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A NEW TYPE OF HEPATITIS C VIRUS IN PATIENTS IN THAILAND*

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Partial nucleotide sequences in the tentative NS5 region of the hepatitis C viral genome obtained from patients with chronic hepatitis in Thailand were analyzed by reverse transcription followed by the polymerase chain reaction. Of ten samples studied, four showed low homologies to any known type of HCV: the homologies of the nucleotide sequences of these clones with HCV-J, -US, -K2a and -K2b were 66.5-69.1%, 66.5-68.2%, 61.2-64.1% and 64.4-66.2%, respectively, and the homologies of their deduced amino acids sequences were 71.7-75.2%, 71.7-75.2%, 69.0-72.6% and 69.9-73.5%, respectively. These four clones were classified a new distinct type of HCV, named HCV-T. Moreover, the nucleotide and amino acid sequence homologies of the four HCV-T clones showed that the HCV-T type could be classified into two genotypes, HCV-Ta and HCV-Tb.

Hepatitis C virus(HCV) is a major causative agent of post-transfusion non-A non-B hepatitis(1,2). Recently nearly the complete genome of HCV was sequenced by three groups(3,4,5).

* The nucleotide sequences of clones T-1, T-7, T-9 and T-10 will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases with the accession numbers D10078, D10079, D10080 and D10081, respectively.

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Comparison of the sequence of HCV-J, the major isolate in Japan, with that of the US isolate HCV-US showed about 23% difference in nucleotide sequences and HCVs about 15% difference in amino acid sequences. Therefore, clones were classified into two different genotypes(3-6). Sequence diversity within the same type of HCV is less than 10%(7-10). Other types of HCV in Japan were demonstrated by two groups, who analyzed different regions of the HCV genome, the putative non-structural(NS) 5 region and NS3 region(11-12). These types were termed HCV-K2 and HCV-GII. HCV-K2 was further classified into two subtypes, HCV-K2a and HCV-K2b. However, a molecular epidemiological study suggested that these two HCVs could be the same type and that HCV-GII could be further classified into two subtypes, HCV-GIIa and HCV-GIIb, which are equivalent to HCV-K2a and HCV-K2b, respectively(13). In fact, recently nucleotide sequence of an HCV genome that has high homology to both HCV-K2 and HCV-GII was reported(14).

To obtain information on the distribution of genotypes of HCV outside Japan, especially in Southeastern Asia, we analyzed nucleotide sequences in the putative NS5 region using the RT-PCR with genotype specific primers. In this way, we detected several isolates of a distinct type of HCV differing from known genotypes such as HCV-J, -US, -K2a and -K2b. Furthermore, two distinct genotypes of this new type of HCV could be distinguished.

MATERIALS AND METHODS

Samples. Plasma samples were obtained from patients in Thailand with chronic non-A, non-B hepatitis. All patients had antibody for C-100(2).

Synthetic oligonucleotides. Oligonucleotide primers for the PCR were synthesized in an Applied Biosystems, model 380A(CA, U.S.A.). The sequences of primers 166 and 167R which were used for the first PCR, correspond to positions nt8230 to 8260 and the complementary sequence to nt8601 to 8630 of HCV-J, respectively(3). The sequences of these primers are well conserved in HCV-J and HCV-US. Primers in the second PCRs for detection of HCV-J, -US and -K2 were 192, 194 and 151 as sense primers, and 193R, 195R and 153R as antisense primers. These primers were designed from the data of Enomoto et al.(11), and all their sequences except those of 192 and 193R are described elsewhere(13). The sequences of 192 and 193R were 5'-TGACATCCGTGTTGAGGAGT -3', and 5'-CAGGCCGCAGAGGCCTTCAA-3', respectively.

RT-nested PCR. RNA was extracted from 0.2ml of plasma as described(6) and was dissolved in 20 μl of distilled water. A sample of 2 μl of the RNA solution was used for cDNA synthesis followed by the nested PCR as described previously(7,15) using the genotype specific primers described above. The first PCR was carried out for 35 cycles of steps for 1 min. at 94 °C, 1 min. at 40 °C and 1 min. at 72 °C. Then using one fiftieth of the first PCR product, the second PCR was performed for 35 cycles of steps for 1 min. at 94 °C, 45 sec. at 55 °C and 1 min. at 72 °C. The amplified products were separated by electophoresis in 3% agarose gel and were detected by ethidium bromide staining.

Cloning and sequencing of PCR products. Products that were amplified by the PCR with the specific primer for HCV-US were cloned into the pTZ18U or pTZ19R plasmid vector as described(6,7). To reduce the possibility of misreading by *Thermus aquaticus* polymerase, we isolated three clones and determined their nucleotide sequences by the dideoxy-nucleotide chain-termination method(DNA sequencing kit, USB Corp., OH.)

RESULTS

To determine the distribution of HCV genotypes in Thailand, we carried out the RT-nested PCR with the genotype specific primers for HCV-J, -US or -K2 using RNA samples from the sera of patients with hepatitis who were positive for anti-C-100 antibody. We found that 5 of 10 RNA samples were amplified by the PCR only with genotype specific primers for HCV-US. However, three of these were infrequently amplified. The efficiency of amplification of these three samples was increased when a second PCR was performed by shifting the annealing temperature from 55 °C to 40 °C. These results suggested that the HCV genomes in these three specimens have somewhat different sequences from those of the US genotype specific primers. Five amplified DNA fragments were subcloned into plasmid vector and their nucleotide sequences were determined. These sequence data revealed that one clone had the US genotype, whereas the other four clones seemed to belong to a distinct type differing from HCV-J, -US or -K2.

The region of the amplification by the second PCR was further extended by using the same primers as in the first PCR. The amplified products(401bp) were then subcloned and their nucleotide sequences were determined. The nucleotide sequences of the internal 340bp of the 401 bp and the deduced amino acid sequences of these

TABLE I. Homologies of nucleotide sequences in the NS5 region of HCV

	T-7	T-9	T-10	J	US	K2a	K2b
T-1	94.4	78.8	79.7	66.5	66.5	61.5	64.4
T-7		79.4	80.3	67.4	66.5	61.2	64.4
T-9			98.5	69.1	68.2	64.1	65.6
T-10				69.1	67.9	64.1	66.2
J					80.9	65.6	67.1
US						67.4	66.8
K2b							82.4

The 340 base pairs of nucleotide sequences in the NS5 region that were utilized for comparison are shown in Fig. 1.
Percentage homology is shown.

four new clones, designated as T-1,-7,-9 and -10, are shown in Fig. 1A and 1B, respectively. Sequence analyses revealed that the nucleotide sequence homologies between these clones and HCV-J, -US, -K2a and -K2b were 66.5-69.1%, 66.5-68.2%, 61.2-64.1% and 64.4-66.2%, respectively (Table I), and that the deduced amino acid sequence homologies between these clones and HCV-J, -US, -K2a and -K2b were 71.7-75.2%, 71.7-75.2%, 69.0-72.6% and 69.9-73.5%, respectively(Table II). Comparison of the amino acid sequences of

TABLE II. Homologies of deduced amino acid sequences in the NS5 region of HCV

	T-7	T-9	T-10	J	US	K2a	K2b
T-1	95.6	85.0	85.8	71.7	71.7	69.9	69.9
T-7		86.7	87.6	71.7	71.7	69.0	69.9
T-9			99.1	75.2	75.2	72.6	73.5
T-10				75.2	75.2	72.6	73.5
J					87.6	73.5	75.2
US						70.8	74.3
K2a							88.5

The sequences of 113 amino acids in the NS5 region that are utilized for comparison were shown in Fig. 1.
Percentage homology is shown.

these clones with those of HCV-J, -US, -K2a and -K2b, showed that 9 of 113 amino acids were specific to these clones (T-1, -7, -9, -10) (Fig. 1B). Analysis of nucleotide sequence diversity indicates that the sequence diversity of NS5 represents that of the whole genome (10, 14). The nucleotide sequence homology between the HCV-J and HCV-US genomes is about 80%, and these genomes are considered to be different genotypes(10). Therefore, these four clones of HCV were classified as a new type of HCV, designated as HCV-T. Furthermore, HCV-T could be classified into at least two genotypes (HCV-Ta for the T-1 and T-7 clones, and HCV-Tb for the T-9 and T-10 clones), because HCV-Ta and -Tb showed about 20% difference in nucleotide sequences, which is about the same as the difference between the sequences of HCV-J and HCV-US. Genotype specific amino acid sequences were found in HCV-Ta and -Tb(Fig. 1B).

DISCUSSION

In this study we have detected a new type of HCV(HCV-T) in patients with hepatitis in Thailand. Furthermore, we found that this HCV-T could be distinguished into two genotypes, HCV-Ta and HCV-Tb. According to the nucleotide sequence diversities in the NS5 region of HCV, the phylogenic relationship between the various types of HCV could be shown in Fig. 2. Probably, a common ancestor evolved into the ancestors of HCV-J/-US, HCV-K2 and HCV-T, and these then evolved into the present HCVs.

Recently, Houghton et al. proposed the classification of known genotypes of HCV into three basic groups; group I as HCV-US, group II as HCV-J and group III as HCV-K2(16). But this classification seems inappropriate, because group III includes two genotypes, HCV-

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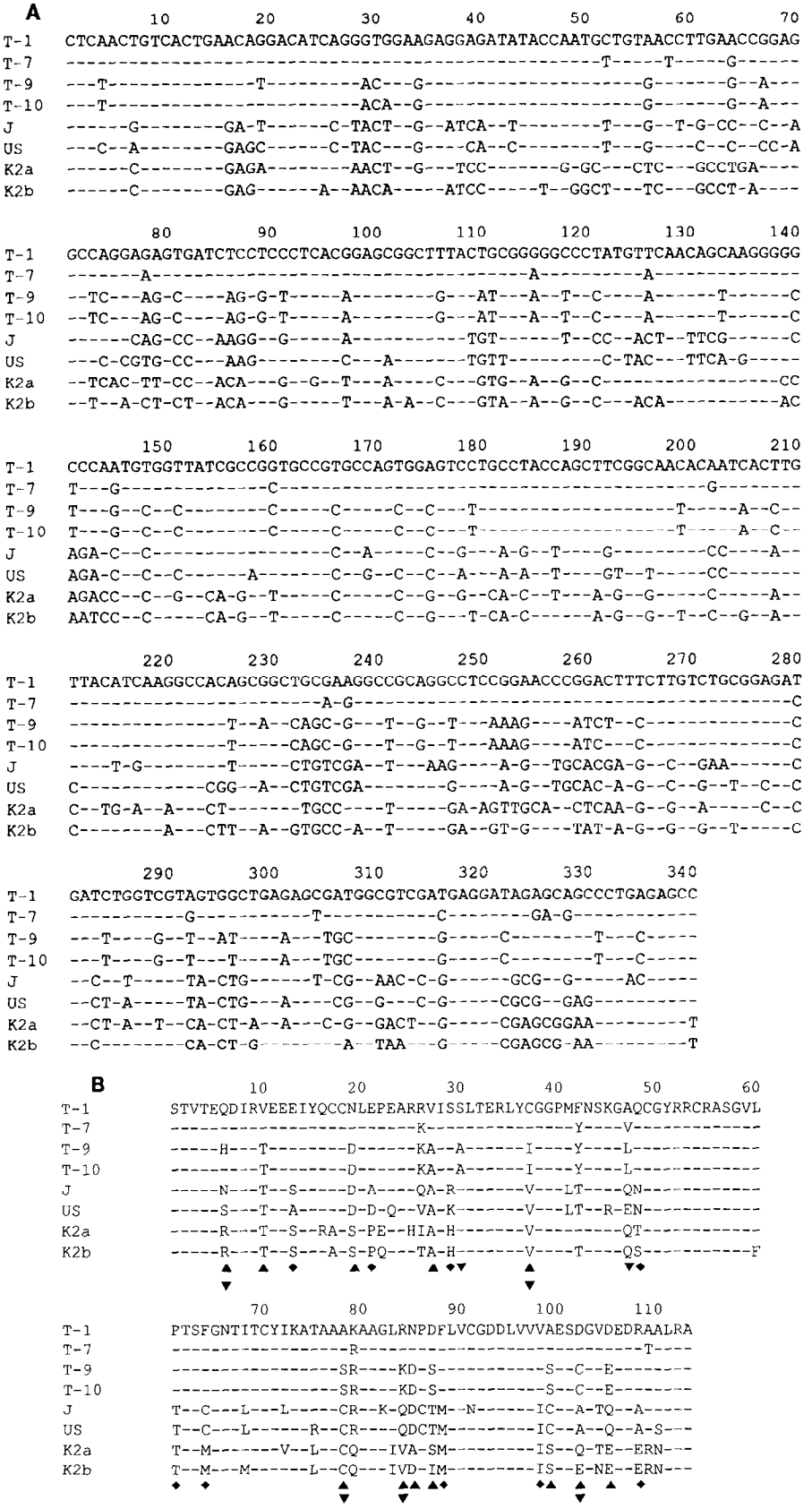
Figure 1. The Nucleotide sequences of cDNAs(A) and deduced amino acid sequences(B) of amplified regions of the respective types of HCV. The sequences of HCV-J, HCV-US and HCV-K2 are cited from Kato et al. (3), Choo et al.(4) and Enomoto et al.(11), respectively. The first nucleotide of HCV-J corresponds to nucleotide position 8273 in the sequence of Kato et al.(3), that of HCV-US corresponds to nucleotide position 7935 in the sequence of Choo et al.(4), and that of HCV-K2 corresponds to nucleotide position 1 in the sequence of Enomoto et al.(11). Amino acid sequences are indicated by the single letter code. ◆, specific amino acid for both HCV-Ta and -Tb; ▲, specific amino acid for HCV-Ta only; ▼, specific amino acid for HCV-Tb only.


A
B

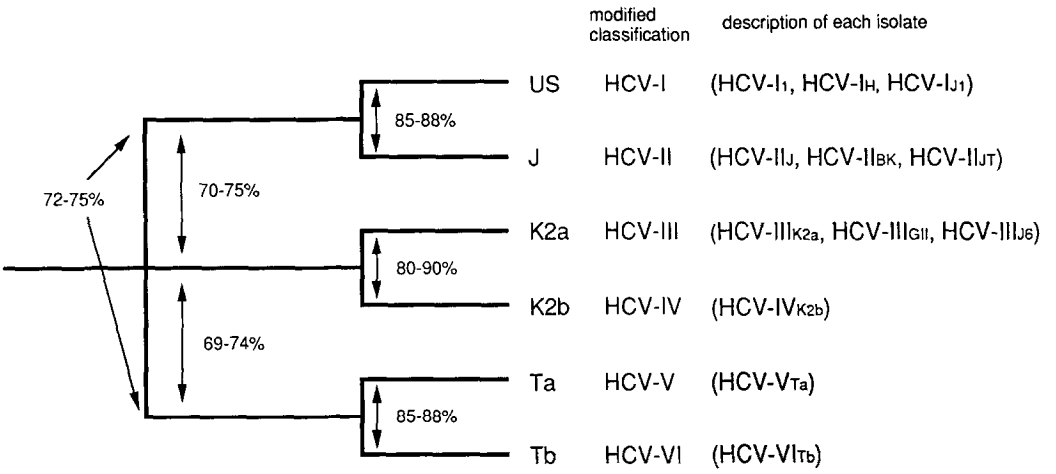


Figure 2. Proposed phylogenic tree and classification of HCV. Values indicate percentage homologies between each type and each genotype. Roman numerals on the right of each genotype are those in a modified classification of HCV.

K2a and K2b. Therefore, we propose the following modified classification of HCV that is more adequate: HCV-I as HCV-US, HCV-II as HCV-J, HCV-III as HCV-K2a, HCV-IV as K2b, HCV-V as HCV-Ta and HCV-VI as HCV-Tb(Fig. 2). Furthermore we consider that different types of isolates should be indicated by a suffix; e.g.) HCV-II_J for the previous HCV-J and HCV-I_1 for the previous HCV-1.

With regard to the distribution of HCV in Japan, we have reported that the prevalence of HCV-I, -II and [-III + -IV] are 3%, 84% and 29%, respectively(13), and Nakao et al. reported that those of -I, -II, -III and -IV are 2%, 74%, 16% and 8%, respectively(17). From these data, HCV-II is considered to be the dominant genotype in Japan(7), On the other hand, HCV-I may be the representative genotype in the US(8). A German group reported that European isolates of HCV were closely related to the prototype HCV(HCV-I) (18). In France, a genotype closely related HCV-I was found as a major, or even predominant genotype(19). In Italy, Pozzato et al. found at least two HCV types(HCV-II and an other unknown type)(20). HCV-V, and -VI has been detected only in Thailand, but its worldwide distribution should be examined.

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REFERENCES

1. Choo, Q.-L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. W., and Houghton, M., (1989) Science 244, 359-362.
2. Kuo, G., Choo, Q.-L., Alter, H. J., Gitnick, G. L., Redeker, A. G., Purcell, R. H., Miyamura, T., Dienstag, J. L., Alter, M. J., Stevens, C. E., Tegtmeier, G. E., Bonino, F., Colombo, M., Lee, W.-S., Kuo. C., Berger. K., Shuster, J. R., Overby, L. R., Bradley, D. W., and Houghton, M., (1989) Science 244, 362-364.
3. Kato, N., Hijikata, M., Ootsuyama, Y., Nakagawa, M., Ohkoshi, S., Sugimura, T., and Shimotohno, K., (1990) Proc. Natl. Acad. Sci. USA 87, 9524-9528.
4. Choo, Q.-L., Richman, K. H., Han, J. H., Berger, K., Lee, C., Dong, C., Gallegos, C., Coit, D., Medina-Selby, A., Barr, P. J., Weiner, A. J., Bradley, D. W., Kuo, G., and Houghton, M., (1991) Proc. Natl. Acad. Sci. USA 88, 2451-2455.
5. Takamizawa, A., Mori, C., Fuke, I., Manabe, S., Murakami, S., Fujita, J., Onishi, E., Ando, T., Yoshida, I., and Okayama, H., (1991) J. Virol. 65, 1105-1113.
6. Kato, N., Ohkoshi, S., and Shimotohno, K., (1989) Proc. Japan Acad. 65B, 219-223.
7. Kato, N., Hijikata, M., Ootsuyama, Y., Nakagawa, M., Ohkoshi, S., and Shimotohno, K., (1990) Mol. Biol. Med. 7, 495-501.
8. Weiner, A. J., Brauer, M. J., Rosenblatt, J., Richman, K. H., Tung, J., Crawford, K., Bonino, F., Saracco, G., Choo, Q.-L., Houghton, M., and Han, J. H., (1991) Virology 180, 842-848.
9. Hijikata, M., Kato, N., Ootsuyama, Y., Nakagawa, M., Ohkoshi, S., and Shimotohno, K., (1991) Biochem. Biophys. Res. Commun. 175, 220-225.
10. Tanaka, T., Kato, N., Nakagawa, M., Ootsuyama, Y., Cho, M.-J., Nakazawa, T., Hijikata, M., Ishimura, Y., and Shimotohno, K., (1992) Virus Res. (in press)
11. Enomoto, N., Takada, A., Nakao, T., and Date, T., (1990) Biochem. Biophys. Res. Commun. 170, 1021-1025.
12. Tsukiyama-Kohara, K., Kohara, M., Yamaguchi, K., Maki, N., Toyoshima, A., Miki, K., Tanaka, S., Hattori, N., and Nomoto, A., (1991) Virus Genes 5, 243-254.
13. Kato, N., Ootsuyama, Y., Ohkoshi, S., Nakazawa, T., Mori, S., Hijakata, M., and Shimotohno, K., (1991) Biochem. Biophys. Res. Commun.181, 279-285.
14. Okamoto, H., Okada, S., Sugiyama, Y., Kurai, K., Iizuka, H., Machida, A., Miyakawa, Y., and Mayumi, M., (1991) J. General Virol. 72, 2697-2704.
15. Kinoshita, T., Shimoyama, M., Tobinai, K., Ito, M., Ito, S., Ikeda, S., Tajima, K., Shimotohno, K., and Sugimura, T., (1989) Proc. Natl. Acad. Sci. USA 86, 5620-5624.
16. Houghton, M., Weiner A., Han, J., Kuo, G., and Choo, Q.-L., (1991) Hepatology 14, 381-388.

17. Nakao, T., Enomoto, N., Takada, N., Takada, A., and Date, T., (1991) J. General Virol. 72, 2105-2112.
18. Fuchs, K., Motz, M., Shreier, E., Zachoval, R., Deinhardt, F., and Roggendorf, M., (1991) Gene 103, 163-169.
19. Li, J.-S., Tong, S.-P., Vitvitski, L., Lepot, D.,Trépo, C., (1991) Gene 105, 167-172.
20. Pozzato, G., Moretti, M., Franzin, F., Crozè, L., S., Tiribelli, C., Masayu, T., Kaneko, S., Unoura, M., Kobayashi, K., (1991) Lancet ii, 509.