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Genetic variability and characterization of non-structural region 5 of hepatitis C virus genome from Chinese patients

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Abstract: Sequence variation in the putative nonstructural region 5b (NS5b) of hepatitis C virus (HCV) was analyzed in China. Complementary DNA fragments from sera of 49 Chinese patients were amplified by polymerase chain reaction (PCR) and the products were cloned and sequenced. Based on the comparison in NS5b of 33 clones of genotype 1b and 16 clones of genotype 2a, Chinese isolates of HCV belong to the same subtype as HCV-J, and HC-J6 from Japan. There does exist, however, some heterogeneity in the primary structure of the nucleotide acid. Higher homology was found among Chinese isolates than among Chinese isolates and Japanese isolates. Furthermore, among Chinese isolates, we found some conserved nucleotide acid positions different from those of Japanese isolates. Comparison of average homology among the 33 clones of genotype 1b and the 16 clones of genotype 2a indicated that the average homology among genotype 2a was lower than that among genotype 1b. In addition, a deletion of three nucleotide acids and a frame-shift, resulting in the introduction of an in-frame stop codon, were first observed in the NS5b region. These results indicated geographical differences in the distribution of individual HCV isolates, and the existence of a local variant in the same subtype. Our findings also suggested the need for further study on the sequence of genotype 2a, to improve diagnosis and help to advance the development of a vaccine.

Key words: hepatitis C virus, nonstructural region 5, sequence analysis, China

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Introduction

Since the identification of hepatitis C virus (HCV) as the major causative agent of post-transfusion non-A, non-B hepatitis,[1,2] several full-length HCV sequences, as well as many partial sequences, have been reported.[3] Analysis of these sequences has shown that there are at least nine types of HCV (types 1, 2, 3, 4, 5, 6, 7, 8, and 9) and each type also has several subtypes (1a, 1b, 2a, 2b, 3a, 4a, and so on) to which individual isolates belong.[3–7] In the United States and Europe, the predominant HCV genotype is type 1a, whereas type 1b is the most frequently found type in Japan.[3] Our previous work also indicated that genotypes 1b and 2a were the most prevalent in China.[8,9] These studies suggest that HCV types and subtypes have different geographical distributions.[10–12] However, it is unknown whether there is geographical distribution of individual HCV isolates, there is in the case of genotypes. Because heterogeneity among different HCV isolates may have implications for the progression of the disease and for the creation of diagnostic reagents, it should be addressed.

At present, we know little about the characterization of genotype 2a HCV, as only one complete sequence and few partial sequences have been published.[13–19] The Lack in wide understanding of the genomic organization of genotype 2a HCV may be responsible for discordant results when analyses of different assays for genotyping HCV are compared. For this reason, more data on the variability of genotype 2a of HCV is necessary.

To clarify these points, we analyzed the nonstructural 5b (NS5b) gene of different Chinese isolates and compared these sequences with reported genotype 1b and 2a sequences. The present study outlines the characterization of Chinese isolates and the difference among distinct isolates in genotype 2a. A deletion mutation and a frame-shift mutation were first reported in this paper.

Table 1. Primers for reverse transcription and polymerase chain reaction (PCR) amplification

Primers	Position of HCV-1[22]	Sequences (5–3)
51	7864~7883	GAGTTCCTGGTGAATACCTG
52	8335~8354	GATGTTATCAGCTCCAGGTC
53	7918~7933	GCTGAATTCGACACCCGCTGTTTTG
54	8277~8293	CAGAAGCTTTAGTCATAGCCTCCGTG

Underlines indicate sequences of endonuclease EcoR I and Hind III.

Patients and methods

Patients

Forty-nine patients (31 men and 18 women) with chronic hepatitis C (aged 22–61 years) were investigated. All were positive for anti-HCV and HCV RNA, but were hepatitis B surface antigen (HBsAg)-negative. The patients had different clinical and histological stages of the disease, including those no sign of clinical onset.

Preparation of nucleic acid, cDNA synthesis Polymerase chain reaction (PCR), and typing

RNA from 50 µl serum was extracted by the acid/guanidium/phenol/chloroform method.[20] After being precipitated with ethanol, the RNA was resuspended in 5 µl of water containing 10 units of RNase inhibitor (RNasin; Promega Corporation, Madison, WI, USA) the RNA obtained was denatured at 70°C for 1 min, chilled quickly on ice, and used as a template for cDNA synthesis. Reverse transcription was carried out at 42°C for 60 min in 10 µl of Tris-HCl buffer (50 mM, pH 8.4) containing 8 mM $MgCl_2$, 30 mM KCl, 1 mM dithiothreitol, 50 pmol primer 52, 10 mM each of the four deoxyribonucleoside triphosphates (dNTPs) (Promega), 2 units avian myeloblastosis virus reverse transcriptase (Promega). The cDNA was amplified in two stages in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CN, USA). Both stages were carried out in a final volume of 50 µl, containing 67 mM Tris-HCl, pH 8.8, 1.5 mM $MgCl_2$, 1.5 units of Taq DNA polymerase (Promega), 10 mM each of the four dNTPs, and 50 pmol of each primer). Both stages consisted of 35 cycles. In the first stage of PCR, each reaction cycle involved denaturation at 94°C for 1 min, annealing of primers at 42°C for 1.5 min, and extension at 72°C for 2 min. In the second stage of PCR, the program of the thermal cycle was similar to the first stage, but the annealing of primers was performed at 55°C. Samples from the reaction mixture were subjected to electrophoresis on polyacrylamide gel (Promega) and the DNA species on gel were stained with ethidium bromide for observation under ultraviolet light. The sizes of PCR products were estimated according to the migration pattern of PBR322/Hae III. The primers used for cDNA synthesis and PCR were deduced from the putative nonstructural gene (NS) 5b of the HCV-J isolate[21] and the HC-C2 isolate;[9] the sequences of these primers are listed in Table 1.

Before being cloned and sequenced, sera positive for HCV RNA were subjected to genotype analysis, using Nakao's[16] and Okamoto's[23] methods.

Cloning and sequencing

Specific second-stage PCR products were gel-purified[24] and cloned into the EcoR I and Hind III sites of pUC18. Recombinant clones were identified by ampicillin resistance and restriction enzyme digestion. Positive clones were sequenced with Sequenase (Bio-Rad, Hercules, CA, USA) and optimal conditions were used for the sequencing of double-strand plasmid DNA. For each of the samples, at least two clones were sequenced in both directions.

Results and discussion

HCV is characterized by a high degree of nucleotide sequence heterogeneity. To date, a large amount of genomic sequence data for HCV has been reported world wide. Comparison and analysis of these sequences have revealed that HCV exists as multiple and distinct genotypes.[3] These observations have been shown to be essential for diagnosis, determination of pathogenesis, and treatment. However, comparisons among different Chinese isolates of common genotypes have not been addressed, and there is a need for more knowledge of genomes of genotype 2a. Therefore, we sequenced and compared HCV genes isolated from Chinese patients with hepatitis C.

In general, full-length sequence is necessary for comparison among different isolates. However, analysis based on partial HCV genes could represent characterization of the whole genome. For example, isolates of genotype 1b, as well as isolates of genotype 2 and 3,

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                                                                                                       8017
                *                   *         *         *         *         *         *         *         *         *
HCV-J  gacacccgctgttttgACTCAACGGTCACTGAGAATGACATCCGTACTGAGGAATCAATTTACCAATGTTGTGACTTGGCCCCCGAAGCCAGGCAGGCCA
HC-C2                  -------------C----------------T------G-------------------------------------AA--A----
HC-P1                  -----------------T-C---------GT------G-----C--------------------------------AG------
HC-P2                  -------------------C---------GT------G--------------------------------G-----A-------
HC-P3                  -------------------C---------GTA-----G--------------------------------G-----A-------
HC-P4                  -------------------C---------GTA-----G--------------------------------------A-------
HC-P5                  -------------C-----C---------GT------G--------------------------------G-----A-G--T--
HC-P6                  ----------G------TG-T-TCC-G--GT-T----G-----G-T--------A---------------GC-AG-CAT-----
HC-P7                  --------------C----C----------T------G--------------------------------G---C-A--A----
HC-P14                 ---------C-T-C----------------T------G--------------------------------------A--A----
HC-P15                 -------------C------------T---T------G--------------------------------------A--A----
HC-P16                 -------------C----------------T------G-----------------------T--------T-----A--A----
HC-P17                 ----------T-------G----------GT------G-----C--------------------------------A-------
HC-P18                 -------A--T-------------------T------G-----C-----------------------T--------A-------
HC-P19                 -------A--T-------------------T------G-----C-----------------------T--------A-------
HC-P20                 -------A--T-------G----------GT------G-----C--------------------------T-----A-------
HC-P21                 -------------C----------------T------G-----------------------T--------------A--A----
HC-P22                 -------------C----------------T------G-----------------------T--------------A--A----
HC-P23                 -------------CC-------T-------T------G-----------------------T-A------------A--A----
HC-P24                 -------------C-----C---------GT------G--------------------------------------A--A----
HC-P25                 -------------C----------------T------G--------------------------------------A--A----
HC-P26                 -------------C----------------T------G--------------------------------------A--A----
HC-P27                 -------------C---------------GT------G-----------------------T--------T-----A-------
HC-P28                 -------------C---------------GT------G--------------------------------T-----A-------
HC-P29                 -------------C---------------GT------G--------------------------------T-----A-------
HC-P30                 -------------C---------------GT------G--------------------------------T-----A-------
HC-P31                 -------------C---------------GT------G--------------------------------T-----A-------
HC-P32                 -------------C---------------GT------G--------------------------------T-----A-------
HC-P33                 -------------C----------------T------G--------------------------------------A-------
HC-P34                 -------------C----------------T------G--------------------------------T-----A-------
HC-P35                 -------------C----------------T------G--------------------------------T-----A-------
HC-P36                 -------T-----C----------------T------G--------------------------------T-----A-------
HC-P37                 -------------C----------------T------G--------------------------------------A-------
HC-P38                 -------------C---------------GT------G--------------------------------------A-------
HC-P39                 -------------------C---------GT------G-----C--------------------------------A-------
```

Fig. 1. Nucleotide sequences of 33 independent nonstructural region 5b (NS5b) clones from genotype II/1b hepatitis C virus (HCV), and homologic comparison with HCV-J and HC-C2. *Dashes* indicate sequence identity. *Slashes* denote deletion. Frame-shifts are in *italics*. In-frame stop codons are *underlined*

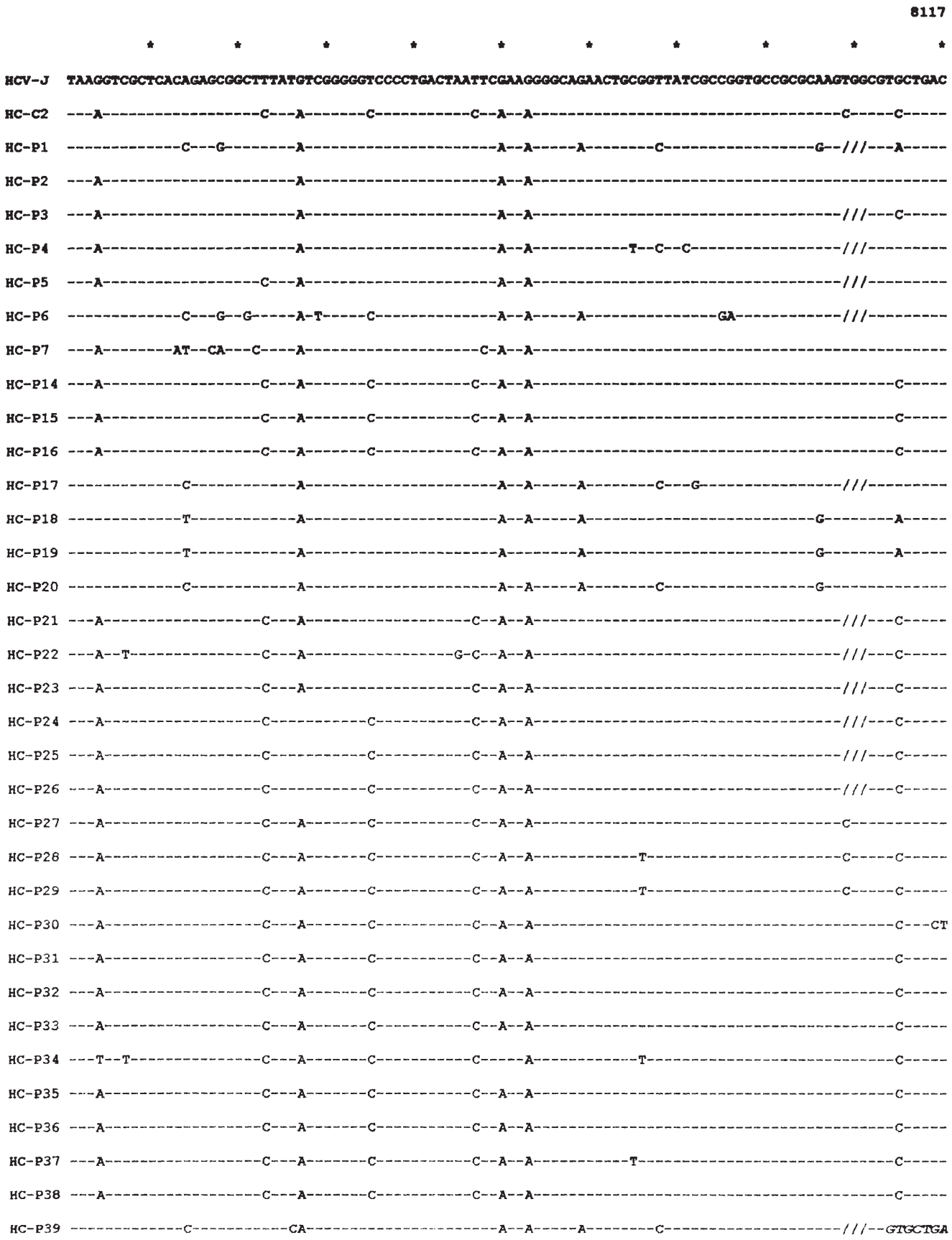

Fig. 1. Continued

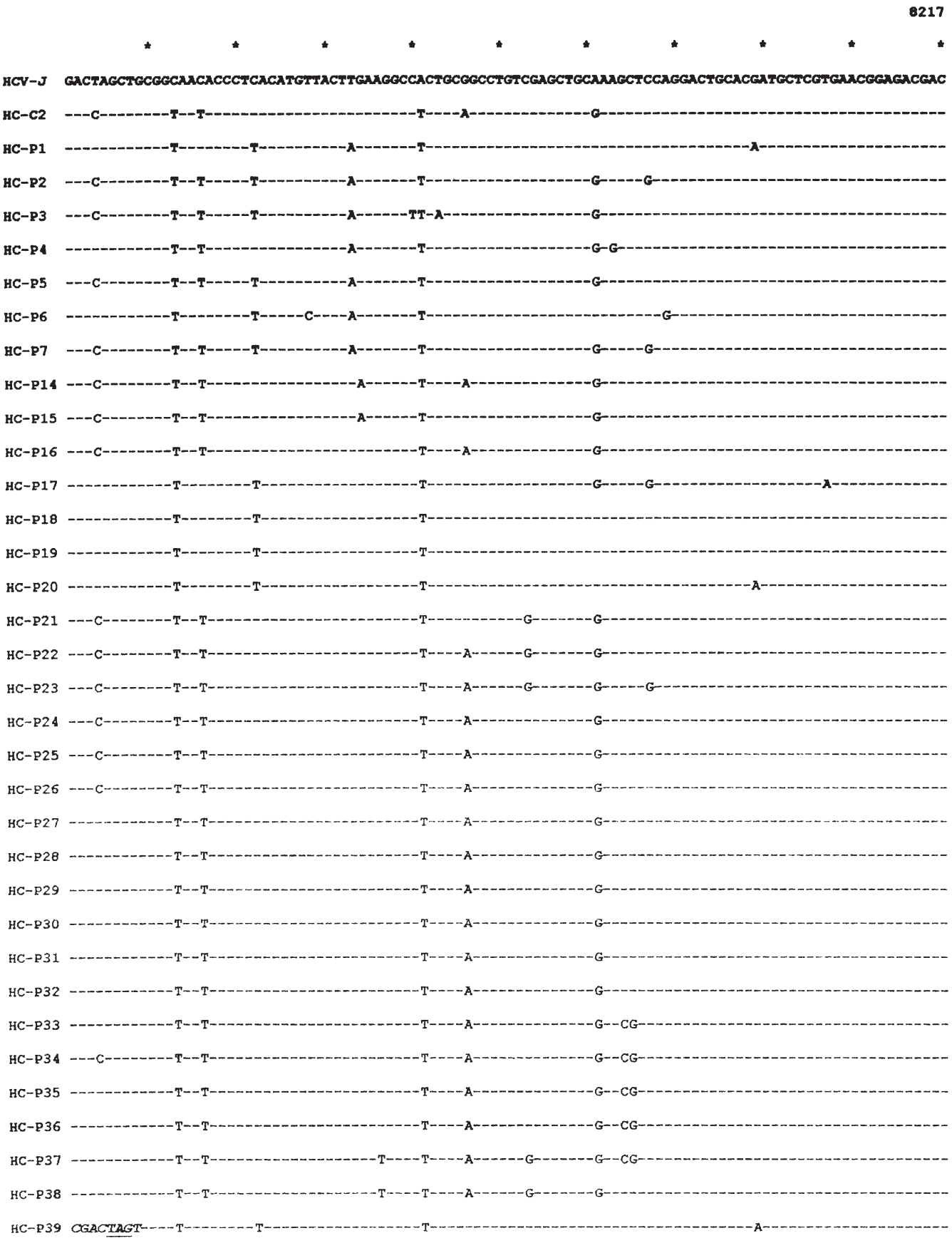

Fig. 1. Continued

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                                                                                                                8287
                        *           *           *           *           *           *           *
HCV-J   CTTGTCGTTATCTGTGAGAGTGCGGGAACCCAGGAGGATGCGGCGGCCCTACGAGCCTTcacggaggctatgacta
HC-C2   -----------------A---------------------------AG--------T---
HC-P1   ------A------T------C------------A----------AAG--------T---
HC-P2   -----------------A--C--------T---A-----------AG--------T---
HC-P3   -----------------A--C------------A----C-A----AG--------T---
HC-P4   -----------------A--C------------A----C-A----AG------------
HC-P5   -----------------A--C--------T---A-----------AG--------T---
HC-P6   ------A-------------C--------T---A-----------AG--------T-T-
HC-P7   -----------------A--C--------T---A-----------AG--------T---
HC-P14  -----------------A--C-----------------------AG--------T---
HC-P15  -----------------A--C-----------------------AG--------T---
HC-P16  -----------------A--C-----------------------AG--------T---
HC-P17  -----------------A--C-----------A------------AG--------T---
HC-P18  ------A-------------C-T---------A------------AG--------T---
HC-P19  ------A-------------C-T---------A------------AG--------T---
HC-P20  ------A-------------C-----------A------------AG------------
HC-P21  -----------------A--C-----------------------AG--------T---
HC-P22  -----------------A--C--------------------T---AG--------T---
HC-P23  -----------------A--C--------------------T---AG--------T---
HC-P24  -----------------A--C-----------A------------AG--------T---
HC-P25  -----------------A--C-----G------------------AG--------T---
HC-P26  -----------------A--C-----------------------AG--------T---
HC-P27  -----------------A--C-----------A------------AG--------T---
HC-P28  -----------------A--C-----------A-GA---------AG------------
HC-P29  ----A---A--------A--C-----------------------AG--------T---
HC-P30  ---------------------C--------T--A-T------AA-AAG--------T---
HC-P31  -----------------A--C-----------A-T----------AG---G----T--A
HC-P32  -----------------A--C-----------------------AG--------T---
HC-P33  -----------------A--C-----G------------------AG--------T---
HC-P34  -----------------A--C-----G------------------AG--------T---
HC-P35  -----------------A--C-----G------------------AG--------T---
HC-P36  -----------------A--C-----G------------------AG--------T---
HC-P37  -----------------A--C-----G------------------AA--------T---
HC-P38  -----------------A--C-----G------------------AG--------T---
HC-P39  ------A-------------C-----------A------------AG------------
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Fig. 1. Continued

were first identified by sequence analysis of the partial NS5b region.[15,25,26] In addition, the NS5b region was variable (up to 44% sequence divergence between some isolates of HCV), could be readily amplified by PCR, and there are also a number of previously published sequences.[10] Thus, based on the above considerations, the partial NS5b region was chosen to examine the genetic heterogeneity of HCV in the present study.

Forty-nine samples were all proven to be HCV RNA-positive by PCR amplification of the 5'NCR region; 33 were identified as genotype 1b (67.35%), and 16 as genotype 2a (32.65%). No 1b/2a mixed type was found.

Thirty-three clones derived from genotype 1b were sequenced. The sequences were compared with HCV-J[21] from Japan, as well as HC-C2[9] from China, and are listed in Fig. 1. Based on comparison in the NS5b of 33 Chinese isolates of genotype 1b, the Chinese isolates belonged to the same subtype as the isolate HCV-J from Japan. There did exist, however, some heterogeneity in the primary structure of the nucleotide acid. The sequence homology among the 33 Chinese isolates and HCV-J was 85.42%–93.39% (91.54% ± 1.55% on average). However, the homology reached 85.13%–98.54% (95.34% ± 3.11% on average) among the 33 Chinese isolates and HC-C2. The average homology of the 33 isolates was higher with HC-C2 than with HCV-J ($P < 0.05$). Of the 33 isolates, 28 (84.85%) showed higher homology with HC-C2 than with HCV-J, and only 4 isolates (12.12%) showed higher homology with HCV-J than with HC-C2. Identical homology was found in the one remaining isolated. The 33 Chinese isolates shared identical nucleotide acids in 7964, 7971, 8010, 8021, 8044, 8067, 8070, 8103, 8133, 8158, 8178, 8235, 8238, 8263, 8264, and 8273 positions (HCV-1 site) the same as HC-C2, but different from HCV-J. These results suggested that these positions might be conserved sites of Chinese isolates of genotype 1b. The HCV isolates of genotype 1b prevalent in mainland China seemed to have a unique characterization, although they shared the same subtype as the HCV isolate from Japan.

The sequences of the 16 clones identified as genotype 2a were compared with HC-J6,[13] and are shown in Fig. 2. The 16 Chinese isolates of genotype 2a had sequence homology of 83.67%–93.88% (90.85% ± 2.50% on average) to HC-J6. There were almost the same nucleotide acids, but they differed from HC-J6 in the 7962, 7989, 8046, 8154, 8158, 8185, 8214, 8237, and 8247 positions (HCV-1 site) in these 16 isolates. This results suggested that all these sites were conserved positions of genotype 2a in the Chinese isolates.

It is clear that the distribution of HCV genotypes shows marked geographic variability, as does that in HBV. The analysis and comparison presented in this study showed that there was higher nucleotide acid homology among the Chinese isolates than between the Chinese isolates and the Japanese isolates, although all these isolates belong to the same subtype. Furthermore, there were some conserved nucleotide acids in the Chinese isolates. These results imply geographical differences in the distribution of individual HCV isolates, as is the case with genotypes. For this reason, it is necessary to understand the consensus sequences of local HCV isolates; this would help to provide more information for the designing of diagnostic reagents and for the development of HCV vaccines in each country.

We next compared the average homology between the 33 genotype 1b isolates and the 16 genotype 2a isolates. The homology was 93.87% ± 3.34% on average, ranging from 84.26% to 99.71%, among the 33 genotype 1b isolates, and 89.73% ± 4.47% on average (range, 76.68%–99.42%) among the 16 genotype 2a isolates. The average homology among HCV isolates of genotype 1b was higher than that among HCV isolates of genotype 2a ($P < 0.05$). In addition, the more conserved nucleotide acids were found in the genotype 1b isolate, but not in the genotype 2a isolate. These findings suggest the notion that there may be higher nucleotide acid heterogeneity among genotype 2a isolates than among genotype 1b isolates. In a previous study, Giannini and co-workers[27] analyzed hepatitis C virus genotype, using genotype-specific PCR primers in the capsid region and a genotype-specific probe-based assay in the 5′ untranslated region (LiPA). It was found in that study that more discordant results were obtained using genotype-specific PCR primers, especially in the presence of type 2a. Another investigation indicated that the sensitivity of the branched DNA amplification assay, widely used for measuring the level of HCV viremia at present, may differ according to the genotype of HCV analyzed, with less sensitivity often being found along with type 2a.[28] These observations may support our notion above. One possible explanation for this issue is that only one complete sequence and a few partial sequence of type 2a have been published, whereas at least ten full-length sequences of genotype 1b have been reported so far.[3] Under these circumstances, the fewer sequences and the genetic heterogeneity of genotype 2a could be taken into consideration when diagnostic reagents, including primers or probes, are designed, because genetic heterogeneity has important implications in diagnosis, as well as in vaccine development. Therefore, there is a need for elucidation of more full-length sequences of genotype 2a to overcome this problem. We believe that, at least, the related gene region of genotype 2a should be extensively investigated before the development

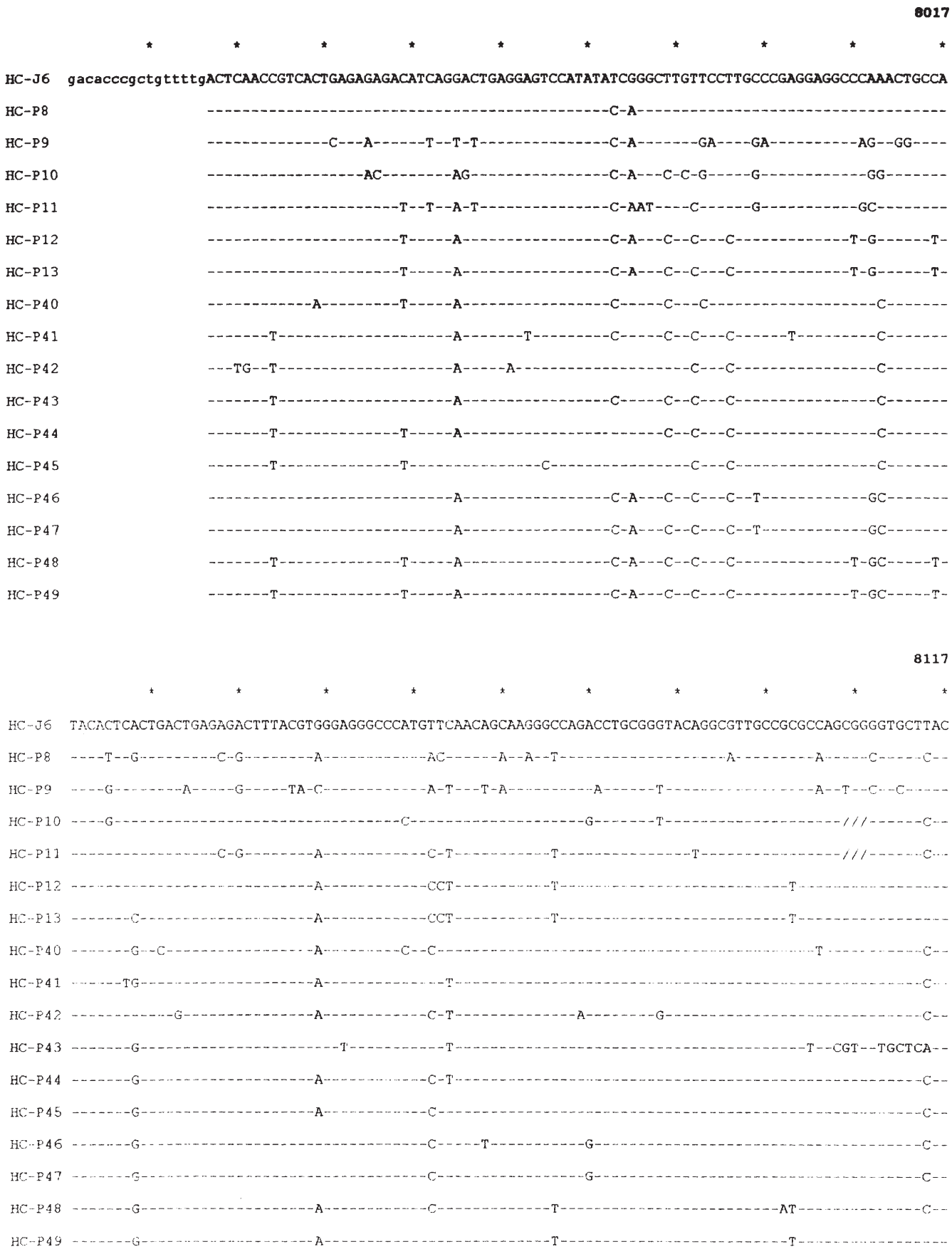

Fig. 2. Nucleotide sequences of 16 independent NS5b clones from genotype III/2a HCV, and homologic comparison with HC-J6. *Dashes* indicate sequence identity with the *top* sequences. *Slashes* indicate deletion

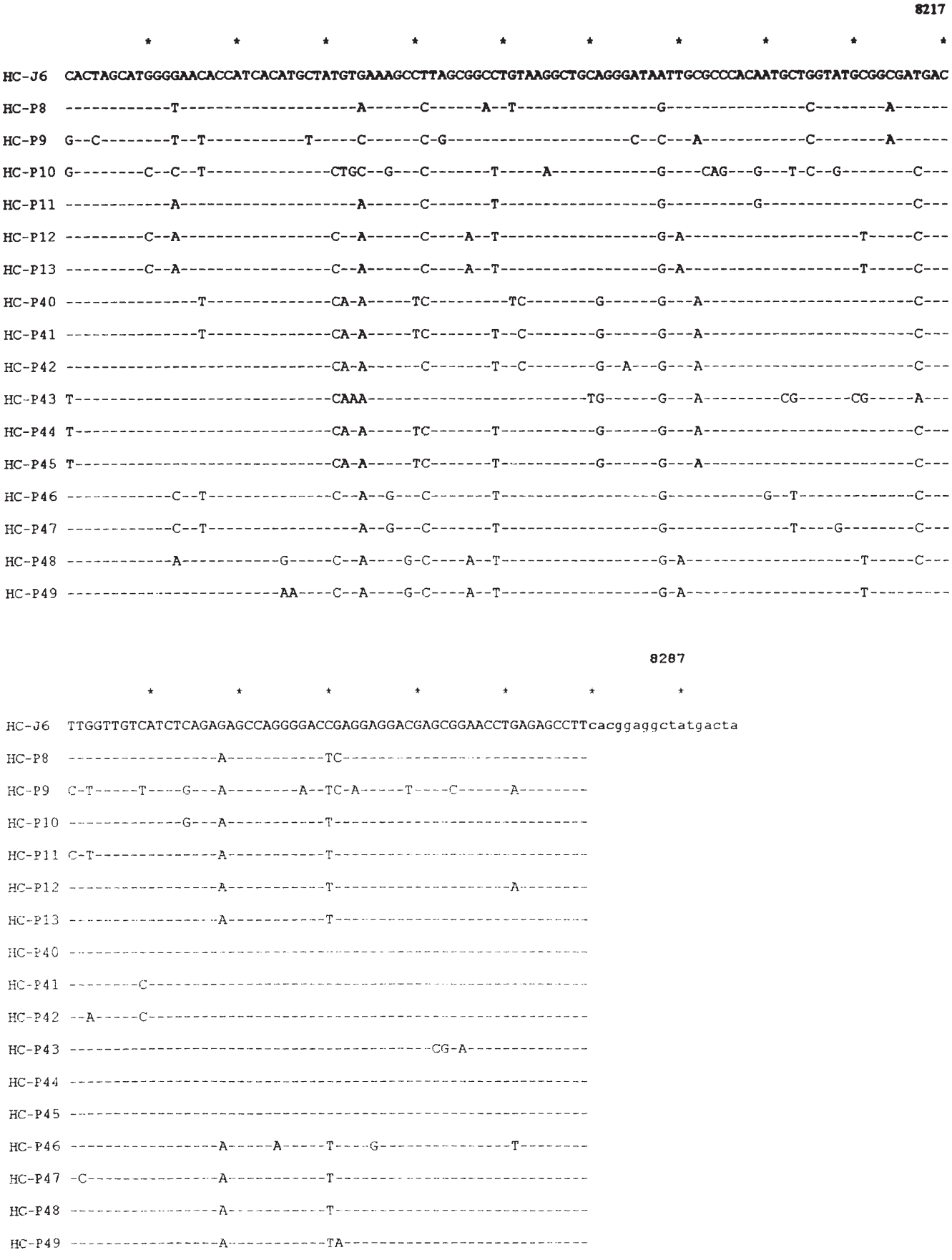

Fig. 2. Continued

of a vaccine and the design of diagnostic reagents are undertaken.

In the present study, a deletion of three nucleotide acids was first found in the NS5 region. The deletions occurred from nt 8106 to nt 8108. resulting in TGG deletion; 13 of the 33 clones derived from sera containing genotype 1b HCV and 2 of the 16 clones of genotype 2a possessed this deletion. Some previous studies have reported that the nucleotide insertions and/or deletions were observed only in the E2 and NS5 genes of HCV, and the number of insertion or deletion was often three or multiples of three.[3,13] This deletion was also of three nucleotide acids. Because three nucleotide acids were deleted, only two codons were involved, and no change was brought about in later sequences. In addition, the clone HC-P39 also possessed a frame-shift mutation, resulting in the introduction of an in-frame stop codon. In two previous studies,[29,30] the introduction of an in-frame stop codon was observed in the NS3/NS4 and E2/NS1 regions. The in-frame stop codon caused by the frame-shift mutation observed in the present study was located in the NS5 region. To ensure that this change was not due to the incorporation of errors using Taq polymerase lacking proofreading of the 3′–5′exonuclease in amplification, HC-P39 was cloned and sequenced from three individual PCR products, and the sequences were found to be absolutely identical. Therefore, this finding confirms that the sequence was not an artifact.

Based on the findings of Martell et al.[29] and Higashi et al.[30] and our findings in this study, it is reasonable to assume that viruses exist containing other defective genes. It is now generally believed that defective viruses require the standard virus for their reproduction, and that they no longer replicate when they lack sufficient numbers of helper virus. Therefore, the proportion of intact and defective viruses fluctuates during the course of an infection.[30] Because defective viruses are involved in the development of disease, there may be a wealth of data indicating how a defective RNA genome restricts viral replication in vivo.

In the NS5b region observed in this study, HCV isolates of genotype 1b contained eight cysteine residues, and those of genotype 2a contained six cysteine residues. All the cysteine residues were virtually conserved among the different HCV isolates. This situation may suggest that the epitopes borne by these cysteine residues are dependent on the molecular conformation and may be relevant in the activity of the putative RNA-direct-RNA polymerase.

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