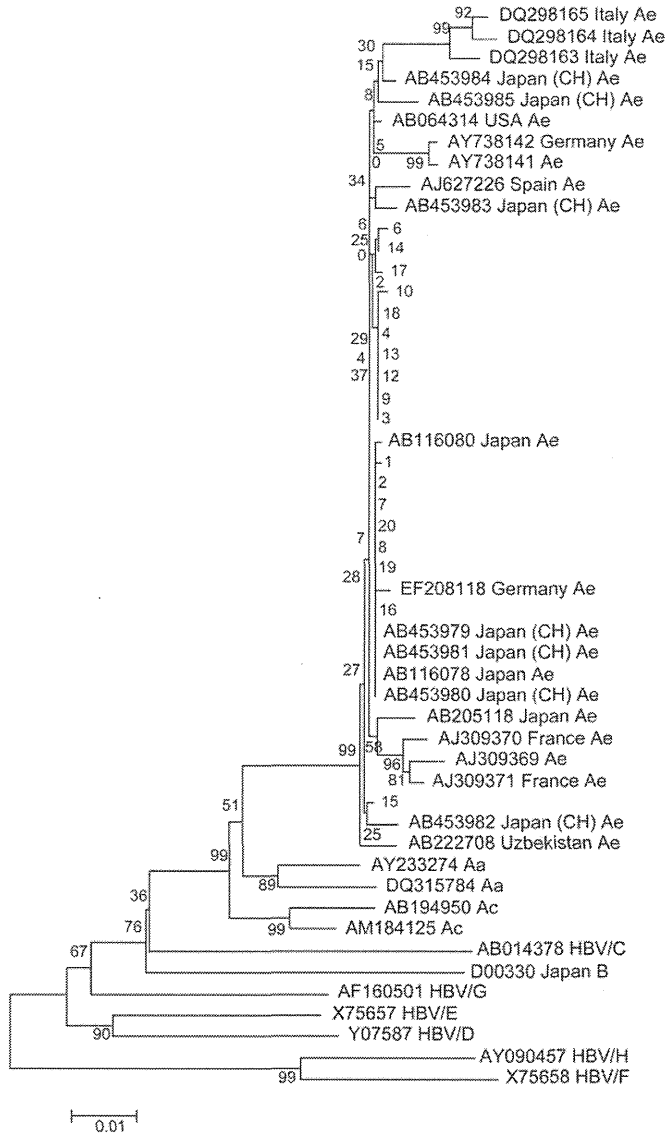

Figure 1. Evolutionary relationships of 86 hepatitis B virus genotype A taxa, including 20 from the present cases. The evolutionary history, inferred using the neighbor-joining method, shows that all 20 samples had similar nucleotide sequences close to previously reported genotype A2 sequences from Western countries.

the onset was useful for distinguishing group 3 or 4 from group 1 or 2. Likewise, HBV DNA levels at 8 weeks from the onset were useful for discriminating between group 4 and group 3, as well as for distinguishing group 3 or 4 from group 1 or 2.

Levels of HBsAg and HBV DNA for Predicting Persistent Infection

As the levels of HBsAg at 12 weeks and HBV DNA at 8 weeks from the onset were useful for distinguishing group 4 from the other groups, we evaluated the appropriate levels for predicting persistent infection in patients with genotype A. When we set the cutoff value of HBsAg at 1000 IU/mL based on the ROC analysis, both the positive predictive value and the negative predictive value were 100% with high sensitivity (100%) and specificity (98.1%). Likewise, when we set the cutoff value of HBV DNA at 10^6 log IU/mL based on the ROC analysis, both the positive predictive value and the negative predictive value were 100% with high sensitivity (100%) and specificity (96.4%). Therefore, HBsAg at 12 weeks >1000 IU/mL or HBV DNA at 8 weeks >10^6 log copies/mL is useful for predicting persistent infection.

DISCUSSION

In Japan, as shown in Table 1, the dominant HBV in acute hepatitis has been shifting from genotype C to A [3, 5, 14, 18]. The fact that nucleotide sequences of HBV/A isolates from patients

Table 2. Baseline Characteristics and the Duration of Hepatitis B Surface Antigen in Patients With Acute Hepatitis B Virus With Different Hepatitis B Virus Genotypes

	HBV Genotypes					
Features	A (n = 113)	B (n = 26)	C (n = 73)	D (n = 1)	E (n = 1)	F (n = 1)
Age, y	30.8 ± 9.5	32.3 ± 9.5	33.3 ± 10.9	27	26	58
Male	106 (93.8%)[a]	21 (80.7%)[b]	29 (39.7%)[a,b]	0	0	1 (100%)
Transmission routes Identified	102 (90.2%)	21 (80.8%)	53 (72.6%)	1 (100%)	1 (100%)	1 (100%)
Heterosexual	70 (68.6%)	19 (90.4%)	47 (88.7%)	1 (100%)	1 (100%)	1 (100%)
MSM	32 (31.4%)[c,d]	1 (4.8%)[c]	6 (11.3%)[d]	0	0	0
ALT, IU/L	2126 ± 938[e,*]	2394 ± 820	2857 ± 1668[e]	4180	1175	1533
Bilirubin, mg/dL	7.1 ± 6.4[f*]	4.8 ± 3.3[f,g]	9.0 ± 7.5[g]	6.8	3.9	3.5
HBV DNA, log copies/mL	6.3 ± 1.7[h,*]	5.5 ± 2.3	4.9 ± 1.5[h]	5.2	7.4	4.8
HBeAg	95/121 (77.3%)[i,*]	24/28 (88.5%)	37/58 (65.5%)[i]	1/1 (100%)	1/1 (100%)	1/1 (100%)
Anti-HIV	7/72 (9.7%)	0/7 (0%)	0/23 (0%)	Not tested	0/1 (0%)	Not tested
Duration of HBsAg*						
Group (mo)						
1 (<3)	35 (42.2%)	16 (64.0%)	31 (64.6%)	0	1	1
2 (3–6)	34 (41.0%)	8 (32.0%)	11 (22.9%)	1	0	0
3 (>6–12)	9 (10.8%)	0	6 (12.5%)	0	0	0
4 (>12)	5 (6.0%)	1 (4.0%)	0	0	0	0

Abbreviations: ALT, alanine aminotransferase; anti-HIV, antibody to human immunodeficiency virus; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; MSM, men who have sex with men.

[a] $P < .001$.

[b] $P < .001$.

[c] $P = .017$.

[d] $P = .002$.

[e] $P = .002$.

[f] $P = .003$.

[g] $P < .001$.

[h] $P < .001$.

[i] $P = .036$.

* *Data from anti-HIV-positive patients are excluded.*

with acute hepatitis B in this study were very close to one another suggests that most HBV/A strains were imported recently and have spread rapidly, which may be attributed to the features of HBV/A in transmission routes and viral kinetics. We have reported that patients with genotype A tend to have multiple sexual partners [5]. Consequently, chances of secondary transmission of HBV/A would be higher than those of other genotypes, which may increase the number of patients who contract HBV/A infections. On the other hand, HBsAg persisted longer in patients with genotype A than B or C, which is consistent with the in vivo experiment using chimera mice carrying human hepatocytes showing that proliferation of HBV starts later and lasts longer in genotype A than in B or C infection [19].

Our results have shown that 6% of the patients with genotype A develop persistent infection. Because liver cirrhosis or hepatocellular carcinoma can develop in a substantial population of HBV carriers [20, 21], it is important to distinguish the patients in whom HBV infection becomes chronic, and follow them carefully. Although polymorphisms in host genes may be useful for identifying patients who are prone to develop chronic HBV infection [22], simple surrogate markers for the outcome have not been reported. Our data indicate that it would be difficult to predict the clinical outcome based on serum levels of viral markers at the first visit alone. This is understandable, because the dose of infecting virus, as well as the interval between infection and the first visit, can vary widely. Hence, we set out to analyze changes in serum levels of viral markers.

As seen in Figure 2, HBsAg levels at 12 weeks from the onset were most useful for discriminating among groups 2, 3, and 4 in the genotype A infection. Therefore, the outcome of acute hepatitis B may be predictable at this time point. Of note is the reelevation of HBsAg observed in group IV (Supplementary Figure 1*A*). Reelevation of viral markers suggests prolonged viral proliferation in the liver, and may be useful to identify the patients who may develop chronic infection.

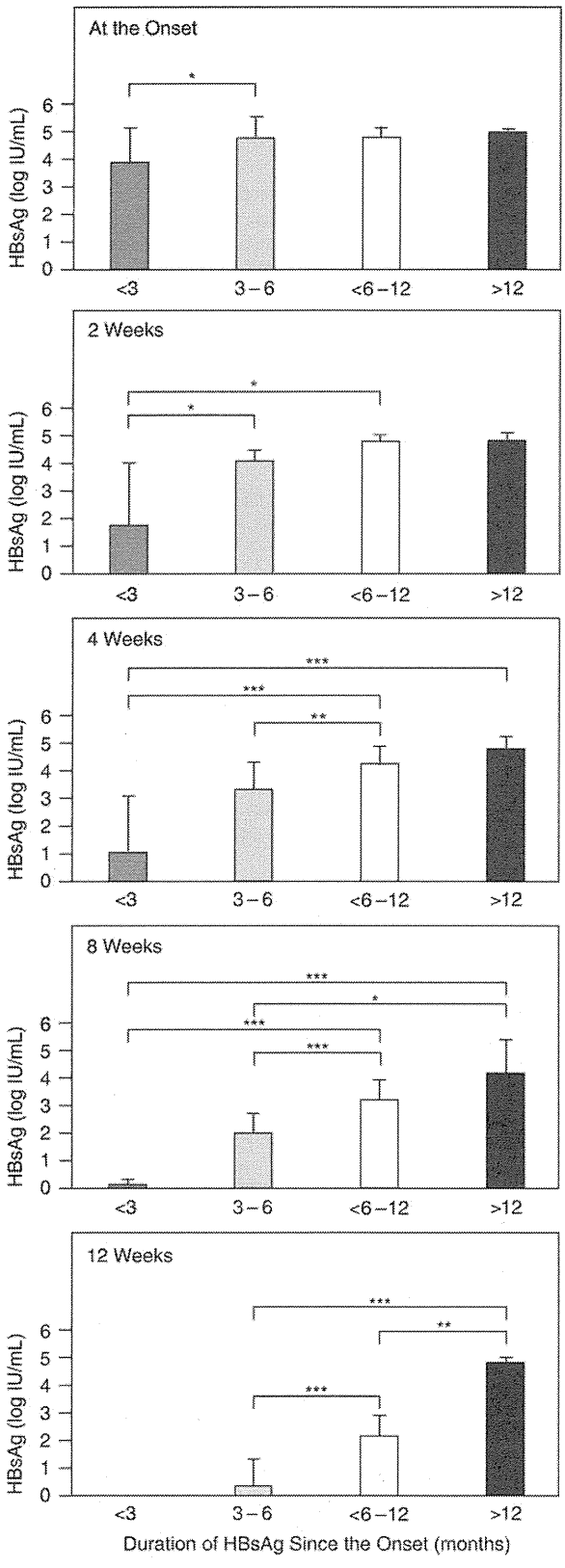

Figure 2. Levels of hepatitis B surface antigen in patients with different durations of infection compared at various weeks after the onset of acute hepatitis B genotype A *P< .05; **P< .01; ***P< .001. Abbreviation: HBsAg, hepatitis B surface antigen.

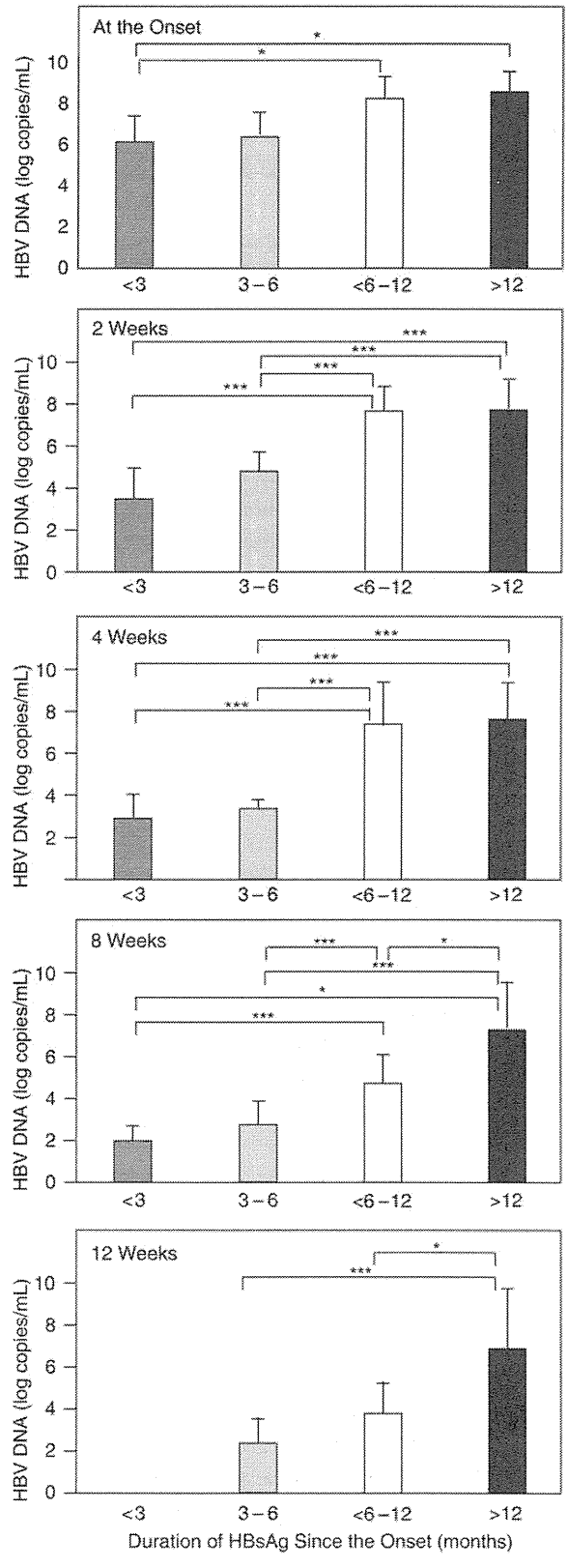

Figure 3. Levels of hepatitis B virus DNA in patients with different durations of infection compared at various weeks after the onset of acute hepatitis B genotype A. *P< .05; **P< .01; ***P< .001. Abbreviations: HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus.

As shown in Figure 3, HBV DNA levels at 4 weeks from the onset can discriminate groups 1/2 from groups 3/4. Furthermore, HBV DNA levels at 8 weeks from the onset can distinguish group 4 from group 1, 2, or 3. Therefore, the combination of HBV DNA levels at weeks 4 and 8 would be useful for predicting the outcome. For the prediction of a chronic outcome, HBV DNA level at 8 weeks from the onset is a useful surrogate marker of the outcome as well as HBsAg level at 12 weeks. There were differences in viral kinetics among groups 1, 2, 3, and 4.

Our present study showed that 15 of the 215 patients (7.0%) cleared HBsAg from >6 to 12 months after the onset. Sixty percent of the 15 patients had HBV/A. Although these patients met the criteria of chronic infection, they finally cleared HBsAg from the sera. Therefore, we would like to propose that transition to chronic infection in acute hepatitis B be judged at 12 months from onset in patients with genotype A; further studies in larger cohorts are necessary. One reason for our proposal is the indication of antiviral treatment. Antiviral treatment in patients with acute hepatitis B is not indicated because previous studies failed to show the efficacy of antiviral treatments in the patients with acute hepatitis B [23, 24]. However, if patients who actually develop chronic infection can be identified and treated by antiviral treatment, the number of those who develop secondary infection may be markedly reduced. Evaluation of the efficacy of antiviral treatments by prospective studies, based on surrogate markers for the outcome, should be conducted as the next step. HBeAg, which was reported to be useful as a surrogate marker for chronicity, should also be assessed as a surrogate marker [25, 26].

Our study has some limitations. First, the lack of data in early stages made it difficult to study viral kinetics precisely. Second, viral kinetics in the infection with each HBV genotype were obtained from a restricted number of patients, not large enough to establish the usefulness of changes in viral markers in earlier stages of HBV infection. Third, anti-HIV was not checked in all patients due to the lack of informed consent. Fourth, HBsAg and HBV DNA were not determined 24 weeks after onset when discrimination between groups 3 and 4 may be possible more easily. Fifth, the maximum levels of ALT and bilirubin may be affected by the time of blood test. Validation studies in larger cohorts are necessary to evaluate the feasibility of our hypotheses.

In conclusion, we have shown that viral kinetics and the clinical outcome are different among patients with acute hepatitis B who are infected with HBV of distinct genotypes. HBsAg levels at 12 weeks and HBV DNA at 8 weeks after the onset would be useful to predict the clinical outcome of patients with acute hepatitis B.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online (http://cid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Original Article

Novel hepatitis B virus strain developing due to recombination between genotypes H and B strains isolated from a Japanese patient

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Aim: In Japan, genotypes B and C are the predominant genotypes isolated from patients with chronic hepatitis B, while genotype A predominates in patients with acute hepatitis B. Globalization, however, appears to have changed the distribution of the hepatitis B virus (HBV) genotypes. Thus, the viral characteristics of HBV genotypes other than genotypes A, B and C were examined.

Methods: Screening of genotypes was performed by enzyme immunoassay and/or polymerase chain reaction INVADER method in 222 patients with HBV. The full-length nucleotide sequences of unusual strains were compared to those in the database, followed by construction of a phylogenetic tree.

Results: Unusual HBV strains were isolated from two patients: a 27-year-old Japanese bisexual man with acute hepatitis B with HIV co-infection and a 52-year-old Japanese man with chronic hepatitis B. The former strain was classified as genotype H, showing an overall identity of 99.8% to the Thailand strain (EU498228), while the nucleotide sequence of the latter strain showed similarity to the genotype B strains isolated in Malaysia (JQ027316) and Indonesia (JQ429079) between DR2 and DR1 in the X region, with identities of 96.9%. However, this strain was classified as genotype H by full-length sequence analysis, and the sequence between nt2023 and nt2262 showed no similarity to that in any previously reported strains.

Conclusion: HBV strains showing recombination between genotype B and H strains were found even in chronic hepatitis patients in Japan. Globalization may yield HBV strains of possible novel genotypes containing novel nucleotide sequences in the precore/core region.

Key words: genotype, globalization, hepatitis B virus, nucleotide sequence, recombination

INTRODUCTION

HEPATITIS B VIRUS (HBV) infection is a global health problem with an estimated 400 million people worldwide showing persistent infection.[1] These patients are at a serious risk of developing the complication of liver cirrhosis and hepatocellular carcinoma (HCC),[2] and approximately 1 million deaths per year are attributed to cirrhosis and HCC caused by HBV infection.[3] In Japan, more than 30 000 people die of HCC each year,[4] and in 15% of these cases, the etiology has been shown to be HBV infection.[5] On the other hand, patients with persistent HBV infection serve as a source of HBV transmission to the healthy population, resulting in the occurrence of acute liver diseases with fatal outcomes. According to a nationwide survey of fulminant hepatitis and late-onset hepatic failure in Japan, acute liver failure is caused by HBV infection, either transient infection or acute exacerbation of persistent infection, in approximately 40% of cases.[6–8]

Hepatitis B virus is a double-stranded DNA virus belonging to the *Hepadnaviridae* family; the genome is composed of approximately 3200 nucleotides organized into four open reading frames (ORF) for the P, C, S and X genes.[9] According to the results of full-length nucleotide sequence analysis of the entire genome, HBV has been classified into at least eight genotypes, A–H,

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showing nucleotide differences of more than 8% from each other.[10] The frequency of each genotype among isolates from patients with HBV infection differs depending on the geographic area of the world;[11] genotype A HBV strains prevail in Africa, Europe and India, while genotype B and C strains are frequent in Asia, and genotype E strains in sub-Saharan Africa. On the other hand, genotype D strains are distributed all over the world, and genotype F and H strains are found exclusively in Central and South America. It has been demonstrated that the clinical features of patients with HBV infection, including their responses to antiviral therapies, differ depending on the genotype of the viral strain causing the infection,[12] suggesting that identification of the HBV genotype causing the infection, in addition to determination of the serum HBV DNA levels and mutation profile of the viral genome is crucial to establish the therapeutic strategy in patients with both acute and chronic liver diseases caused by HBV.

However, it has been reported recently that globalization of the world may have altered the geographic distribution of HBV genotypes, including in Asian countries. In Japan, genotypes B1/Bj and C2 strains are the predominantly isolated strains from patients with both acute and chronic liver diseases caused by HBV infection; the distribution of the HBV genotypes has been reported to differ depending on the geographic areas even within Japan; genotype B strains are found more frequently in Okinawa islands and northeastern areas of Honshu island, while genotype C strains are more prevalent in other areas of Japan.[13] It has been suggested that such a distribution pattern may be upset in the near future, because genotype A strains have begun to be isolated more frequently from patients with acute liver diseases caused by HBV infection in Japan, especially in metropolitan cities such as Tokyo, Osaka and Nagoya,[14,15] and this genotype strain is known to produce persistent infection even in elderly patients contracting the infection.[16] Furthermore, the occurrence of recombination among different genotypes may also influence the geographic distribution patterns. HBV strains resulting from genome recombinations among genotype A, C and G strains have been found in Laos and Vietnam, and been tentatively proposed as "genotype I" strains.[17,18] Moreover, a HBV strain positioned between the human and ape genotypes on the phylogenetic tree has been isolated from a Japanese patient with HCC who had previously lived in Borneo.[19]

Thus, we screened the genotypes of the HBV strains isolated from patients with acute and chronic liver diseases caused by HBV, and the full-length nucleotide sequences of the strains other than genotype A, B and C strains found in the screening examination were analyzed and compared with those in the database. In the present paper, we report on the viral characteristics of such unusual strains detected in Japanese patients with HBV infection.

METHODS

Patients and experimental designs

THE SUBJECTS WERE 222 Japanese patients with acute or chronic hepatitis seen first between May 2011 and December 2012 at the outpatient clinic of Saitama Medical University Hospital. All the patients tested positive for serum hepatitis B surface antigen (HBsAg), and the HBV genotypes were screened by enzyme immunoassay (EIA)[20,21] or the polymerase chain reaction (PCR)-INVADER method.[22] The full-length nucleotide sequence was analyzed when genotypes other than A, B or C were identified from the patients. The screening examinations for the HBV genotypes were done under the assurance of national health insurance coverage. Written informed consent was obtained from each of the patients prior to the analysis of the full-length nucleotide sequences of the isolated HBV strains. The characteristics of the viral genotypes other than A, B or C identified through the screening examination were analyzed after obtaining the approval of the institutional review board of Saitama Medical University Hospital.

DNA extraction and direct nucleotide sequencing of the HBV strains

Nucleic acids were extracted from 200 μL of serum samples QIAamp MinElute Virus Spin Kits (Qiagen, Tokyo, Japan). The virus DNA was eluted in RNase-free water at a volume of 100 μL and maintained at −20°C until use. To obtain a full-length nucleotide sequence of HBV DNA, a long-distance nested PCR was performed to amplify two overlapping fragments according to the methods of Takahashi *et al.*[23] using oligonucleotide primers shown in Table S1.

A fragment with a length of 3040 bases (WA2) corresponding to oligonucleotides from 1908–1780 nt of a standard genotype C HBV isolate was amplified using two primer sets, external WA-L (1859–1882 nt) and WA-R (1805–1828 nt) primers and internal WA2-L (1887–1908 nt) and WA2-R (1780–1801 nt) primers, and PrimeSTAR GXL DNA Polymerase (TaKaRa, Shiga, Japan) with the primer annealing at 60°C for 35 cycles

in the first PCR and 30 cycles in the second PCR. A fragment with a length of approximately 378 bases (gN2) corresponding to the residue from 1702–2081 nt was amplified similarly using two primer sets, external gN1-L (1606–1625 nt) and gN1-FR/gN1-HR (2160–2179 nt) primers and internal gN2-L/gN2-HL (1683–1702 nt) and gN2-FR/gN2-HR (2081–2100 nt) primers, and TaKaRa Ex Taq Hot Start Version (TaKaRa) with the primer annealing at 55°C for 35 cycles in the first PCR and 30 cycles in the second PCR. PCR conditions for PrimeSTAR GXL DNA Polymerase and PrimeSTAR GXL DNA Polymerase were specified according to the protocol of the manufacturer.

Both WA2 and gN2 fragments were purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced using the BigDye Teminator version 3.1 Cycle Sequence Kit (Applied Biosystems, Foster City, CA, USA) using the internal primers shown in Table S1, according to the protocol of the manufacturer. The nucleotide sequences of the amplified products were directly sequenced with a 3130 Genetic Analyzer (Applied Biosystems), and the obtained data for nucleotide sequences were connected using ATGC version 7 (GENETYX, Tokyo, Japan).

Whole-genome cloning of HBV strains

To obtain a whole-genome clone of HBV strains, an additional PCR and In-Fusion reactions were performed. The WA2 and gN2 fragments were amplified using Prime STAR MAX DNA Polymerase (TaKaRa) and primer sets, WA2-Sap I-L (1943–1960 nt) and WA2-Sap I-R (1689–1708 nt) primers and gN2-Sap I-L (1704–1723 nt) and gN2-Sap I-R (1940–1957 nt) primers, respectively (Table S1), with the primer annealing at 55°C for 35 cycles. T-Vector pMD20 (TaKaRa) was amplified using a primer set, pMD20-Sap I-L (1705–1708 nt) and pMD20-Sap I-R (1704–1707 nt) primers, at conditions similar to that in amplification of both fragments. All PCR conditions were specified according to the protocol of the manufacturer. Both fragments and the vector were purified using the QIAquick PCR Purification Kit (Qiagen). WA2-Sap I fragment (100 ng), 50 ng of gN2-Sap I fragment and 100 ng of T-Vector pMD20-Sap I were mixed in a tube with In-Fusion HD Enzyme Premix (Clontech, Mountain View, CA, USA) at a total volume of 10 μL. The reaction mixture was incubated at 50°C for 15 min, and then transferred to ice. Reaction mixture (2.5 μL) was transformed into Stellar Competent Cell (Clontech) followed by mini-prepping and was subjected to nucleotide sequencing. Both conditions for In-Fusion reaction and transformation were specified according to the protocol of the manufacturer.

SimPlot analysis and construction of the phylogenetic tree

The complete full-genome sequences of the isolated HBV strains were compared with those of the 35 reference sequences retrieved from the DNA Data Bank of Japan (DDBJ)/European Molecular Biology Laboratory (EMBL)/GenBank database. The full-genome sequences of the following HBV strains shown in the database (represented by their accession numbers) were used in the SimPlot analysis, followed by construction of the phylogenetic tree: genotype A, *AB076678*, *AF090838* and *M57663*; genotype B, *AB010291*, *AB033554*, *AF121249*, *D00329* and *D50521*; genotype C, *AB049609*, *AB049610*, *AB112063*, *AB112066*, *AB112471* and *AB115417*; genotype D, *AB033559*, *AB126581* and *Z35716*; genotype E, *AB091255*, *AB106564* and *X75657*; genotype F, *AB166850*, *AY090459* and *X69798*; genotype G, *AB056513*, *AB064310* and *AF160501*; genotype H, *AB179747*, *AY090454*, *AY090457* and *AY090460*; genotype I, *EU833891*, *GU357844*, *JF899337* and *JF899338*; and genotype J, *AB486012*.

The nucleotide sequences were multiple-aligned using GENETYX for Windows version 11 software (GENETYX) and the genotype was specified using Kimura's two-parameter method.[24] A phylogenetic tree was constructed by the neighbor-joining method.[25] To confirm the reliability of the phylogenetic tree analysis, bootstrap resampling and resampling were carried out 1000 times. The subtypes of the strains used for the comparison were obtained from published articles.[26,27] Moreover, the recombination of the HBV genomes among strains of different genotypes was examined by the SimPlot program (available at http://sray.med.som.jhmi.edu/SCRoftware/) and boot scanning analysis.[25,28]

RESULTS

Genotypes of HBV strains obtained from patients with acute and chronic liver diseases

THE HBV STRAINS isolated from the 222 patients were classified according to the screening examinations carried out by EIA and/or the PCR-INVADER method as follows: genotype A, 21 (9.4%) strains; genotype B, 66 (29.7%) strains; and genotype C, 112 (50.5%) strains. The HBV genotype was indeterminate in 21 patients (9.4%) due to the low titers of serum HBsAg and/or HBV DNA. When the total subject population was stratified further, genotypes A, B, C and the

indeterminate genotype were found in 15 (50.0%), three (10.0%), 11 (36.7%) and zero (0%) of the 30 patients with acute liver diseases, and six (3.1%), 63 (32.8%), 101 (52.6%) and 21 (11.0%) of the 192 patients with chronic liver diseases, respectively. In contrast, one each of the patients (1.0%) with acute (case 1) and chronic (case 2) liver diseases had a HBV genotype other than A, B or C. The demographic and clinical features of the two patients were as follows.

A 27-year-old bisexual man (case 1) working in the adult entertainment industry was diagnosed as having acute hepatitis caused by HBV, and the genotype of the infecting HBV strain was identified as genotype H by the PCR-INVADER method. He received highly active antiretroviral therapy because of co-infection with HIV, and the serum HBV DNA titers decreased to less than the detectable level, with positivity for serum anti-HBs antibody developing 25 months later.

A 57-year-old man (case 2) was diagnosed as having chronic hepatitis caused by HBV, and the infecting HBV strain was classified as genotype F by the PCR-INVADER method, despite the genotype being classified as indeterminate by the EIA method. His deceased father had lived in Brazil in his youth and his elder brother had been diagnosed as being a HBV carrier at another hospital. He received oral entecavir at a daily dose of

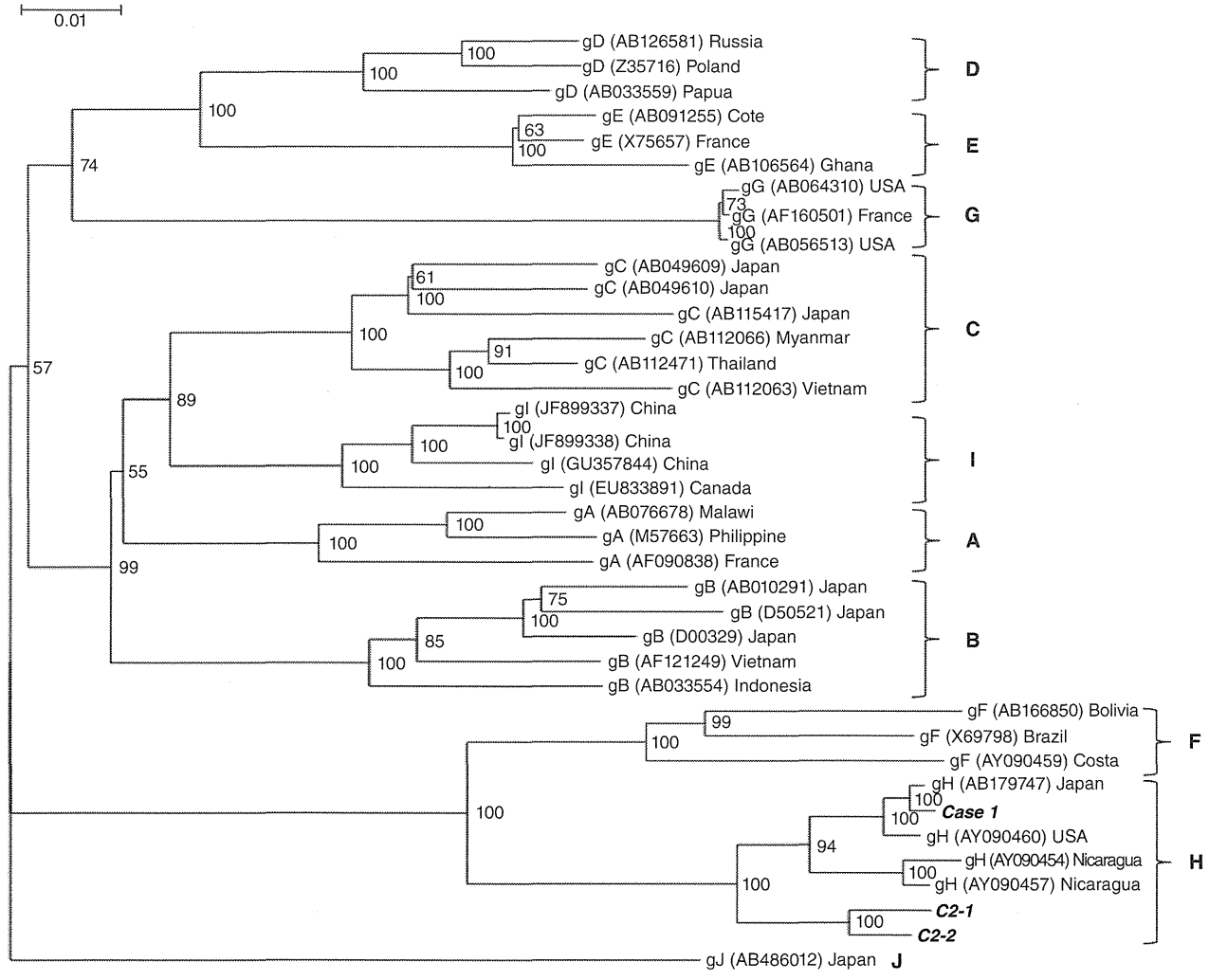

Figure 1 A phylogenetic tree constructed based on the full-length sequence of hepatitis B virus (HBV) strains isolated from case 1 and case 2 in comparison with that of 35 reference strains. The bootstrap values are indicated at each tree root and the genotypes are on the right. The horizontal bar provides a genetic distance.

0.5 mg, and the serum HBV titers decreased from 5.3 log copies/mL to a level less than 2.1 log copies/mL by 3 months of treatment.

Full-length nucleotide sequences of the isolated HBV strains that were different from genotypes A, B and C

The nucleotide sequences of the HBV strains isolated from cases 1 and 2 were analyzed. A phylogenetic tree constructed based on the full-length sequence of HBV genome led to classification of the HBV strain isolated from case 1 as genotype H, showing an overall identity of 99.8% (3210/3215 bp) to the Thailand strain of genotype H (EU498228) (Figs 1,2). A similar analysis using a phylogenetic tree led to classification of the HBV strain isolated from case 2 as genotype H (Figs 1,3) despite it being classified as indeterminate and genotype F by EIA and PCR-INVADER assay, respectively. The full-length nucleotide sequence analysis showed an overall identity of 97.1% (3125/3218 bp) to genotype H strain isolated from a patient in Mexico (AB375164).

The nucleotide sequence of the HBV strains isolated from case 2 was further analyzed depending on the ORF, because the identity of the full-length nucleotide sequences to that of previously reported strains was less in case 2 than that in case 1. Consequently, the nucleotide sequence between DR2 (1590–1600 nt) and DR1 (1824–1834 nt) in the X region showed a similarity to that of the corresponding region of a genotype B strain isolated in Malaysia (JQ027316) and Indonesia (JQ429079), with identities of 98.4% (241/245 bp) and 98.0% (240/245 bp) (Fig. 4a). Moreover, analysis of the nucleotide sequence between 2023 and 2262 nt in the precore/core regions revealed that several different clones existed as quasispecies among HBV strains isolated from case 2, and two major clones, C2-1 and C2-2, were separated following cloning and sequencing of whole-genome nucleotides. Both C2-1 and C2-2 clones

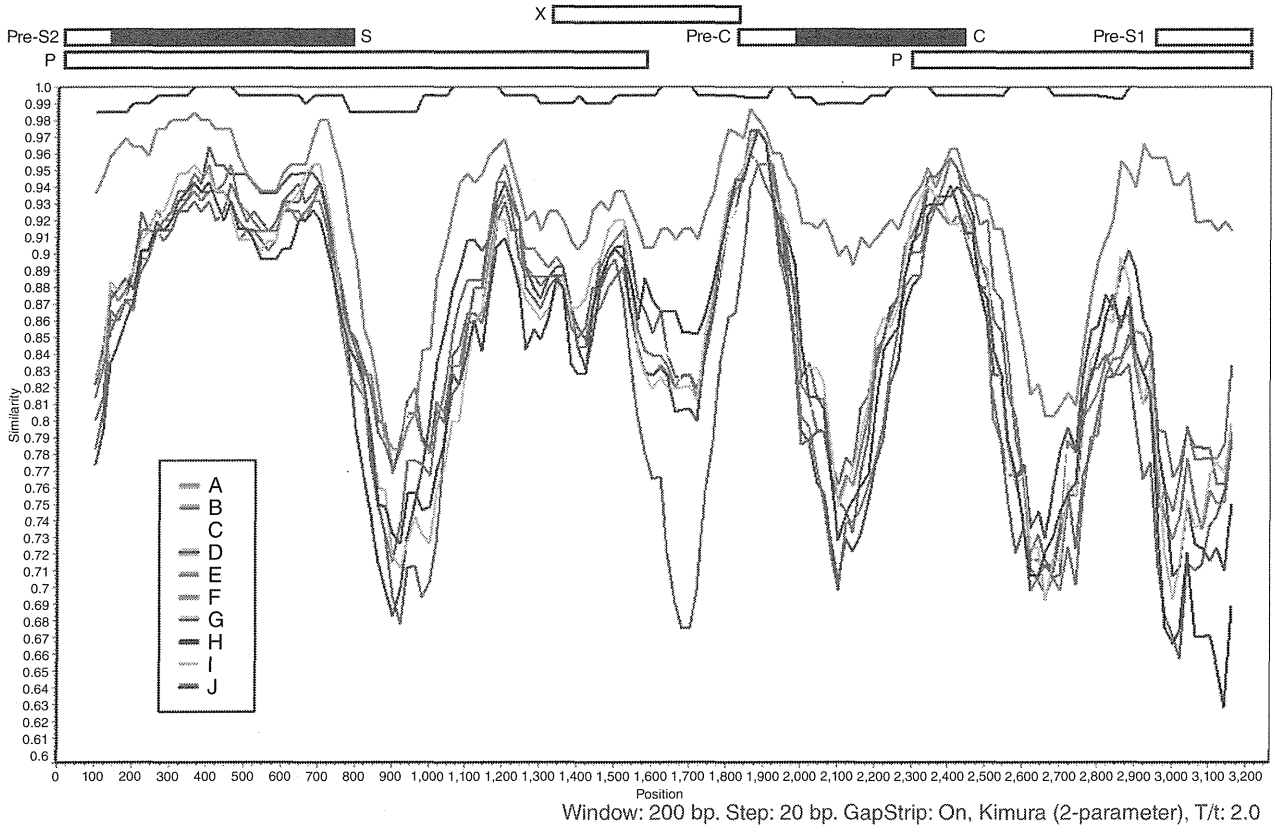

Figure 2 Nucleotide similarity comparison of a full-length sequence of hepatitis B virus (HBV) strains isolated from case 1 in reference to previously reported HBV genotypes A–J. The parameters used for the analysis are shown at the bottom of the figure (200-bp window size, 20-bp step size and gap-stripped alignments).

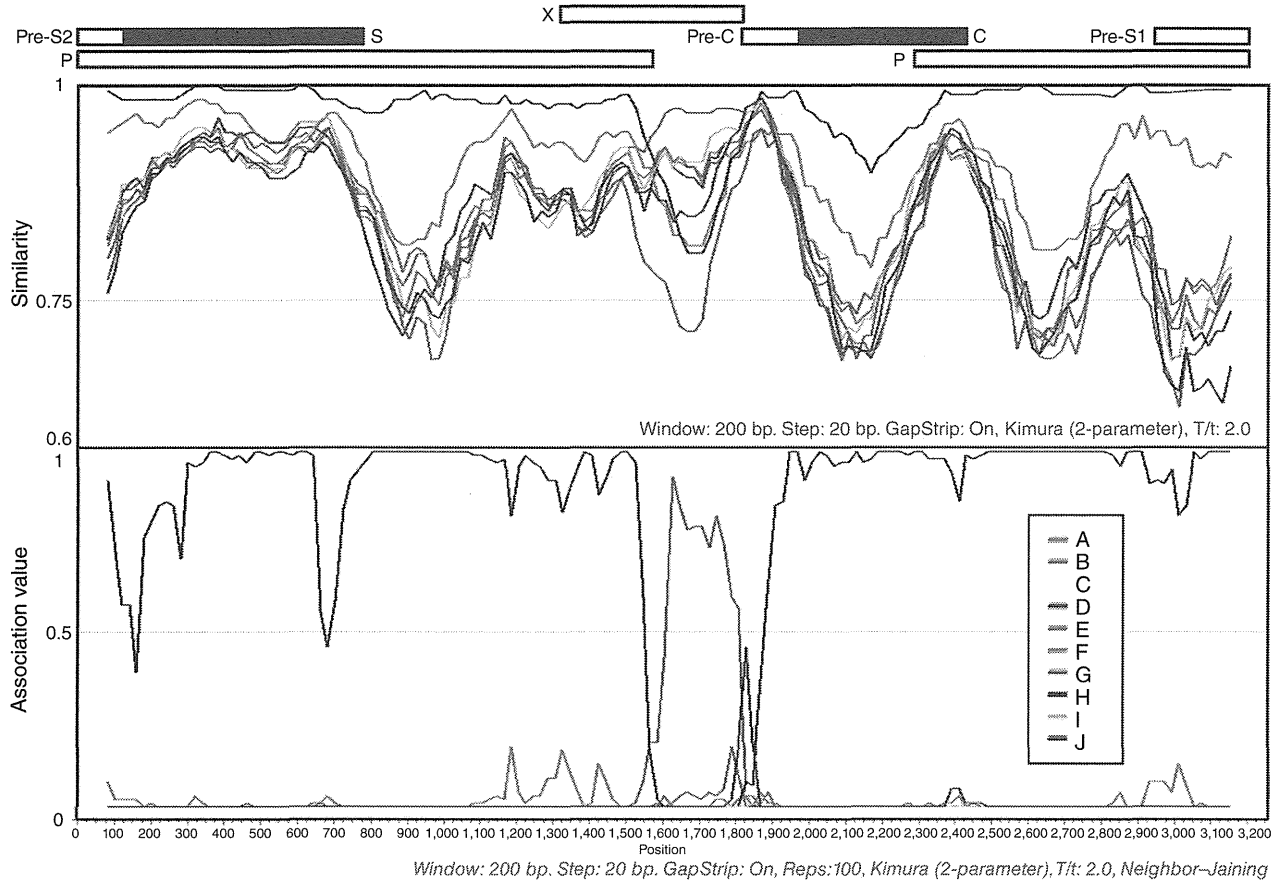

Figure 3 Nucleotide similarity comparison of the full-length sequence of the hepatitis B virus (HBV) strain isolated from case 2 in reference to previously reported HBV genotypes A–J. The parameters used for the analysis are shown at the bottom of the figure (200-bp window size, 20-bp step size, 100 bootstrap replicates, gap-stripped alignments and neighbor-joining algorithm).

were classified as genotype H according to full-length nucleotide sequence analysis, with an identity of 96.4% to 95.8% to each other, and as genotype B based on analysis of the nucleotide sequence between DR2 and DR1, with an identity of 96.9% to 95.8%, respectively. However, the nucleotide sequence between 2023 and 2262 nt in the precore/core regions showed no similarity to that of any previously reported HBV strains. In these regions, the C2-1 and C2-2 clones showed nucleotide sequences with an identity of 98.6% to each other, and the nucleotide divergences in comparison to strains of genotypes A–J ranged 9.6–30.0% in the C2-1 clone and 8.1–28.5% in the C2-2 clone (Table 1). A phylogenetic tree constructed based on these regions revealed that both strains may be classified into the novel cluster of HBV (Fig. 4b). Also, the amino acid sequence divergences from previously reported HBV strains ranged from 18.1% to 27.9% in the C2-1 clone and 17.1% to 26.9% in the C2-2 clone.

The nucleotide sequence data reported in the present study will appear in the DDBJ/EMBL/GenBank databases under accession number AB818694 for case 1, AB819065 for the C2-1 and AB819066 for the C2-2 strain.

DISCUSSION

IN THE PRESENT paper, the genotypes of the HBV strains isolated from 222 patients with acute and chronic hepatitis B were evaluated by EIA and/or PCR-INVADER assay, and HBV genotype A strains, commonly isolated in Africa, Europe and India, were found in 9.4% of the patients; genotype A strains were isolated from 50.0% of patients with acute liver diseases and 3.1% of patients with chronic liver diseases. These values were almost in line with those reported from other institutions in Japan.[11–13] HBV genotype A strains are known to be frequently isolated from patients with

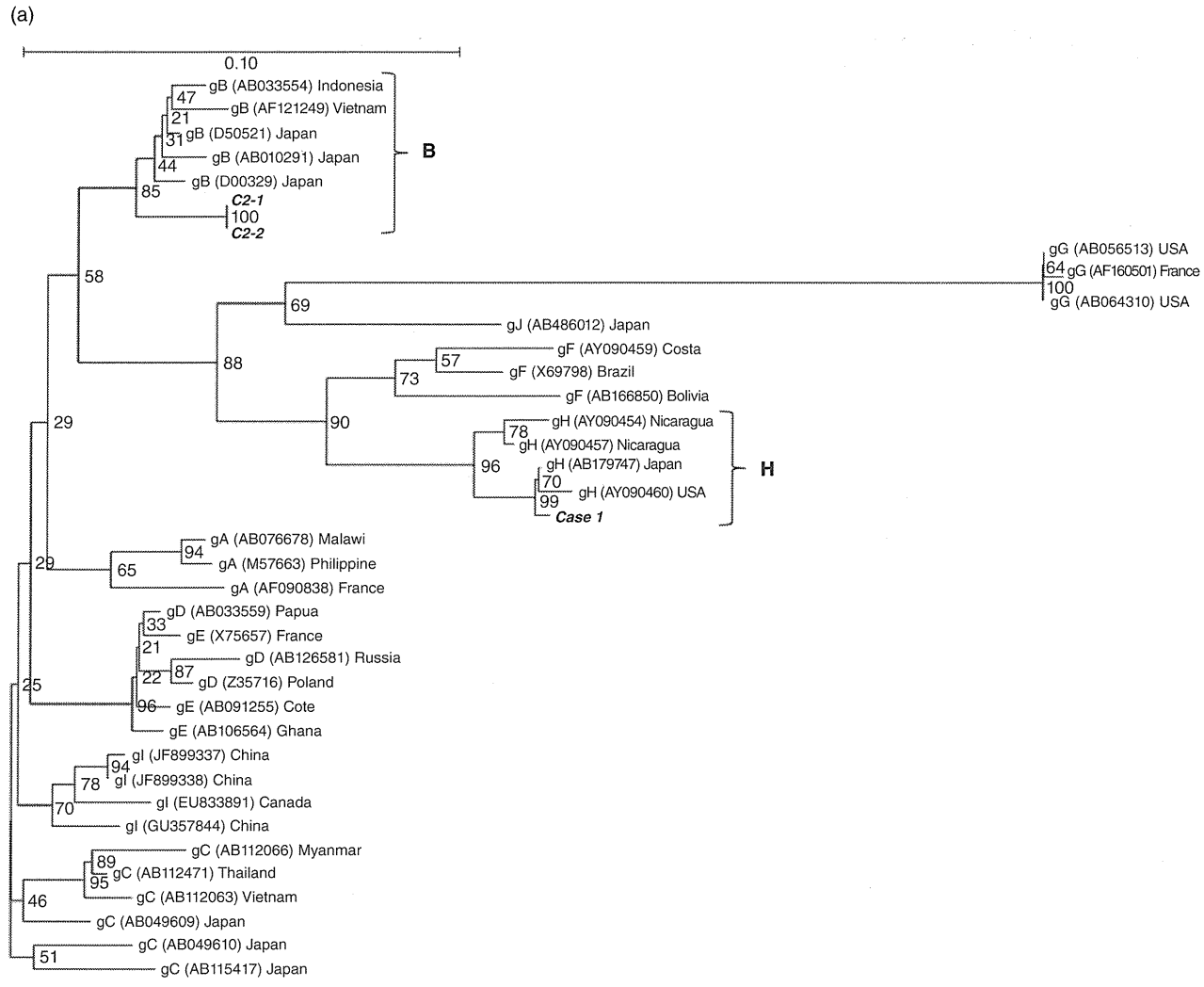

Figure 4 A phylogenetic tree constructed based on the sequence of the hepatitis B virus (HBV) strain isolated from case 2 in comparison with that of 35 reference strains. The bootstrap values are indicated at each tree root and the genotypes are on the right. The horizontal bar provides a genetic distance. The regions included in the analysis were: (a) nucleotide sequence between DR2 (1590 nt) and DR1 (1834 nt) in the X region, (b) between 2023 and 2262 nt in the precore/core region.

acute liver diseases caused by HBV, especially in urban areas as compared to the countryside,[29] suggesting that globalization and diversification of the sex industry may change the distribution pattern of the HBV genotypes in Japan, including in Saitama Prefecture, the area around our institution.

To our surprise, HBV genotype H strains, which are mainly prevalent in Central America, were isolated from two patients, one each with chronic and acute liver diseases. The HBV strain isolated from the patient with acute liver disease (case 1) showed a nucleotide sequence with 99.8% identity to the Thailand strain (EU498228), which has recently been reported to be isolated from Japan as well as Central America.[30] Considering that case 1 was a bisexual male with HIV co-infection contracted as a result of sexual activities with a number of unspecified Japanese partners, the HBV strain isolated from this patient may be resident in Japanese persons engaging in unusual sexual activities. On the other hand, HBV genotype A strains, especially the genotype A2/Ae strain, have been isolated increasingly frequently from patients with HBV and HIV co-infection.[31] These observations prompted us to postulate that HBV genotype H strains as well as genotype A

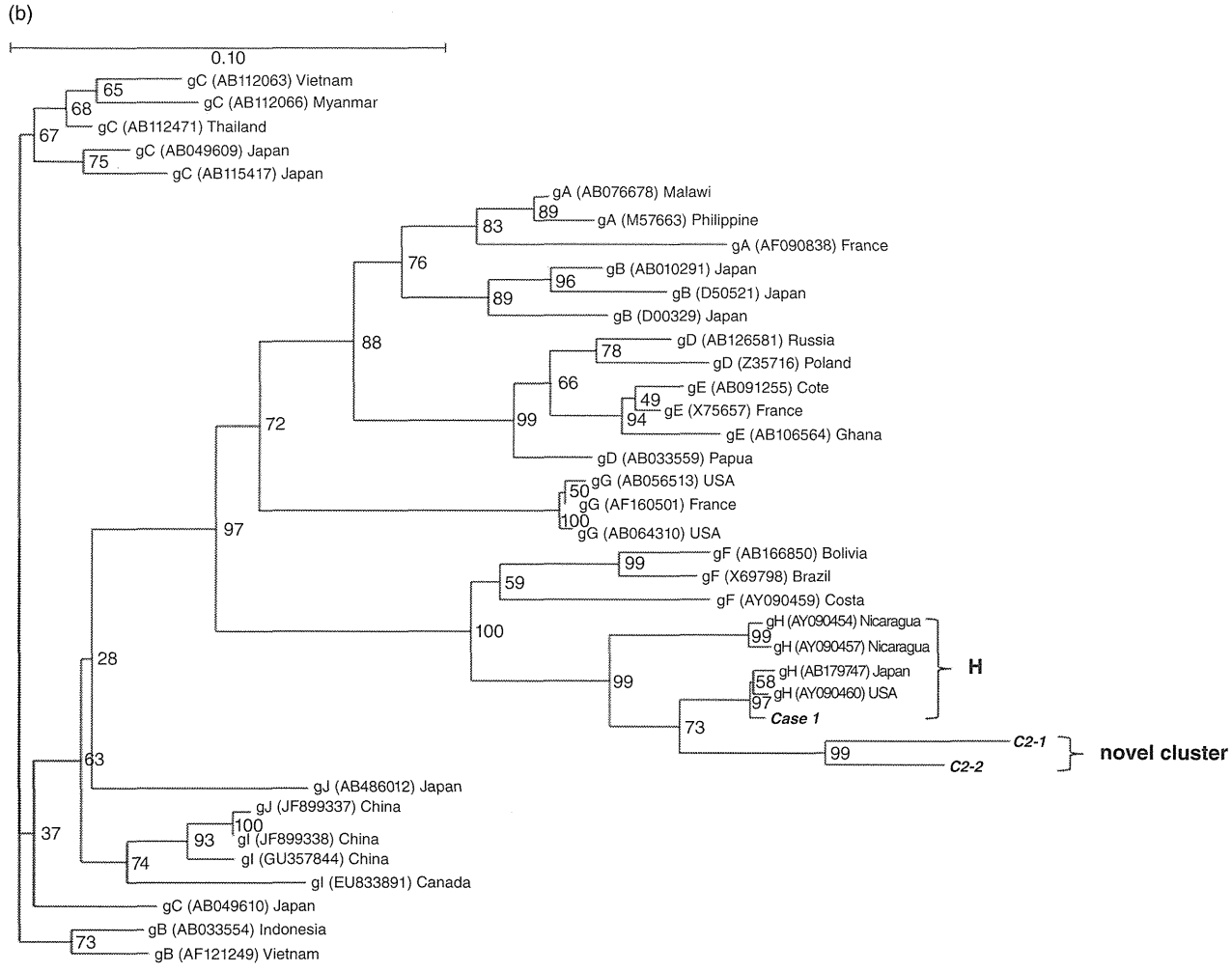

Figure 4 *Continued*

strains seem to spread among Japanese persons with unusual sexual habits. Previously, Tanaka *et al.* reported a HIV-infected patient in whom co-infection of both HBV genotype H and G strains was observed.[32] In case 1, however, co-infection of HBV genotype G strain was not detected.

It is noteworthy that HBV genotype H strains were isolated even from a Japanese patient with chronic liver disease (case 2), which showed recombination with a genotype B strain. The recombination breakpoint was estimated at positions 1590 and 1834 nt, located between DR2 and DR1 in the X region (Fig. 5): the nucleotide sequence in the X region of this strain showed an identity of 97.2% to that of genotype B strains in Malaysia (JQ027316) and Indonesia (JQ429079) despite the full-length nucleotide sequence showing 97.1% identity to a genotype H strain isolated from Mexico (AB375164). In the present study, nucleotide sequences were analyzed using two fragments (WA2 and gN2), suggesting that the possible recombination points exist in the overlapping regions of both fragments. However, the possibility that both genotypes B and H HBV strains existed as quasispecies in case 2 was neglected, because the sequences of the overlapping regions (1702–1780 and 1908–2081 nt) showed 100% identity between WA2 and gN2 fragments. It is well known that a HBV genotype B2/Ba strain, widely prevalent in Asian countries, shows nucleotide sequences identical to genotype C strains in the precore/core region due to the inter-genotype recombination

Table 1 Percentages of differences in the nucleotide and amino acid sequences of hepatitis B virus (HBV) strains isolated from case 2 (C2-1 and C2-2) and representative strains of genotypes A–J HBV

		Percentages of differences to representative HBV strains of genotypes									
		A (3)	B (5)	C (6)	D (3)	E (3)	F (3)	G (3)	H (4)	I (4)	J (1)
C1-1	Nucleotide	25.9–30.0	25.6–28.6	24.4–26.9	26.9–29.6	28.5–29.8	17.6–17.9	26.2–26.7	9.6–13.0	24.8–26.5	26.1
	Amino Acid	18.6–25.7	21.3–25.1	23.8–27.9	22.8–25.5	24.2–25.7	18.1–18.2	22.8–24.2	18.1–19.4	22.7–27.3	24.6
C2-2	Nucleotide	24.4–28.5	24.1–27.1	22.9–25.4	25.4–28.1	27.0–28.3	16.1–16.4	24.7–25.2	8.1–11.5	23.3–25.0	24.6
	Amino Acid	17.6–24.7	20.3–24.1	22.8–26.9	21.8–24.5	23.3–24.7	17.1–17.2	21.8–23.3	17.1–18.4	21.7–26.3	23.6

Values in parenthesis indicate the number of HBV strains.

between B and C strains.[33] Also, HBV strains developing as a consequence of the inter-genotype recombination between A and D, A and E, A and C, C and D, and C and G have been reported from Africa, Vietnam, Tibet and Thailand.[34–37] Moreover, recombination among HBV strains of the same genotype, the so-called intra-genotype recombination, has been proposed to occur especially in HBV genotype A, D, F and H strains.[38] However, HBV genotype H strains showing recombination with other genotype strains have not ever been reported. Considering the fact that the father of case 2 had lived in Brazil in his youth, the sequences of genotype H in case 2 strains might have originated in Brazilian strains. In Brazil, genotypes A and D HBV strains are predominantly distributed with frequencies of 49.5% and 24.3%, respectively, while genotype B HBV strains are only 2.9%.[39] Thus, the recombination event with the genotype B HBV strain might have developed following the emigration of his father to Japan. To clarify the area and era in which the recombination developed, the full-length nucleotide sequence of the HBV strain isolated from the elder brother of case 2 needs to be evaluated, but, unfortunately, the brother, receiving medical examination at another institution, rejected further viral genome analysis.

Although the mechanisms involved in the development of inter-genotype and intra-genotype recombination of the HBV genomes remains unclear, several observations reported in previous publications prompted us to postulate the "non-random pathway"; DR1 (1830 nt) in the X gene, a possible origin of viral replication, is considered to be a hot spot that may be responsible for recombination of HBV genomes among different strains.[40,41] Hino *et al.* reported, based on *in vitro* recombination assay, that HBV DNA fragments containing the region spanning DR1 increased the recombination events reproducibly in the presence of extracts from actively dividing HCC cells.[40] Also, Pineau *et al.* revealed that the integration sites of covalently closed circular HBV DNA were usually located in the nucleotide sequence between 1600 and 2000 nt, when the HBV genomes chromosomally integrated in the host genomes were evaluated in human HCC tissues.[41] These *in vitro* and *in vivo* observations were consistent with the results obtained from the analysis of the HBV strains isolated from case 2, showing that the genome of the HBV genotype B strains were integrated in that of the HBV genotype H strain between DR2 and DR1.

Hepatitis B virus strains isolated from case 2 were classified as quasispecies in accordance with the nucleotide sequence between 2023 and 2262 nt in the precore/

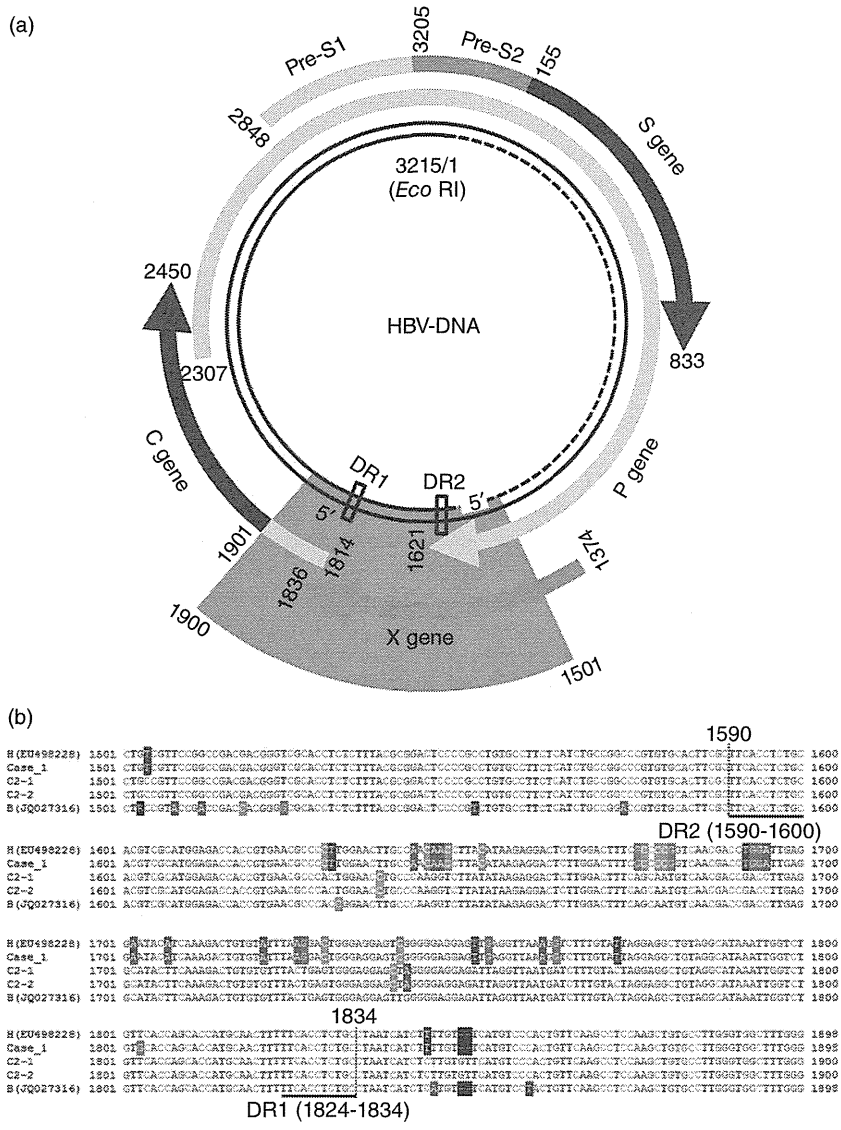

Figure 5 Hepatitis B virus (HBV) genome and the open reading frame. (a) The sequence region (shaded in red) includes the recombination breakpoint at position 1590 and 1834 nt, located between DR2 and DR1 in X region. (b) Nucleotide alignments over the sequences spanning 1501–1900 nt in case 1, C2-1, C2-2 and reference strains of HBV genotype H (accession no. EU498228) and B (JQ027316). Dashed lines at 1590 and 1834 nt represent the recombination breakpoint.

core regions. Thus, the nucleotide sequences were analyzed following cloning of the HBV genome, and two major clones, C2-1 and C2-2, were isolated. Neither clone showed any similarity to any of the previously reported strains in the precore/core regions, and a phylogenetic tree constructed based on these regions revealed that these strains may be classified into the novel cluster of HBV; sequence divergences of nucleotides in the range of 8.1–30.0% and of amino acid in the range of 17.1–27.9% as compared to previously reported genotype A–J strains. The possibility that inter-genotype recombination of the HBV genome between H and B strains may provoke mutation of the nucleotide sequence in the precore/core regions leading to development of a possible novel genotype HBV strain needs to be evaluated in the future.

In conclusion, HBV genotype H strains, which are prevalent in Central American countries, were isolated from Japanese patients with chronic as well as acute liver diseases. HBV strains isolated from the chronic liver disease patient showed recombination of the genome between genotype H and B strains, and no similarity was found in the nucleotide sequences of the precore/core regions in comparison with those of the previously reported HBV strains. Thus, globalization may promote development of a possible novel genotype of HBV through recombination between Central American and East Asian strains.

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SUPPORTING INFORMATION

ADDITIONAL SUPPORTING INFORMATION may be found in the online version of this article at the publisher's website:

Table S1 Hepatitis B virus DNA-specific oligonucleotide primers used in the study.

TITLE PAGE

Title

OSTEOPONTIN BINDING TO LIPOPOLYSACCHARIDE LOWERS TUMOR NECROSIS FACTOR-α AND PREVENTS EARLY ALCOHOL-INDUCED LIVER INJURY IN MICE

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Keywords

Alcoholic liver disease, macrophages, inflammation, steatosis, oxidative stress, bile, gut

FOOTNOTE PAGE

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List of abbreviations

ADH, alcohol dehydrogenase; ALD, alcoholic liver disease; ASH, alcoholic steatohepatitis; BSA, bovine serum albumin; CYP2E1, cytochrome P450 2E1; H&E, hematoxylin and eosin; IHC, immunohistochemistry; IOD, integrated optical density; IRS1, insulin receptor substrate-1; LBP, lipopolysaccharide binding protein; LDL, low-density lipoproteins; LPS, lipopolysaccharide; m-OPN*, biotinylated human milk osteopontin; m-OPN, milk osteopontin; OPN, osteopontin; *Opn*$^{-/-}$, osteopontin knockout mice; *Opn*^{HEP} Tg, transgenic mice overexpressing osteopontin in hepatocytes; RNS, reactive nitrogen species; rOPN, recombinant OPN; ROS, reactive oxygen species; TG, triglycerides; TLR4, toll-like receptor-4; TNFα, tumor necrosis factor-α; VLDL, very low-density lipoproteins; WT, wild-type; αSMA, α-Smooth muscle actin

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