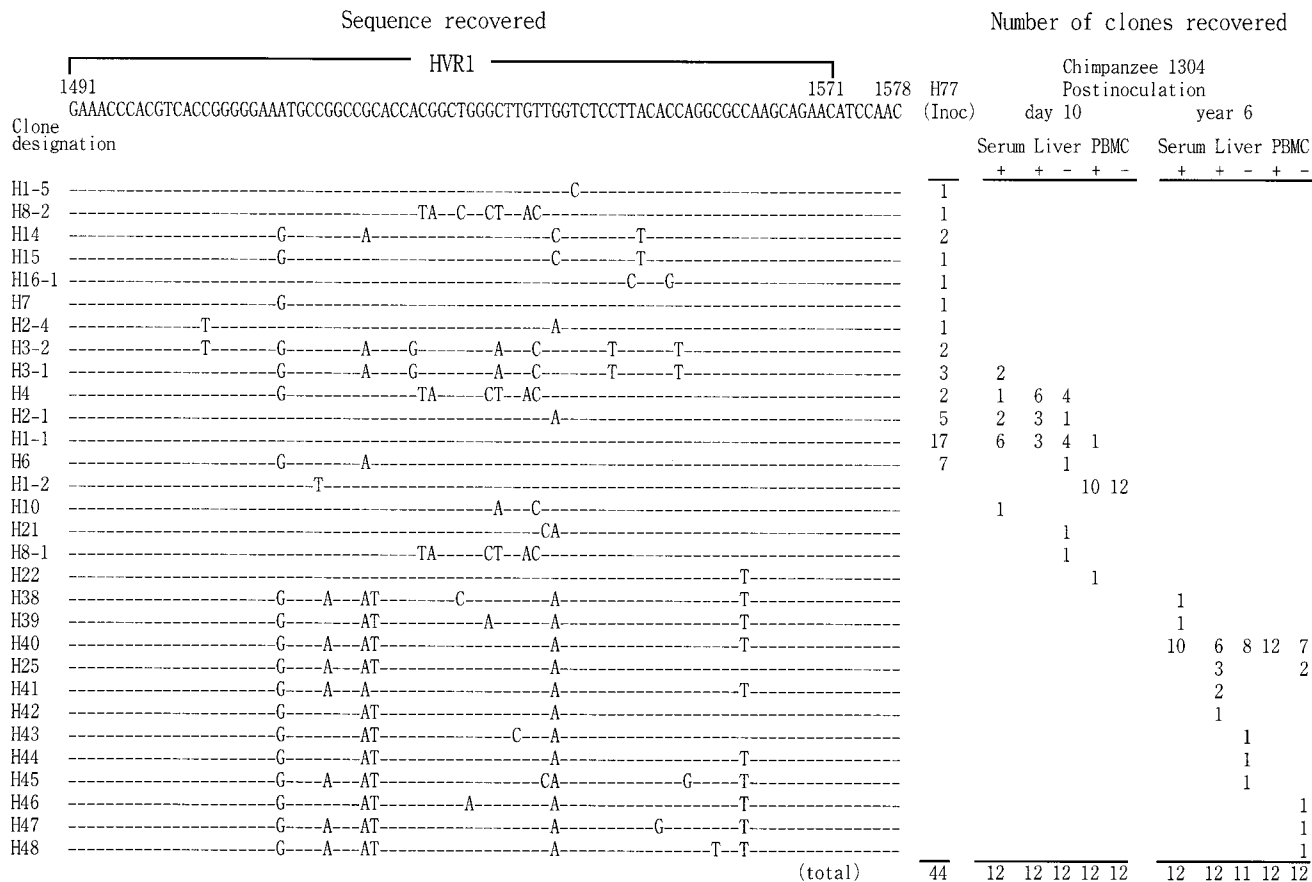


FIG. 2. HVR1 nucleotide sequences of HCV and numbers of clones recovered from the serum, liver, and PBMC of chimpanzee 1304 on day 10 and 6 years after inoculation with plasma H77. Horizontal bars indicate nucleotides identical to those of the published sequence of strain H (7). *, data from previous work (12); Inoc, inoculum; +, positive-strand HCV RNA; -, negative-strand HCV RNA.

cleotide positions 90 to 251) of the HCV genome was carried out. Sample sets were obtained from chimpanzees 1313, 1304, and 177, respectively, 9 and 6 days and 5 years postinfection. As shown in Fig. 4, the same sequence, NC-7, was detected as the negative strand of HCV RNA in PBMC from all of the chimpanzees. From the serum and liver, a different sequence, NC-6, was recovered as the only or predominant sequence. NC-7 was the sequence which persisted in HPBMA10-2 cells and Daudi cells (12).

To determine the relative titers of the HCV genome in tissues samples, samples collected on the same day from the same chimpanzee were subjected to a titration assay. Nucleic acids were extracted from 100 µl of serum, approximately 1 mm³ (about 10⁶ cells) of liver, and 10⁶ PBMC collected from chimpanzee 1313 on day 37 and diluted in 10-fold increments. Each dilution was tested by RT-PCR for the HVR1 sequence of the HCV genome. A positive signal was detected at dilutions of up to 10⁻³ in serum and liver samples and at a dilution of 10⁰ in PBMC (data not shown).

DISCUSSION

Several laboratories have provided evidence of HCV replication not only in the liver but also in the PBMC of infected individuals, and it has been suggested that active replication of HCV in PBMC as an extrahepatic reservoir might play a role in pathogenesis.

To investigate the possibility of the existence of a lympho-

tropic HCV strain, in the present study we employed sequence analysis of the genome of HCV recovered from serum, liver, and PBMC samples obtained from the same chimpanzee on the same day. Of 13 different HVR1 sequences present in the inoculum (plasma H77), the only viral sequence found in PBMC collected during the early stage of infection was H1-2. The detection of sequence H1-2 in PBMC could not be due to contamination by HCV in the serum because H1-2 was not detectable in the serum; sequences detected in the serum samples collected during the early stage were H3-1, H4, H2-1, H1-1, H1-4, H1-6, and H10 (Fig. 1 and 2). When the 5' non-coding region was examined, sequence NC-7 was the only

TABLE 1. Detection of HCV RNA in PBMC taken within 10 days of infection

Chimpanzee and RNA strand detected	Result ^a on postinoculation day:									
	1	2	3	4	5	6	7	8	9	10
1313										
Positive	-	-	-	-	+	-	+	-	+	+
Negative	-	-	-	+	+	-	-	+	+	-
1304										
Positive	-	-	-	+	-	+	-	-	+	+
Negative	-	-	-	-	-	+	-	-	+	+

^a +, detection of HCV RNA; -, nondetection of HCV RNA.

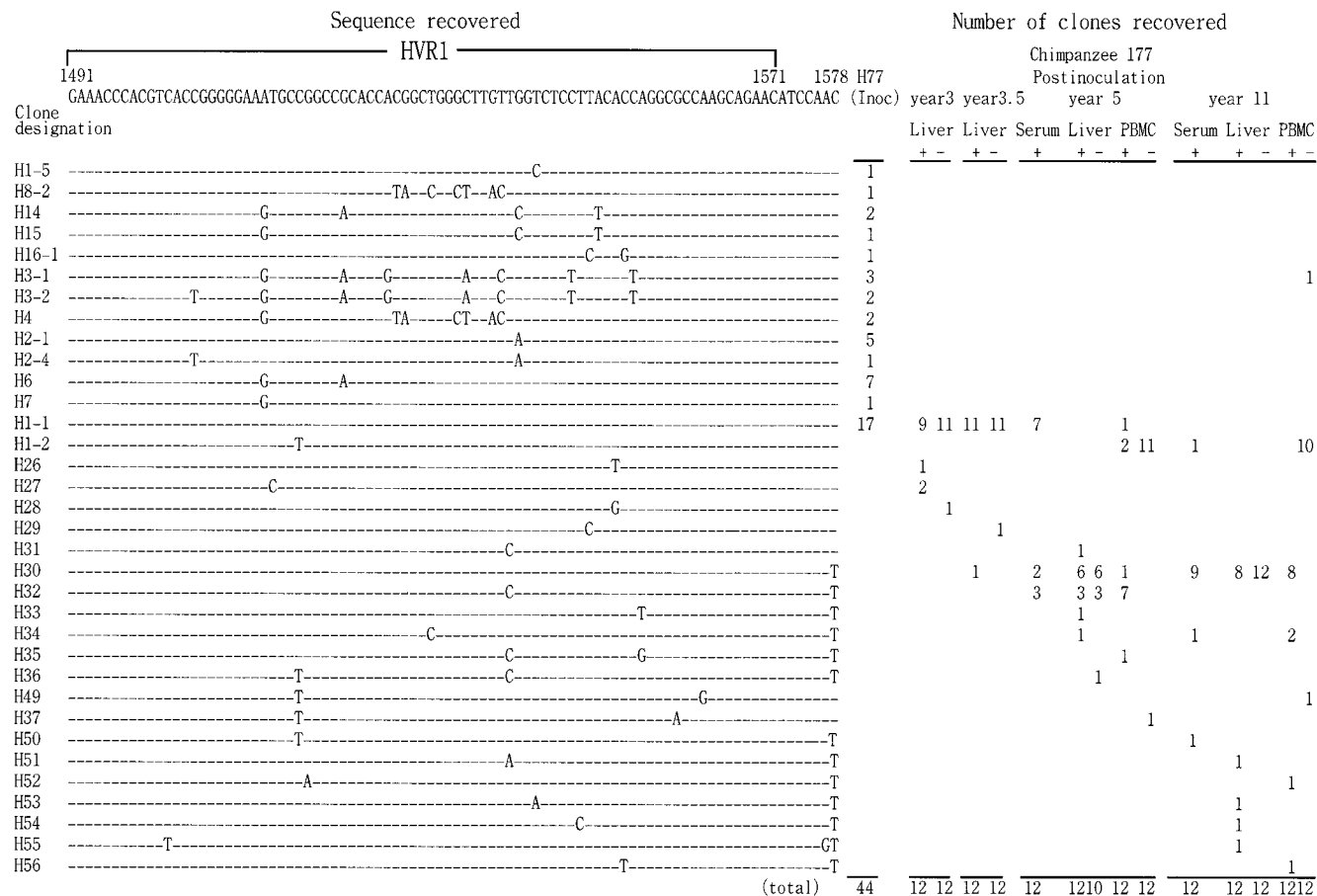


FIG. 3. HVR1 nucleotide sequences of HCV and numbers of clones recovered from the serum, liver, and PBMC of chimpanzee 177 at 3, 3.5, 5, and 11 years after inoculation with plasma H77. Horizontal bars indicate nucleotides identical to those of the published sequence of strain H (7). *, data from previous work (12); Inoc, inoculum; +, positive-strand HCV RNA; -, negative-strand HCV RNA.

sequence detected in the PBMC of the chimpanzees while sequence NC-6 was predominant in the serum and liver (Fig. 4). As the H1-2 and NC-7 sequences were the ones which persisted in lymphocytes infected with the same inoculum, our data, taken together, suggest that particular HCV strains had a replication advantage in lymphocytes both in vitro and in vivo. Sequence H1-1 tended to persist in the liver, and this clone may have an affinity for the liver.

The sequence of negative-strand HCV RNA was also checked in the present study. The specificity of detection of negative-strand HCV RNA has, however, been questioned; detection of the negative strand by conventional RT-PCR could be compromised by false priming of the incorrect strand during RT in some cases. To circumvent this problem, Gungi et al. developed a strand-specific RT-PCR strategy combined with chemical modification of RNA samples at the 3' end (6) and Lanford et al. developed a highly strand-specific rTth method of RT-PCR (8). Our studies described above had been carried out before these methods were developed, and most of the samples were not available for reexamination. However, we could test the PBMC sample collected from chimpanzee 1313 on day 5 by the rTth method. Both the positive and negative strands of HCV RNA were detected in the PBMC by this method, and the HVR1 sequence recovered was H1-2 for both strands, confirming that H1-2 actually replicated in the PBMC.

To confirm further the presence of lymphotropic HCV, we carried out sequence analysis of paired samples of serum and PBMC from a human patient with chronic hepatitis C. The sequences of negative-strand HCV RNA detected by rTth RT-PCR in the PBMC were different from those of positive-strand HCV RNA found in the serum (data not shown).

Recently, Cabot et al. analyzed the quasispecies of HCV in paired liver and serum samples from human patients with chronic hepatitis C and demonstrated that the HCV strain

Recovered 5'NC sequence	Number of clones recovered									
	Chimp1313			Chimp1304		Chimp177				
	Day 9			Day 6		Year 5				
	Serum	Liver	PBMC	Serum	PBMC	Serum	Liver	PBMC		
nt. 90	+	-	-	+	-	+	-	-		
G	12	12	0	12	0	10	12	0		
NC-6	0	0	12	0	12	2	0	12		
NC-7										
(total)	12	12	12	12	12	12	12	12		

FIG. 4. Nucleotide (nt.) sequence of the 5' noncoding (NC) region and numbers of clones recovered from chimpanzees 1313, 1304, and 177 9 and 6 days and 5 years, respectively, after inoculation with plasma H77. From nucleotide positions 90 to 251, sequences NC-6 and NC-7 were the same, except at position 107; NC-6 had a G nucleotide, and NC-7 had an A nucleotide. +, positive-strand HCV RNA; -, negative-strand HCV RNA.

detected in the serum does not necessarily reflect the strain replicating in the liver (3). Gretch et al. reported the emergence of viral strains which have reduced hepatotropism after liver transplantation in patients infected with HCV (5). Based on our data, it is possible that replication of HCV in PBMC as an extrahepatic site may account for these observations.

The pattern of HVR1 sequence distribution in the serum, liver, and PBMC remained relatively unchanged up to 37 days in chimpanzee 1313. However, 6 years after inoculation, as in chimpanzee 1304, the pattern became entirely different. In the serum, liver, and PBMC, the predominant sequence was H40; no tissue compartmentalization of the HCV genome was observed. It is probable that during *in vivo* replication associated with mutation and selection, HCV strains with the most advantageous character, i.e., those which can escape the host immune response and replicate in any tissues, may persist.

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