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Identification of Hepatitis C Viruses with a Nonconserved Sequence of the 5′ Untranslated Region

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The nucleotide sequence of the 5′ untranslated region of hepatitis C virus (HCV) has been shown to be conserved. In contrast, we have detected more sequence variation in this region in several HCV isolates than hitherto expected. The nucleotide sequences of the 5′ untranslated regions of these isolates differ significantly from that of the prototype but are very similar to each other. We believe that these isolates belong to the same type of HCV. Among 240 HCV RNA polymerase chain reaction-positive specimens that we examined, 7 belong to this type. The results suggest that the HCV variants that we detected represent a different type of HCV and that they are responsible for approximately 3% of HCV infections in patients that we have examined.

Hepatitis C virus (HCV), a causative agent of posttransfusion hepatitis, was previously referred to as non-A, non-B hepatitis virus because diagnosis of its infection was achieved by exclusion of hepatitis A and B viruses (7). Recently, an enzyme-linked immunoassay using HCV antigen C100-3 to detect HCV infections directly has been developed (16). HCV is an RNA virus (2, 3, 5), and cDNA fragments corresponding to different portions of the HCV genome have been cloned (1, 5, 13, 19–21). With these clones, the nucleotide sequences of the entire genomes of at least two HCV isolates have been determined (6, 20). On the basis of sequence comparison, HCV was found to be distantly related to flavivirus and pestivirus (18). Similar to that of flavivirus or pestivirus, the HCV genome has 5′ and 3′ untranslated (UT) regions and encodes a single large open reading frame (6, 11, 20). This open reading frame is thought to produce a polyprotein which is then cleaved to become the envelope, the capsid, and nonstructural proteins NS2, NS3, NS4, and NS5.

In addition to the entire genomes of the two HCV isolates, the nucleotide sequences of portions of many other HCV isolates have been reported (1, 8, 13–15, 17, 19, 21, 22). A comparison of these sequences revealed that HCV sequences of different isolates are quite variable (11, 19, 20). For example, the putative envelope region of HCV BK was found to have an average homology of only 76.6% with that of another isolate, HC-J1 (20). Recently, the nucleotide sequences of the 5′ UT regions of 12 HCV isolates from different geographical locations were compared and found to be remarkably conserved (9). In this communication, we report the identification of several HCV isolates of which the sequences of the 5′ UT regions differ significantly from that of the prototype, HCV1.

Polymerase chain reaction (PCR) was performed to detect HCV RNA in serum samples from patients with hepatitis. The serum specimens were obtained from the Clinical Pathology Laboratory of the Indiana University Medical Center and were aliquots of the specimens that were sent to the Clinical Pathology Laboratory for liver enzyme analyses. One specimen per patient was used in this study, and the specimens were collected in 1990 and 1991 over a period of about a year. HCV RNA was isolated by using the method of Chomczynski and Sacchi (4). Five hundred microliters of serum was mixed with an equal volume of RNA extraction buffer containing 4 M guanidinium isothiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol. The HCV RNA was purified by extraction with a mixture of phenol, chloroform, and isoamyl alcohol (25:24:1) and then precipitated with isopropanol. After the RNA pellet was washed with 75% ethanol, the RNA was dried and dissolved in water that had been treated with 0.1% diethylpyrocarbonate. This RNA solution was heated to 65°C for 10 min and then to 95°C for 5 min to destroy the secondary structures of the purified RNA.

To perform PCR, an HCV cDNA was first synthesized by reverse transcription. This reaction was performed in a 12.5-μl reaction mixture containing reverse transcription buffer (50 mM Tris-HCl [pH 8.3], 75 mM KCl, 3 mM $MgCl_2$), each deoxynucleoside triphosphate at 0.5 mM, 10 mM dithiothreitol, 100 pmol of the antisense oligonucleotide PCR primers, 2.5 U of RNasin (Promega, Madison, Wis.), and 20 U of Moloney murine leukemia virus reverse transcriptase (BRL, Gaithersburg, Md.). The mixture was incubated at room temperature for 10 min and then at 42°C for 1 h. The reverse transcriptase was then inactivated by heating the reaction mixture at 95°C for 5 min.

HCV PCR was performed in a 50-μl mixture containing all of the above cDNA product, PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatin), 100 pmol of each PCR primer, and 2 U of *Taq*I DNA polymerase (Perkin Elmer-Cetus, Norwalk, Conn.). The mixture was overlaid with 100 μl of mineral oil to prevent evaporation during thermal cycling. The PCR was performed in three stages. The initial stage was a low-stringency step which consisted of 5 cycles of 94°C for 2 min, 50°C for 2 min, and 72°C for 3 min. The second stage was a high-stringency step including 30 cycles of 94°C for 1.5 min, 55°C for 1.5 min, and 72°C for 2 min. The final stage was a 5-min extension at 72°C. The PCR products were electrophoresed in a 6% polyacrylamide gel to determine the sizes of the amplified products.

Two pairs of PCR primers were used: a modified JHC93 (5′-GGCGACACTCCACCAT-3′) and JHC51 (5′-CCCAA CACTACTCGGCTA-3′), which amplified the 5′ UT region between nucleotides 18 and 268 (9) and produced a 251-bp fragment. The nucleotide numbers of the 5′ UT region used

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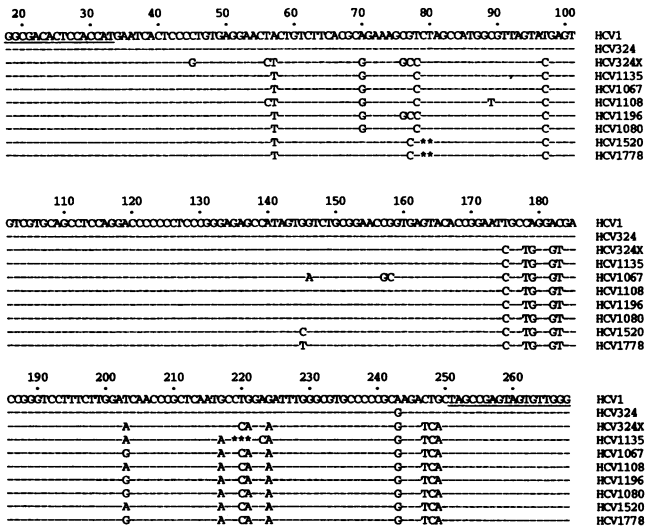

FIG. 1. Comparison of nucleotide sequences of the 5′ UT regions of HCV isolates. The 5′ UT regions of different HCV isolates between positions 18 and 268 were amplified by PCR, and the PCR products were cloned and sequenced. The nucleotide sequences of these clones were compared with that of HCV1. Identical sequences are indicated with dashes. Different sequences are as shown. Missing nucleotides are represented with asterisks. The nucleotide numbers used were those designated by Han et al. (9). The underlined regions of the HCV1 sequence were the PCR primer-binding sites.

here and hereafter were the same as those designated by Han et al. (9). JK3794 (5′-CTTCCTTACATCGAACAAGG-3′) and JK3951 (5′-TCCACATGTGCTTCGCCCAG-3′) produced a 158-bp DNA fragment of the NS3 region (12), corresponding to nucleotides 3794 to 3951 of the Chiron HCV genome (10). Most of the samples we have examined yielded visible and correct size bands of PCR products with both primer sets after electrophoresis in a 6% polyacrylamide gel. We have cloned one of the 251-bp 5′ UT PCR products into pCR1000 (Invitrogen, San Diego, Calif.). The insert of this clone was designated C324. The nucleotide sequence of C324 was then determined by the dideoxy-chain termination method of Sanger et al. (19a) and found to be almost identical to that of prototype HCV1 at the corresponding region; only one nucleotide, located at 243, was different (Fig. 1). Some of the samples yielded visible PCR product bands on gels with the JHC93-JHC51 primer set but produced no visible PCR product bands with the JK3794-JK3951 primer set, even after 40 cycles of PCR. Since these samples gave results that were different from those of the majority of other samples, we speculated that the HCV present in these samples may be different from the prototype HCV1. To prove this hypothesis, we cloned and sequenced one of the PCR products of the 5′ UT region from these samples, and the sequence of this clone (C324X) was found to be quite different from that of HCV1 or C324, which was isolated from a different patient, in the corresponding areas. Among the 251-bp regions that we compared (Fig. 1), 21 nucleotides were different from those of HCV1. We considered this isolate an HCV variant and designated it HCV324X.

To determine the prevalence of HCV324X, the PCR products of 240 serum samples from different patients were probed with C324X or C324 by the dot blot hybridization technique. A 10-μl aliquot of each PCR product was mixed with 90 μl of 0.3 N NaCl, boiled for 5 min, and then quickly chilled on ice. One hundred microliters of ice-cold 2 M ammonium acetate was added to each sample, and the samples were dot blotted onto Nytran membranes in duplicate. The membranes were prehybridized at 55°C overnight in a solution containing 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt's solution, 0.05% sodium PP_i, 0.5% sodium dodecyl sulfate (SDS), and 100 μg of calf thymus DNA per ml. Hybridization was performed at 55°C overnight in a hybridization solution containing 6× SSC, 1× Denhardt's solution, 0.05% sodium PP_i, 100 μg of calf thymus DNA per ml, and DNA probes labelled with [α-^{32}P]dNTP by the primer extension technique (oligolabelling). The membranes were washed with 1× SSC–0.1% SDS twice at room temperature for 20 min each time and then with 0.1× SSC–0.1% SDS at 70°C for 30 min. The membranes were then blot dried and exposed to X-ray film overnight.

TABLE 1. Properties of HCV isolates

HCV isolate[a]	Relative hybridization intensity[b]			
	C324 probe[c]	C324X probe[d]	JHC PCR[e]	JK PCR[f]
324	+++	+	+++	+++
324X	+	+++	+++	−
1067	+	+++	++	±
1135	+	++	++	−
1196	+	+++	++	±
1080	−	+++	+++	±
1108	−	+++	+++	−
1520	−	++	+++	−
1778	−	++	+++	−

[a] HCV isolates were identified by specimen numbers.
[b] For the C324 and C324X probes, the number of plus signs indicates the relative intensity of the hybridization signal; for the JHC and JK probes, it indicates the relative intensity of the PCR product bands in a 6% polyacrylamide gel after electrophoresis. A minus sign indicates negative results, and a plus-or-minus sign indicates equivocal results.
[c] The PCR products of the 5′ UT region were probed with the C324 probe.
[d] The PCR products of the 5′ UT region were probed with the C324X probe.
[e] PCR was performed with the modified JHC93-JHC51 primer set.
[f] PCR was performed with the modified JK3794-JK3951 primer set.

Of the 240 PCR products, 7 hybridized strongly with C324X but weakly with the C324 probe. These seven isolates were designated HCV1135, HCV1067, HCV1108, HCV1196, HCV1080, HCV1520, and HCV1778 (Table 1). To determine whether these seven isolates were HCV variants, the 5′ UT region PCR products of the isolates were cloned and sequenced. The nucleotide sequences of these clones were then compared with those of the corresponding regions of HCV1 and HCV324. As seen in Fig. 1, the nucleotide sequences of these clones differ significantly from that of HCV1 or HCV324. Among the 251 bp nucleotides compared, 18 to 21 were different. In other words, the nucleotide sequence homologies between the variants and HCV1 were 89.2 to 91%. However, the nucleotide sequences of these clones are almost identical to each other. Only two to nine nucleotides were found to differ (Fig. 1). The nucleotide sequence homologies between the variants were 95.8 to 99%, suggesting that these variants represent a certain type of HCV.

The results of this study indicate that the nucleotide sequence of the 5′ UT region of HCV can be more variable than previously described (9). Since the 5′ UT region may regulate HCV replication and production of HCV proteins, it

is conceivable that differences in the 5′ UT region may produce differences in the behavior of these variants. Although only the 5′ UT region was sequenced, the observation that these variants gave either negative or equivocal PCR results with primers of the NS3 region (Table 1) suggested that their nucleotide sequences in other regions of the genome could be quite variable.

REFERENCES

1. **Arima, T., H. Nagashima, S. Murakami, C. Kaji, J. Fujita, H. Shimomura, and T. Tsuji.** 1989. Cloning of a cDNA associated with acute and chronic hepatitis C infection generated from patients serum RNA. Gastroenterol. Jpn. **24:**540–544.
2. **Bradley, D. W., J. E. Maynard, H. Popper, E. H. Cook, J. W. Ebert, K. A. McCaustland, C. A. Schable, and H. A. Field.** 1983. Posttransfusion non-A, non-B hepatitis: physicochemical properties of two distinct agents. J. Infect. Dis. **148:**254–265.
3. **Bradley, D. W., K. A. McCaustland, E. H. Cook, C. A. Schable, J. W. Ebert, and J. E. Maynard.** 1985. Posttransfusion non-A, non-B hepatitis in chimpanzees. Gastroenterology **88:**773–779.
4. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolated by acid guanidium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
5. **Choo, Q.-L., G. Kuo, A. J. Weiner, L. R. Overby, D. W. Bradley, and M. Houghton.** 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science **244:**359–362.
6. **Choo, Q.-L., K. H. Richman, J. H. Han, K. Berger, C. Lee, C. Dong, C. Gallegos, D. Coit, A. Medina-Selby, P. J. Barr, A. J. Weiner, D. W. Bradley, G. Kuo, and M. Houghton.** 1991. Genetic organization and diversity of the hepatitis C virus. Proc. Natl. Acad. Sci. USA **88:**2451–2455.
7. **Choo, Q.-L., A. J. Weiner, L. R. Overby, G. Kuo, M. Houghton, and D. W. Bradley.** 1990. Hepatitis C virus: the major causative agent of viral non-A, non-B hepatitis. Br. Med. Bull. **46:**423–441.
8. **Enomoto, N., A. Takada, T. Nakao, and T. Date.** 1990. There are two major types of hepatitis C virus in Japan. Biochem. Biophys. Res. Commun. **170:**1021–1025.
9. **Han, J. H., V. Shyamala, K. H. Richman, M. J. Brauer, B. Irvine, M. S. Urdea, P. Tekamp-Olson, G. Kuo, Q.-L. Choo, and M. Houghton.** 1991. Characterization of the terminal regions of hepatitis C viral RNA: identification of conserved sequences in the 5′ untranslated region and poly(A) tails at the 3′ end. Proc. Natl. Acad. Sci. USA **88:**1711–1715.
10. **Houghton, M., et al.** May 1989. European patent application 88,310,922.5, publication 318,216.
11. **Houghton, M., A. Weiner, J. Han, G. Kuo, and Q.-L. Choo.** 1991. Molecular biology of the hepatitis C viruses: implication for diagnosis, development and control of viral disease. Hepatology **14:**381–388.
12. **Kaneko, S., M. Unoura, K. Kobayashi, K. Kuno, S. Murakami, and N. Hattori.** 1990. Detection of serum hepatitis C virus RNA. Lancet **335:**976.
13. **Kato, N., M. Hijikata, Y. Ootsuyama, M. Nakagawa, S. Ohkoshi, T. Sugimura, and K. Shimotohno.** 1990. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc. Natl. Acad. Sci. USA **87:**9524–9528.
14. **Kato, N., S. Ohkoshi, and K. Shimotohno.** 1989. Japanese isolates of the non-A, non-B hepatitis viral genome show sequence variations from the original isolate in the USA. Proc. Jpn. Acad. Ser. B **65:**219–223.
15. **Kubo, Y., K. Takeuchi, S. Boonmar, T. Katayama, Q.-L. Choo, G. Kuo, A. J. Weiner, D. W. Bradley, M. Houghton, I. Saito, and T. Miyamura.** 1989. A cDNA fragment of hepatitis C virus isolated from an implicated donor of post-transfusion non-A, non-B hepatitis in Japan. Nucleic Acids Res. **17:**10367–10372.
16. **Kuo, G., Q.-L. Choo, H. J. Alter, G. L. Gitnick, A. G. Redeker, R. H. Purcell, T. Miyamura, J. L. Dienstag, M. L. Alter, C. E. Stevens, G. E. Tegtmeier, F. Bonino, M. Colombo, W.-S. Lee, C. Kuo, K. Berger, J. R. Shuster, L. R. Overby, D. W. Bradley, and M. Houghton.** 1989. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science **244:**362–364.
17. **Maéno, M., K. Kaminaka, H. Sugimoto, M. Esumi, N. Hayashi, K. Komatsu, K. Abe, S. Sekiguchi, M. Yano, K. Mizuno, and T. Shikata.** 1990. A cDNA clone closely associated with non-A, non-B hepatitis. Nucleic Acids Res. **18:**2685–2689.
18. **Miller, R. H., and R. H. Purcell.** 1990. Hepatitis C virus shares amino acid sequence similarity with pestiviruses and flaviviruses as well as members of two plant virus supergroups. Proc. Natl. Acad. Sci. USA **87:**2057–2061.
19. **Okamoto, H., S. Okada, Y. Sugiyama, S. Yotsumoto, T. Tanaka, H. Yoshizawa, F. Tsuda, Y. Miyakawa, and M. Mayumi.** 1990. The 5′-terminal sequence of the hepatitis C virus genome. Jpn. J. Exp. Med. **60:**167–177.

19a. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.

20. **Takamizawa, A., C. Mori, I. Fuke, S. Manabe, S. Murakami, J. Fujita, E. Onishi, T. Andoh, I. Yoshida, and H. Okayama.** 1991. Structure and organization of the hepatitis C virus genome isolated from human carriers. J. Virol. **65:**1105–1113.
21. **Takeuchi, K., S. Boonmar, Y. Kubo, T. Katayama, H. Harada, A. Ohbayashi, Q.-L. Choo, G. Kuo, M. Houghton, I. Saito, and T. Miyamura.** 1990. Hepatitis C viral cDNA clones isolated from a healthy carrier donor implicated to post transfusion non-A, non-B hepatitis. Gene **91:**287–291.
22. **Takeuchi, T., Y. Kubo, S. Boonmar, Y. Watnabe, T. Katayama, Q.-L. Choo, G. Kuo, M. Houghton, I. Saito, and T. Miyamura.** 1990. Nucleotide sequence of core and envelope genes of the hepatitis C virus genome derived directly from human healthy carriers. Nucleic Acids Res. **18:**4626.