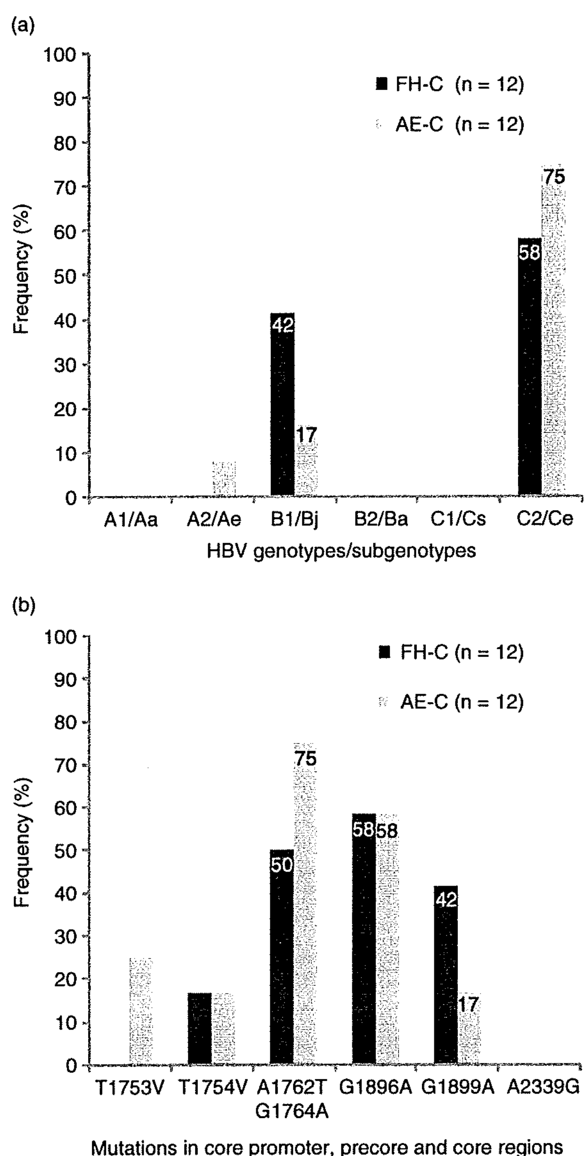

Figure 3 Genotypes/subgenotypes (a) and mutations in core promoter, pre-core and core regions (b) between the 12 transient hepatitis B virus infection (FH-T) and the 12 acute self-limited hepatitis B (AHB) patients.

HBV infection (Table 2). In particular, G1896A mutation was the most important factor associated with the development of FHB. Host responses, represented by T.bil, contributed to the development of FHB as well.

As for HBV genotypes, B1/Bj alone was significantly more frequent in the FH-T patients in univariate analysis. In the patients infected with B1/Bj, G1896A was more frequent in those with FH-T than AHB. In *in vitro* replication analysis, Ozasa *et al.*[15] observed extremely high expressions of intra- and extracellular HBV DNA in culture transfected with an HBV clone of B1/Bj genotype having the G1896A mutation; a high replication would be induced by this pre-core mutation for the induction of FHB. Our clinical results stand in support of this *in vitro* analysis. Taken altogether, chances for developing severe acute or FH would be high in the patients with acute hepatitis who are infected with HBV/B1 having the pre-core mutation. By contrast, in patients infected with C2/Ce, G1896A or A1762T/G1764A, or both was much more frequent in the FH-T patients than AHB. Of note, the co-occurrence of G1896A and A1762T/G1764A mutations was invariably accompanied by either FHB or acute severe hepatitis B in this study. Hence, these pre-core and core-promoter mutations might have addictive or synergetic effects for exacerbating hepatitis, when they emerge in the patients infected with C2/Ce. Such high-risk patients deserve special care and surveillance for signs and symptoms of fulminant or severe acute hepatitis B.

In the present study, serum levels of HBV DNA were significantly higher in the patients with FH-T than AHB. High serum levels of HBV DNA have been reported in patients with FHB;[39] they are followed by rapid decrease as the sequel of virus elimination operated by vigorous immune responses. Because of rapid and extensive elimination of HBV by the host immune system, HBV DNA in serum, in general, has decreased to low levels in patients with FHB at the presentation.[40] HBV DNA levels may be subject to the time that has elapsed from the onset of hepatitis to its measurement.[39] Also, serum levels of core protein (the product of the C gene) closely correlate with serum HBV DNA levels in patients with hepatitis B,[27] and they were compared between the FH-T patients and AHB. The core protein was determined by the newly developed CLEIA method; it is much easier and less expensive than the determination of HBV DNA. The level of core protein has turned out to be marginally higher in the FH-T patients than AHB (Table 1), and therefore might not contribute to an early diagnosis of FHB by transient infection.

Fulminant hepatitis B by AE of ASC is assumed as a different clinical condition from FHB by transient HBV infection. In this study, as there was no case-control study on virological factors associated with FHB for the patients with AE of ASC, we also attempted to identify virological factors associated with the development of FHB in the 12 FH-C and the 12 AE-C patients who were

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matched for age as well as sex. Disappointingly, no differences of virological factors such as HBV genotypes and pre-core mutations, which were strongly associated with the development of FHB by transient infection, were found between the FH-C and AE-C patients (Fig. 3a,b). Furthermore, there were also no significant differences about HBeAg-positive rate and the levels of serum HBV DNA or core protein (Table 3), suggesting that several host factors may play a more important role in the development of FHB in ASC instead of virological factors. In this case-control study, however, there seems to be some problems: a small number of patients, different duration of HBV infection, different clinical stage (ASC or CHB) at the onset of AE, and HBV quasispecies complexity. Further investigations are needed to identify factors associated with FHB precipitating in asymptomatic HBV carriers.

In conclusion, virological factors associated with enhancement of viral replication seemed to be important for the development of FHB in the patients by transient HBV infection. But no virological factors were identified for differentiation of the FH-C patients from the AE-C patients. Hence, the pathogenic mechanism of FHB between transient HBV infection and AE of ASC would be different.

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REFERENCES

1 Fujiwara K, Mochida S, Matsui A, Nakayama N, Nagoshi S, Toda G. Fulminant hepatitis and late onset hepatic failure in Japan. *Hepatol Res* 2008; **38**: 646–57.
2 Norder H, Hammas B, Lofdahl S, Courouce AM, Magnius LO. Comparison of the amino acid sequences of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hepatitis B virus strains. *J Gen Virol* 1992; **73** (Pt 5): 1201–8.
3 Okamoto H, Tsuda F, Sakugawa H *et al.* Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J Gen Virol* 1988; **69**: 2575–83.
4 Stuyver L, De Gendt S, Van Geyt C *et al.* A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J Gen Virol* 2000; **81** (Pt 1): 67–74.
5 Arauz-Ruiz P, Norder H, Robertson BH, Magnius LO. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *J Gen Virol* 2002; **83** (Pt 8): 2059–73.
6 Miyakawa Y, Mizokami M. Classifying hepatitis B virus genotypes. *Intervirology* 2003; **46**: 329–38.
7 Chu CJ, Lok AS. Clinical significance of hepatitis B virus genotypes. *Hepatology* 2002; **35**: 1274–6.
8 Tanaka Y, Hasegawa I, Kato T *et al.* A case-control study for differences among hepatitis B virus infections of genotypes A (subtypes Aa and Ae) and D. *Hepatology* 2004; **40**: 747–55.
9 Sugauchi F, Orito E, Ichida T *et al.* Hepatitis B virus of genotype B with or without recombination with genotype C over the pre-core region plus the core gene. *J Virol* 2002; **76**: 5985–92.
10 Huy TT, Ushijima H, Quang VX *et al.* Genotype C of hepatitis B virus can be classified into at least two subgroups. *J Gen Virol* 2004; **85** (Pt 2): 283–92.
11 Tanaka Y, Orito E, Yuen MF *et al.* Two subtypes (subgenotypes) of hepatitis B virus genotype C: a novel subtyping assay based on restriction fragment length polymorphism. *Hepatol Res* 2005; **33**: 216–24.
12 Lindh M, Andersson AS, Gusdal A. Genotypes, nt 1858 variants, and geographic origin of hepatitis B virus–large-scale analysis using a new genotyping method. *J Infect Dis* 1997; **175**: 1285–93.
13 Sato S, Suzuki K, Akahane Y *et al.* Hepatitis B virus strains with mutations in the core promoter in patients with fulminant hepatitis. *Ann Intern Med* 1995; **122**: 241–8.
14 Omata M, Ehata T, Yokosuka O, Hosoda K, Ohto M. Mutations in the pre-core region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. *N Engl J Med* 1991; **324**: 1699–704.
15 Ozasa A, Tanaka Y, Orito E *et al.* Influence of genotypes and pre-core mutations on fulminant or chronic outcome of acute hepatitis B virus infection. *Hepatology* 2006; **44**: 326–34.
16 Liang TJ, Hasegawa K, Rimon N, Wands JR, Ben-Porath E. A hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. *N Engl J Med* 1991; **324**: 1705–9.
17 Imamura T, Yokosuka O, Kurihara T *et al.* Distribution of hepatitis B viral genotypes and mutations in the core promoter and pre-core regions in acute forms of liver disease in patients from Chiba, Japan. *Gut* 2003; **52**: 1630–7.
18 Sugiyama M, Tanaka Y, Kurbanov F, Nakayama N, Mochida S, Mizokami M. Influences on hepatitis B virus replication by a naturally occurring mutation in the core gene. *Virology* 2007; **365**: 285–91.

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19 Laskus T, Persing DH, Nowicki MJ, Mosley JW, Rakela J. Nucleotide sequence analysis of the pre-core region in patients with fulminant hepatitis B in the United States. *Gastroenterology* 1993; **105**: 1173–8.

20 Liang TJ, Hasegawa K, Munoz SJ *et al.* Hepatitis B virus pre-core mutation and fulminant hepatitis in the United States. A polymerase chain reaction-based assay for the detection of specific mutation. *J Clin Invest* 1994; **93**: 550–5.

21 Feray C, Gigou M, Samuel D, Bernuau J, Bismuth H, Brechot C. Low prevalence of pre-core mutations in hepatitis B virus DNA in fulminant hepatitis type B in France. *J Hepatol* 1993; **18**: 119–22.

22 Karayiannis P, Alexopoulou A, Hadziyannis S *et al.* Fulminant hepatitis associated with hepatitis B virus e antigen-negative infection: importance of host factors. *Hepatology* 1995; **22**: 1628–34.

23 Trey C, Lipworth L, Chalmers TC *et al.* Fulminant hepatic failure. Presumable contribution to halothane. *N Engl J Med* 1968; **279**: 798–801.

24 Ng HJ, Lim LC. Fulminant hepatitis B virus reactivation with concomitant listeriosis after fludarabine and rituximab therapy: case report. *Ann Hematol* 2001; **80**: 549–52.

25 Fujiwara K, Mochida S, Matsui A. [Prospective study for the efficiency of lamivudine for the patients with acute exacerbation of HBV carrier.] *Annual Report of Intractable Liver Disease Study Group of Japan, the Ministry of Health, Welfare and Labor* 2004. (In Japanese.)

26 Kimura T, Rokuhara A, Sakamoto Y *et al.* Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. *J Clin Microbiol* 2002; **40**: 439–45.

27 Kimura T, Rokuhara A, Matsumoto A *et al.* New enzyme immunoassay for detection of hepatitis B virus core antigen (HBcAg) and relation between levels of HBcAg and HBV DNA. *J Clin Microbiol* 2003; **41**: 1901–6.

28 Abe A, Inoue K, Tanaka T *et al.* Quantitation of hepatitis B virus genomic DNA by real-time detection PCR. *J Clin Microbiol* 1999; **37**: 2899–903.

29 Sugauchi F, Mizokami M, Orito E *et al.* A novel variant genotype C of hepatitis B virus identified in isolates from Australian Aborigines: complete genome sequence and phylogenetic relatedness. *J Gen Virol* 2001; **82** (Pt 4): 883–92.

30 Shin IT, Tanaka Y, Tateno Y, Mizokami M. Development and public release of a comprehensive hepatitis virus database. *Hepatol Res* 2008; **38**: 234–43.

31 Chisari FV, Ferrari C Hepatitis B virus immunopathogenesis. *Annu Rev Immunol* 1995; **13**: 29–60.

32 Carman WF, Jacyna MR, Hadziyannis S *et al.* Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. *Lancet* 1989; **2** (8663): 588–91.

33 Buckwold VE, Xu Z, Chen M, Yen TS, Ou JH. Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on pre-core gene expression and viral replication. *J Virol* 1996; **70**: 5845–51.

34 Okamoto H, Tsuda F, Akahane Y *et al.* Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. *J Virol* 1994; **68**: 8102–10.

35 Lamberts C, Nassal M, Velhagen I, Zentgraf H, Schroder CH. Precore-mediated inhibition of hepatitis B virus progeny DNA synthesis. *J Virol* 1993; **67**: 3756–62.

36 Chen MT, Billaud JN, Sallberg M *et al.* A function of the hepatitis B virus pre-core protein is to regulate the immune response to the core antigen. *Proc Natl Acad Sci USA* 2004; **101**: 14913–8.

37 Chen M, Sallberg M, Hughes J *et al.* Immune tolerance split between hepatitis B virus pre-core and core proteins. *J Virol* 2005; **79**: 3016–27.

38 Bocharov G, Ludewig B, Bertoletti A *et al.* Underwhelming the immune response: effect of slow virus growth on CD8+-T-lymphocyte responses. *J Virol* 2004; **78**: 2247–54.

39 Sainokami S, Abe K, Sato A *et al.* Initial load of hepatitis B virus (HBV), its changing profile, and pre-core/core promoter mutations correlate with the severity and outcome of acute HBV infection. *J Gastroenterol* 2007; **42**: 241–9.

40 Tassopoulos NC, Papaevangelou GJ, Roumeliotou-Karayannis A, Ticehurst JR, Feinstone SM, Purcell RH. Search for hepatitis B virus DNA in sera from patients with acute type B or non-A, non-B hepatitis. *J Hepatol* 1986; **2**: 410–8.

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A Genetic Variant of Hepatitis B Virus Divergent from Known Human and Ape Genotypes Isolated from a Japanese Patient and Provisionally Assigned to New Genotype J▽†

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Hepatitis B virus (HBV) of a novel genotype (J) was recovered from an 88-year-old Japanese patient with hepatocellular carcinoma who had a history of residing in Borneo during the World War II. It was divergent from eight human (A to H) and four ape (chimpanzee, gorilla, gibbon, and orangutan) HBV genotypes, as well as from a recently proposed ninth human genotype I, by 9.9 to 16.5% of the entire genomic sequence and did not have evidence of recombination with any of the nine human genotypes and four nonhuman genotypes. Based on a comparison of the entire nucleotide sequence against 1,440 HBV isolates reported, HBV/J was nearest to the gibbon and orangutan genotypes (mean divergences of 10.9 and 10.7%, respectively). Based on a comparison of four open reading frames, HBV/J was closer to gibbon/orangutan genotypes than to human genotypes in the P and large S genes and closest to Australian aboriginal strains (HBV/C4) and orangutan-derived strains in the S gene, whereas it was closer to human than ape genotypes in the C gene. HBV/J shared a deletion of 33 nucleotides at the start of preS1 region with C4 and gibbon genotypes, had an S-gene sequence similar to that of C4, and expressed the *ayw* subtype. Efficient infection, replication, and antigen expression by HBV/J were experimentally established in two chimeric mice with the liver repopulated for human hepatocytes. The HBV DNA sequence recovered from infected mice was identical to that in the inoculum. Since HBV/J is positioned phylogenetically in between human and ape genotypes, it may help to trace the origin of HBV and merits further epidemiological surveys.

Worldwide, an estimated 400 million people are infected with hepatitis B virus (HBV) persistently, of whom three quarters live in the Southeast and Far East Asia, and one million die of decompensated cirrhosis and/or hepatocellular carcinoma (HCC) annually (8, 15). HBV is the smallest animal DNA virus and has a genome made of approximately 3,200 nucleotides (nt) that contains four open reading frames for P, C, S, and X genes; they code for DNA polymerase/reverse-transcriptase, core protein, surface protein, and X protein, respectively (49). The S gene is divided into preS1 and preS2 regions and the small S gene, and the C gene splits into PreC and C.

Eight genotypes of HBV have been recognized by a sequence divergence of >8% in the entire genome and named by capital alphabet letters (A to H) in the order of discovery (3, 26, 29, 42). HBV genotypes are further classified into subgenotypes, such as B1/Bj and B2-5/Ba (44), as well as C1/Cs, C2/Ce, and C3-5 (36). A systematic nomenclature is proposed for designating HBV subgenotypes using Arabic numbers, such as A1, A2, and A3 (25). HBV genotypes have distinct geographical distribution (16, 23). Genotype A is prevalent in Africa, Europe and India, genotypes B and C are common in Asia, and genotype E is common in sub-Saharan Africa. Genotypes F and H are restricted to Central and South American continents, whereas genotype D is distributed all over the world. HBV genotypes have clinical application, and they influence severity and progression of liver disease and the response to antiviral therapies. Previous reports indicate that HCC is more frequent in the patients infected with genotype C than B (7, 47), and interferon is more effective in those infected with genotype B than C in Asia and more effective in those infected with genotype A than D in Europe (18, 34, 51).

Recently, a ninth genotype (I) was tentatively proposed for HBV strains detected in Laos (31). These strains are phylogenetically similar to aberrant Vietnamese strains that display complex recombination over the genome (10). In the present study, an HBV isolate was recovered from a Japanese patient with HCC, who was involved in military actions in Borneo during the World War II. The isolated strain was compared against eight human (A to H) and four ape (chimpanzee, gorilla, gibbon, and orangutan) genotypes and was provisionally designated genotype J. The new genotype was assigned based on a sequence diver-

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TABLE 1. Nucleotide divergence in the full-genome sequence estimated from pairwise comparison between the Ryukyu 34 strain of a provisional genotype J and 1,440 HBV strains from the database entered by September 2008

Genotype	No. of strains	Divergence (%)		
		Range	Mean	SD
A	202	12.1–15.9	13.0	0.4
B	309	11.1–13.6	11.9	0.5
C	396	11.2–13.1	11.9	0.5
D	264	12.6–15.0	13.4	0.2
E	90	12.3–13.4	12.7	0.3
F	56	15.2–16.5	15.6	0.2
G	23	12.8–14.6	13.7	0.3
H	21	15.4–16.3	15.7	0.3
I	16	11.4–12.0	11.7	0.2
Chimpanzee	14	11.6–12.7	12.1	0.3
Gorilla	1	12.2		
Gibbon	34	9.9–11.7	10.9	0.5
Orangutan	12	10.4–11.2	10.7	0.4
Woolly monkey	2	27.2–27.4	27.3	0.1

gence of 10.7 to 15.7% from other genotypes, a unique phylogenetic position between human and ape genotypes, and the absence of strong evidence of recombination.

MATERIALS AND METHODS

Patient. A Japanese man, 88 years old, developed HCC in 2006. He had a history of residing in Borneo during the World War II. No HBV infections were recorded in his family members. In October 1996, he was diagnosed with chronic hepatitis B. Hepatitis B surface antigen (HBsAg) was detected in serum, and the aspartate aminotransaminase and alanine aminotransferase levels were elevated to 83 and 73 U/liter, respectively (normal levels, <30 U/liter for both). Thereafter, the transaminase levels were normalized, and he had been monitored as an asymptomatic HBV carrier. In August 2000, the level of a tumor marker (des-γ-carboxy prothrombin) was elevated to 52 mAU/ml (normal, <40 mAU/ml), while another tumor marker (alpha-fetoprotein) remained within normal range (<10 ng/ml) as alanine aminotransferases. In October 2006, a tumor (4.3 by 4.1 cm) was detected in the liver by ultrasonography, and he received treatment with transarterial embolization. Des-γ-carboxy prothrombin was elevated to 419 mAU/ml, while the aminotransferase levels remained within normal limits. Hepatitis B e antigen (HBeAg) was negative, and the corresponding antibody (anti-HBe) was detected in his serum. The subtype of HBsAg in this serum was *ayw*.

HBV DNA was extracted from his serum specimen obtained in 2006, and the full-length genome sequence was determined for phylogenetic and biological analyses. An informed consent had been obtained from the patient, and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

Markers of HBV infection. HBeAg and anti-HBe were determined by enzyme-linked immunosorbent assay (ELISA) with commercial kits (HBeAg EIA; Institute of Immunology, Tokyo, Japan), and subtypes of HBsAg by ELISA with commercial kits (HBsAg Subtype EIA; Institute of Immunology). Hepatitis B core-related antigen (HBcrAg) was determined by chemiluminescence enzyme immunoassay (13). The method allows more sensitive detection of core protein and, as was shown in previous studies, HBcrAg levels reflect HBV DNA loads and well correlate with intrahepatic covalently closed circular DNA (cccDNA) levels. The measurement of serum HBcrAg is a useful noninvasive tool for monitoring intrahepatic HBV viral status (52). HBV DNA was quantified by the S gene-targeted real-time detection PCR with a sensitivity of 100 copies/ml (equivalent to 20 IU/ml) (1). However, due to small volumes of sera available from the challenged mice, HBV DNA was extracted from 10-fold-diluted specimens, resulting in reduced assay sensitivity in the present study (1,000 copies/ml [200 IU/ml]).

Determination of the complete nucleotide sequence of HBV/J isolate. HBV DNA was extracted by using the QIAamp DNA blood kit (Qiagen, GmbH, Hilden, Germany) from 100 μl of serum that had been stored at −80°C. The complete genome sequence of an HBV/J isolate recovered from the patient was determined by the strategy previously reported (43). In brief, two sets of primers were designed to amplify overlapping fragments (A and B) covering the entire HBV genome (stat not shown). Nested PCR was carried out for 35 cycles (95°C, 30 s; 57°C, 30 s; and 72°C, 2 min) using TaKaRa LA *Taq* polymerase (Takara Biochemicals, Kyoto, Japan). Amplified fragments were inserted into the pGEM-T Easy vector (Promega, Madison, WI), and cloned in DH5a cells (Toyobo, Osaka, Japan). Obtained HBV DNA clones were confirmed to have the sequence identical to the major-clone consensus sequence determined directly on PCR products by Prism BigDye (Applied Biosystems, Foster City, CA) in the ABI 3100 automated sequencer.

Phylogenetic analysis. Full-length sequences of HBV isolates were aligned with use of the CLUSTAL W software program (48) (available at www.ebi.ac.uk), and the alignment was confirmed by visual inspection. Genetic distances were estimated by the six-parameter method, and phylogenetic trees were constructed with the neighbor-joining method (35). To confirm the reliability of phylogenetic trees, bootstrap resampling and reconstruction were carried out 1,000 times using the program

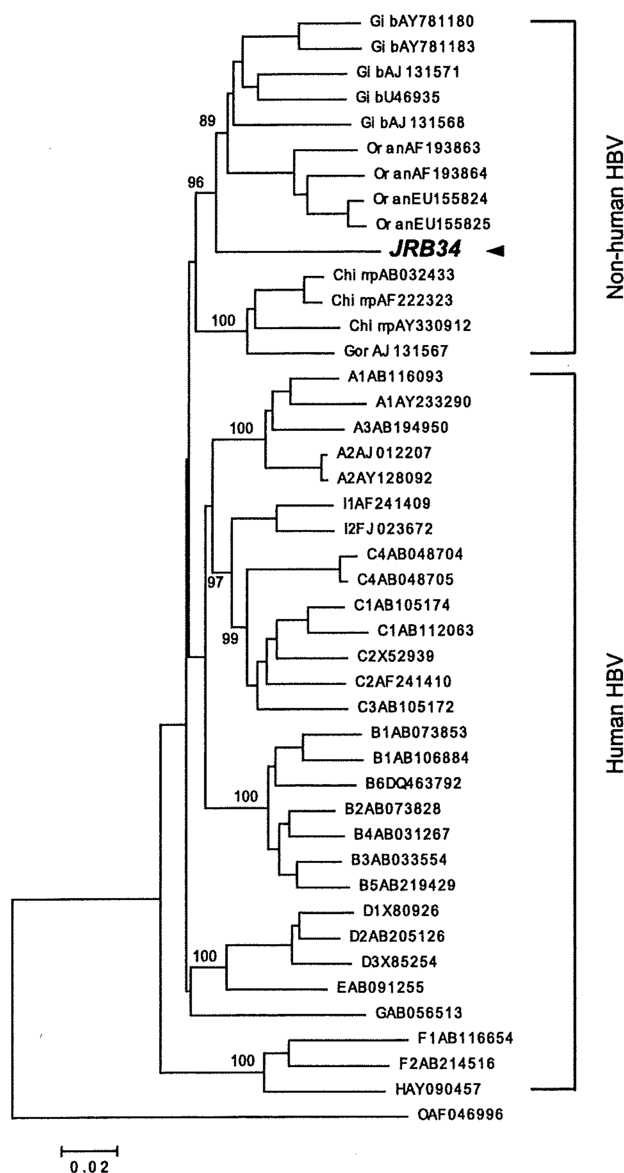

FIG. 1. Phylogenetic tree constructed on the entire genome sequences of 44 HBV isolates representing four ape and eight human genotypes. A woolly monkey HBV isolate serves as an outgroup. The HBV/J isolate (JRB34) is indicated by an arrowhead, and the genetic distance is indicated by a bar below.

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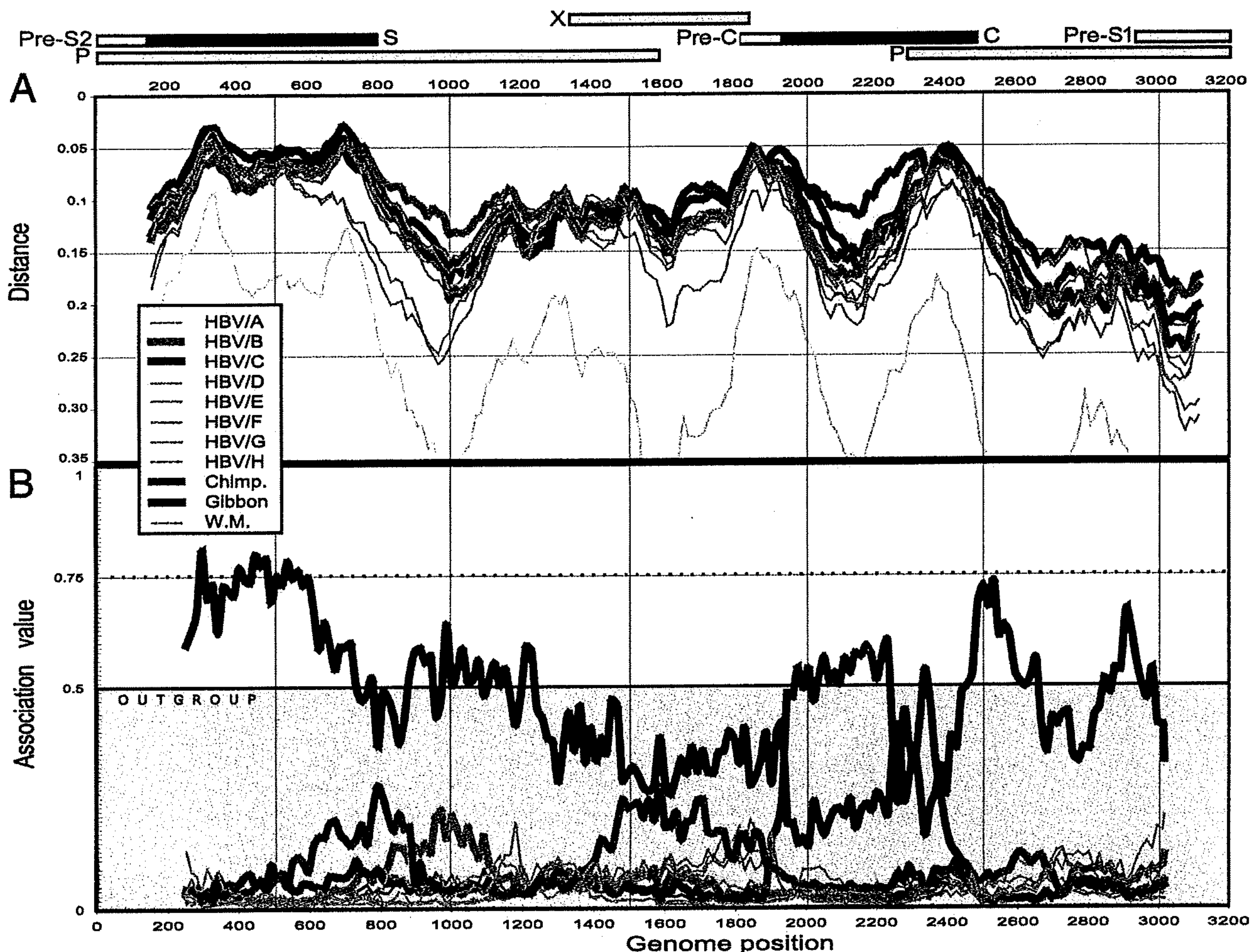

FIG. 2. Complete genome scanning carried by PHYLIP, the phylogeny inference package implemented in the Simmonic software, for the JRB34 strain versus 228 selected nonrecombinant HBV genotypes (HBV/Ba and HBV/I not included) reference strains grouped by genotype. Kimura two-parameter distance model (A) and grouping scan (B) were determined with a 300-nt size window sliding by an increment of 15 nucleotides. The x axis indicates the genome position (corresponding to the midpoint of the scanning fragment), and the y axis indicates the mean distances between JRB34 and reference groups (A). Phylogenetic association (y axis) was evaluated throughout entire HBV genome (x axis) with the same window and step size parameters (B). The association value below 0.5 was considered to represent an outgroup. The open reading frame map is shown schematically at the top of the figure.

of the Hepatitis Virus Database (39). All 1,440 complete genomes available in the DDBJ/GenBank served as references for the initial alignment in the present study. Divergence in the nucleotide sequence between a strain of provisional genotype J and previously reported strains was estimated by using MEGALIGN v.6.00 (Lazergene package; DNASTAR, Inc., Madison, WI).

Examination of recombination evidence. Evidence of possible recombination was investigated by using the software packages Simmonic 2005 v1.6 and SimPlot v3.5.1, both implementing PHYLIP (Phylogeny Inference Package v3.68; J. Felsenstein, Department of Genome Sciences, University of Washington, Seattle [distributed by the authors]) (19, 40).

Inoculation of chimeric mice with the liver repopulated for human hepatocytes. Severe combined immunodeficiency mice transgenic for the urokinase-type plasminogen activator gene ($uPA^{+/+}/SCID^{+/+}$ mice) with the liver repopulated with human hepatocytes (chimeric mice) were purchased from Phoenix Bio Co., Ltd. (Hiroshima, Japan). Human serum albumin was measured by ELISA with commercial assay kits (Eiken Chemical Co., Ltd., Tokyo, Japan) for estimating the extent of repopulation. The research complied with all relevant federal guidelines and institutional policies.

Immunofluorescence. Freshly prepared liver tissues were snap-frozen in isopentane precooled in liquid nitrogen. Frozen specimens were cut at 5 to 6 μm by cryostat, mounted on glass slides, air dried, and fixed in 100% acetone at room temperature for 10 min. Sections were blocked with antibody diluent (Dako, Tokyo, Japan) and stained for hepatitis B core antigen (HBcAg). They were incubated with rabbit anti-HBc (Dako) at room temperature for 1 h, washed in phosphate-buffered saline, and then incubated with goat anti-rabbit immunoglobulin G conjugated with Cy3 (Chemicon International, Inc., Temecula, CA) or goat anti-human albumin antibody labeled with fluorescein isothiocyanate (Bethyl Laboratories, Inc., Montgomery, TX). Sections were washed with phosphate-buffered saline and observed in a fluorescence microscope (Eclipse E800M; Nikon, Tokyo, Japan).

Nucleotide sequence accession numbers. The nucleotide sequence data reported in the present study will appear in the DDBJ/EMBL/GenBank databases under accession no. AB486012.

RESULTS

Composition of the HBV genome of genotype J. HBV DNA was extracted from serum of a patient with HCC. It was named JRB34 ("J" for Japanese; "R" after the southernmost island [Ryukyu] where the patient has spent most of his life now

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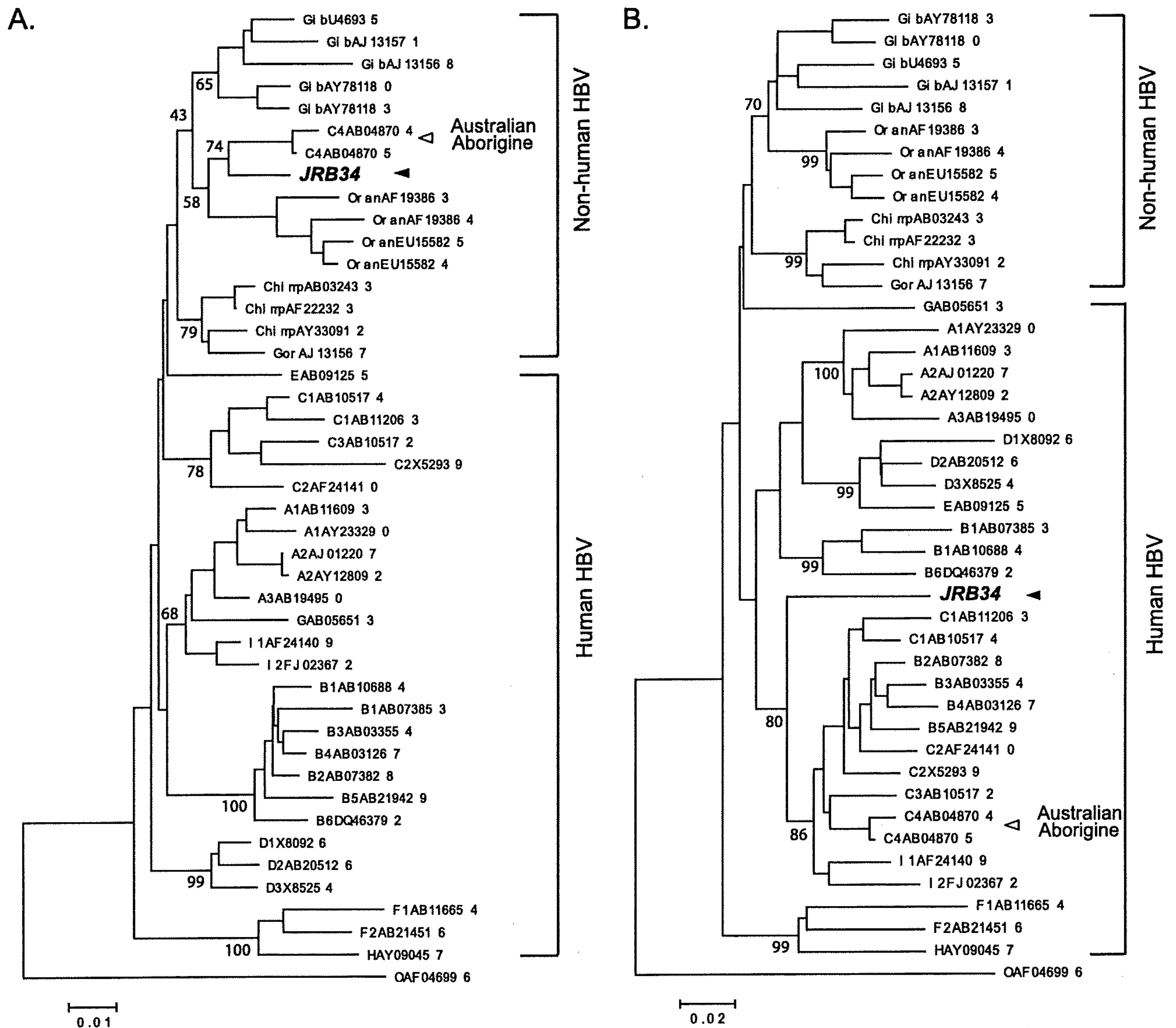

FIG. 3. Phylogenetic tree constructed on the preS/S gene (A) and C gene (B) sequences of 44 HBV isolates representing four ape and eight human genotypes. A woolly monkey HBV isolate serves as an outgroup. The HBV/J isolate (JRB34) is indicated by an arrowhead, and an HBVC4 isolate from Australian aborigine is indicated by an open triangle. The genetic distance is indicated by a bar below.

exceeding 90 years; and "B" for Borneo where he is suspected to have contracted the HBV infection). The entire nucleotide sequence was determined for the JRB34 isolate of genotype J (HBV/J). It had a genomic length of 3,182 nt, which consisted of envelope gene containing preS1 region (nt 2848 to 3171, coding for 108 amino acids [aa]), preS2 region (nt 3172 to 154 [55 aa]), and the small S gene (nt 155 to 835 [226 aa]), X gene (nt 1374 to 1838 [154 aa]), preC region (nt 1814 to 1897 [27 aa]), C gene (nt 1901 to 2452 [183 aa]), and P gene (nt 2307 to 1623 [832 aa]).

Sequence divergence of the JRB34 strain from other genotypes. The complete genome sequence of the JRB34 strain obtained in the present study was compared against those of 1,440 HBV genomes registered in the Viral Hepatitis Database (39). Estimated nucleotide sequence divergence of the JRB34 strain from four ape and nine human genotypes is summarized in the Table 1. The mean divergence by genotypes ranged from 10.7 and 10.9% (from orangutan and gibbon, respectively) to 15.6 and 15.7% (from genotypes F and H, respectively). Surprisingly, the minimum divergence of 9.9% was observed in comparison with a nonhuman HBV isolate from *Hilobates agilis* gibbon confiscated in Taiwan in 1993 (AY330917) (41). Since the sequence divergence from any documented genotypes, including recently proposed genotype I, exceeded 8%, the JRB34 strain was tentatively classified into a novel genotype J of HBV.

Phylogenetic analysis of the entire genomic sequence. In the phylogenetic tree constructed on 1,440 complete genome

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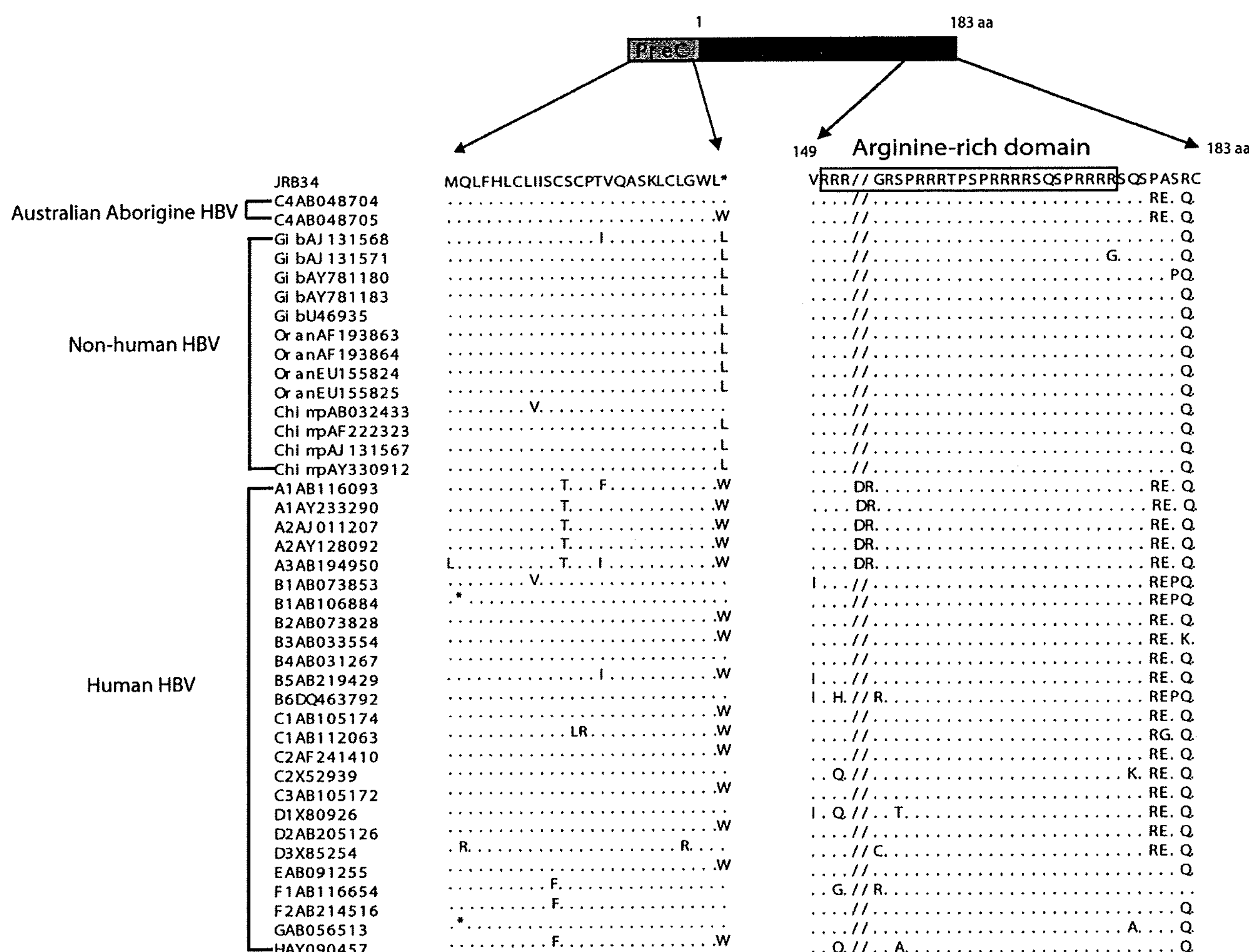

FIG. 4. Comparison of the amino acid sequence in the preC gene and carboxy-terminal amino acid sequences in the C gene of HBV isolates of various genotypes. The sequence of the HBV/J isolate (JRB34) is indicated at the top. Dots represent amino acids shared by JRB34, and a dash indicates the deletion of an amino acid. The sequence of the arginine-rich domain bearing the binding site with HBV DNA is boxed.

EMBL/DDBJ/GenBank database entries, the HBV/J strain was positioned distinctively from all known human genotypes (data not shown). It was closest to the cluster formed by gibbon- and orangutan-derived strains. However, including recombinant strains in such analyses may significantly affect the overall phylogenetic topology. This possibility was ruled out by reconstruction of the phylogeny using nonrecombinant HBV strains that further confirmed the phylogenetic peculiarity of the studied JRB34 strain (see Fig. S1 in the supplemental material). A total of 44 representative reference strains were further selected for establishing the consistency. Thus, phylogenetic topology indicating genotype-specific clustering is shown in the Fig. 1. Hence, using various sets of references, we confirmed that genotype J undoubtedly differed phylogenetically from all other known genotypes.

Lack of significant evidence of recombination with other human or ape genotypes in genotype J. To investigate possible recombination in the JRB34 genome, a window scanning analysis of aligned HBV genomes was performed by means of Simplot and Simmonics software packages. Both Bootscanning by SimPlot and GroupScanning by Simmonics showed similar output results. However, the methodological approach is different between these two software packages; GroupScanning provides more robust analysis of the phylogenetic relation between the examined strain and clusters of reference strains, whereas SimPlot does this comparison between the examined strain and parametrically generated consensus of the reference strains. The results obtained by SimPlot therefore can be significantly affected by selected parameters for the generation of consensus. This is especially undesirable when a new genotype strain (for which no references are available among known genotypes) is being analyzed (40). Figure 2 shows genome-wide distance scanning and GroupScanning plots for the JRB34 strain in comparison with a reference set consisting of 228 nonrecombinant HBV isolates retrieved from the public database (the phylogenetic tree is shown in Fig. S1 in the supplemental material). It is evident that the JRB34 strain was divergent from all known genotypes, and the closest genetic neighbors were estimated by distance and phylogenetic association scanning were the gibbon genotype (in preS, S, and P

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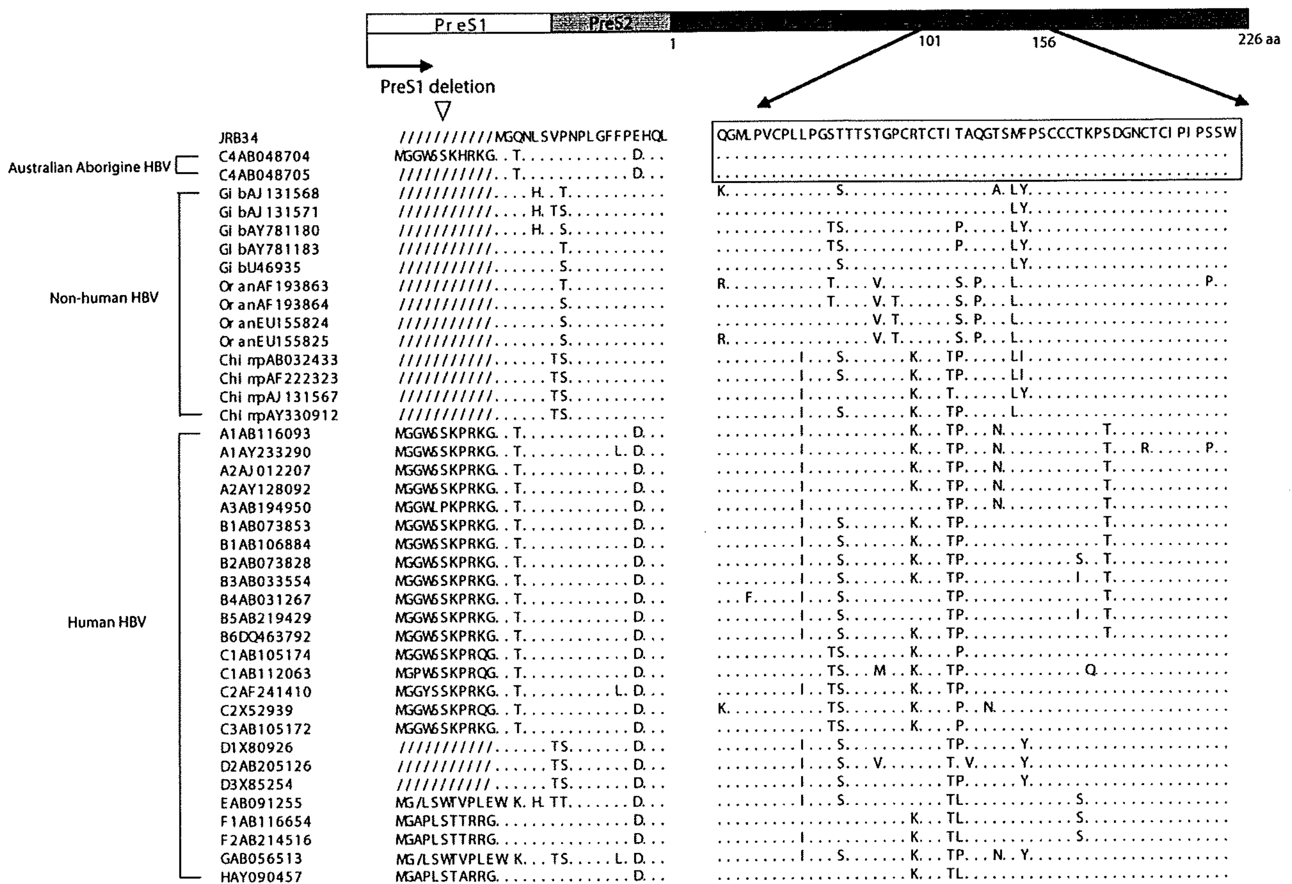

FIG. 5. Comparison of amino acid sequences of the preS/S gene among HBV isolates of various genotypes. The sequence of the HBV/J isolate (JRB34) is indicated at the top. Dots represent amino acids shared by JRB34, and a dash indicates the deletion of an amino acid. The sequence from positions 101 to 156 forming loops, bearing the common antigenic determinants of HBsAg, is boxed.

genes) and genotype C (in the core gene). However, no significant evidence of recombination between these two ape and human genotypes was revealed by the used methods. Homology scan carried out by SimPlot using the same set of reference sequences gave concordant results.

Phylogenetic analyses of the four open reading frames. Phylogenetic relationship between the JRB34 strain and other genotypes was further analyzed in four open reading frames. In the small S gene, subgenotype C4 recovered from Australian aborigines (43) changed its phylogenetic topology from the branch of human genotypes to a branch intermediate between orangutan and gibbon strains (Fig. 3A). Remarkably, genotype J and C4 strains joined together to create a clade between orangutan and gibbon strains. In contrast, genotype J clustered with human genotypes in the phylogenetic analysis of the C gene and was closely related to genotype C; it took a position outside genotype I strains, however (Fig. 3B). Genotype J was closer to gibbon and orangutan genotypes in the phylogenetic trees constructed on P and large S genes (data not shown), demonstrating its topology similar to that in the analysis of the entire genome (Fig. 1).

Amino acid sequence of the HBV/J isolate. The amino acid sequence of HBV/J was compared against those of other genotypes over three different areas of the genome. The amino acid sequence in the preC gene and arginine-rich domain in the carboxy-terminal sequence in the C gene were well conserved by genotype J (Fig. 4). In the preS1 region, genotype J had a deletion of 11 aa as gibbon and chimpanzee genotypes (Fig. 5). This deletion was shared by one of the two HBV/C4 isolates from Australian aborigines, as well as all HBV/D isolates. Amino acid sequence in the S gene of genotype J was the same as those of aborigine isolates of subgenotype C4; they would share antigenic epitopes of HBsAg. Amino acids at codons 122 and 160 were arginine (with G as nt 365) and lysine (with G as nt 479), respectively, which was consistent with subtype *ayw* of HBsAg from this patient (27).

Five domains (A to E) of DNA polymerase/reverse transcriptase in the P gene were preserved well in HBV/J, and it did not have mutations in the Tyr-Met-Asp-Asp motif in the domain C that determines the sensitivity to lamivudine (data not shown). HBV/J possessed A1762T/G1764A double mutations in the core promoter and G1896A stop codon mutation in the preC region, which was compatible with an HBeAg-minus phenotype of HBV recovered from the patient positive for anti-HBe.

Infection with HBV/J in chimeric mice with the liver repopulated for human hepatocytes. Two chimeric mice that had been transplanted with human hepatocytes were inoculated with 10^4 HBV DNA copies of genotype J. In both mice, HBV

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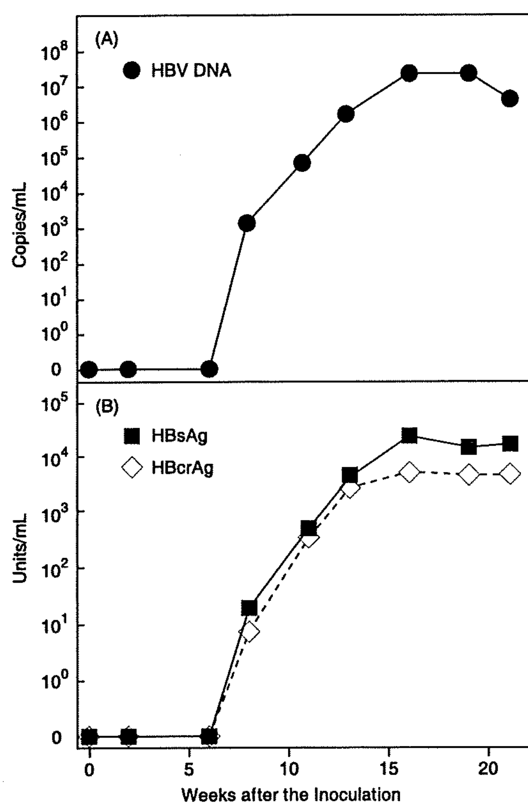

FIG. 6. Markers of HBV infection in two chimeric mice inoculated with the HBV/J isolate (JRB34). The levels of HBV DNA are illustrated in panel A, and those of HBsAg and HBcrAg are illustrated in panel B. Values represent the means for two mice.

DNA in a high titer (10^5 copies/ml) appeared in the circulation at week 7, plateaued at high levels (10^6 to 10^8 copies/ml), and stayed detectable until 22 weeks of observation after the inoculation (Fig. 6A). HBsAg and HBcrAg became detectable at week 7 and kept increasing in concentrations until week 15 when they reached a plateau at high levels (Fig. 6B). HBV strains recovered from mice at the last day of follow-up were identical in the complete genome sequence to the JRB34 strain used for inoculation.

The liver from chimeric mice infected with HBV/J was stained for HBcAg by immunofluorescence (Fig. 7A). The staining for HBcAg was confined to areas where mouse liver had been replaced for human hepatocytes, and the same areas were stained for human albumin (Fig. 7B). Colocalization of HBcAg and human hepatocytes was demonstrated by double staining for HBcAg and human albumin (Fig. 7C). Finally, expression and replication of the JRB34 strain were confirmed by successful detection of cccDNA and HBV RNA in the liver tissue from both sacrificed mice (see Fig. S2A and B in the supplemental material).

DISCUSSION

An HBV isolate (JRB34) was recovered from a male, 88-year-old Japanese patient with HCC and sequenced over the entire genome. In the full-genome sequence, the JRB34 strain had 10.9 to 15.7% divergence from 1,440 HBV strains retrieved from the DDBJ/EMBL/GenBank. The divergence exceeds 8% that has been defined originally for distinguishing between four genotypes (A to D) (29) and later for an additional four genotypes (E to H) (3, 26, 42). Phylogenetically, the sequence of JRB34 was closer to ape than human HBV genotypes. No significant evidence of recombination with eight known human and four ape genotypes was revealed by the GroupScanning analysis (40) and phylogenetic analyses. These lines of evidence have qualified the JRB34 strain to represent a possible new HBV genotype. To further confirm the epidemiological significance of this strain, capable of establishing new infections, two chimeric mice were each inoculated with 10^4 copies of JRB34 HBV DNA. They both were successfully infected with sharp increases in HBV DNA and HBsAg in serum several weeks after the inoculation. Replication in the chimeric mice was also confirmed by detection of cccDNA and HBV RNA in their liver tissues.

Recently, an HBV isolate from Vietnam (VH24 [accession no. AB231908]) was reported as a ninth human genotype (I) (12). However, VH24 differed by only 7.0% ± 0.4% from HBV isolates of genotype C and possessed complex recombination with genotypes A and G in three genomic areas. A number of sporadic HBV isolates have been reported to date that contain recombination between human genotypes (4, 24, 40), as well as between human and ape genotypes (21). Only a few recombinant variants, however, became widely spread in human populations, developing their own specific distributions and epidemiologies. This is particularly demonstrated for the B/C recombinant designated as a distinct subgenotype; Ba/B2-5 now accounts for the majority of genotype B strains in mainland Asia (44). Likewise, the C/D recombinant prevails in Tibet and northern China (50). To avoid assigning a new genotype for every newly discovered sporadic recombinant HBV variant, evidence of intergenotypic recombination should be carefully eliminated (14). However, in some cases, designation of a new genotype is proposed by a potential epidemiological significance of a novel genetic variant. Recently, a study carried out in Laos described a number of strains closely related phylogenetically with the Vietnamese genotype I strains, thereby suggesting their epidemiological significance (31). The JRB34 strain documented in the present study was genetically and phylogenetically distinct from any previously published strains, including those of genotype I from Vietnam and Laos. To avoid possible misconceptions in the future, the strain is provisionally designated genotype J.

HBV of distinct genotypes can infect great apes in the wild, including chimpanzee, gorilla, orangutan and gibbons (9, 20, 37, 51). HBV genotypes of chimpanzee and gorilla, as well as those of orangutan and gibbon, cocluster in agreement with their geographical distribution in Africa and Southeast Asia, respectively (41). Genotype J represented by the JRB34 strain clustered with gibbon/orangutan genotypes. In a phylogenetic analysis of the S region/gene sequence, JRB34 belonged to a nonhuman HBV group but was closely related to an HBV isolate of subgenotype C4 (AB048704) recovered from an Australian aborigine; C4 is most divergent from other subgenotypes of genotype C (43). In the phylogenetic analysis of the C gene, however, JRB34 clustered with human genotypes and closely related to genotype C, including C4, and was positioned

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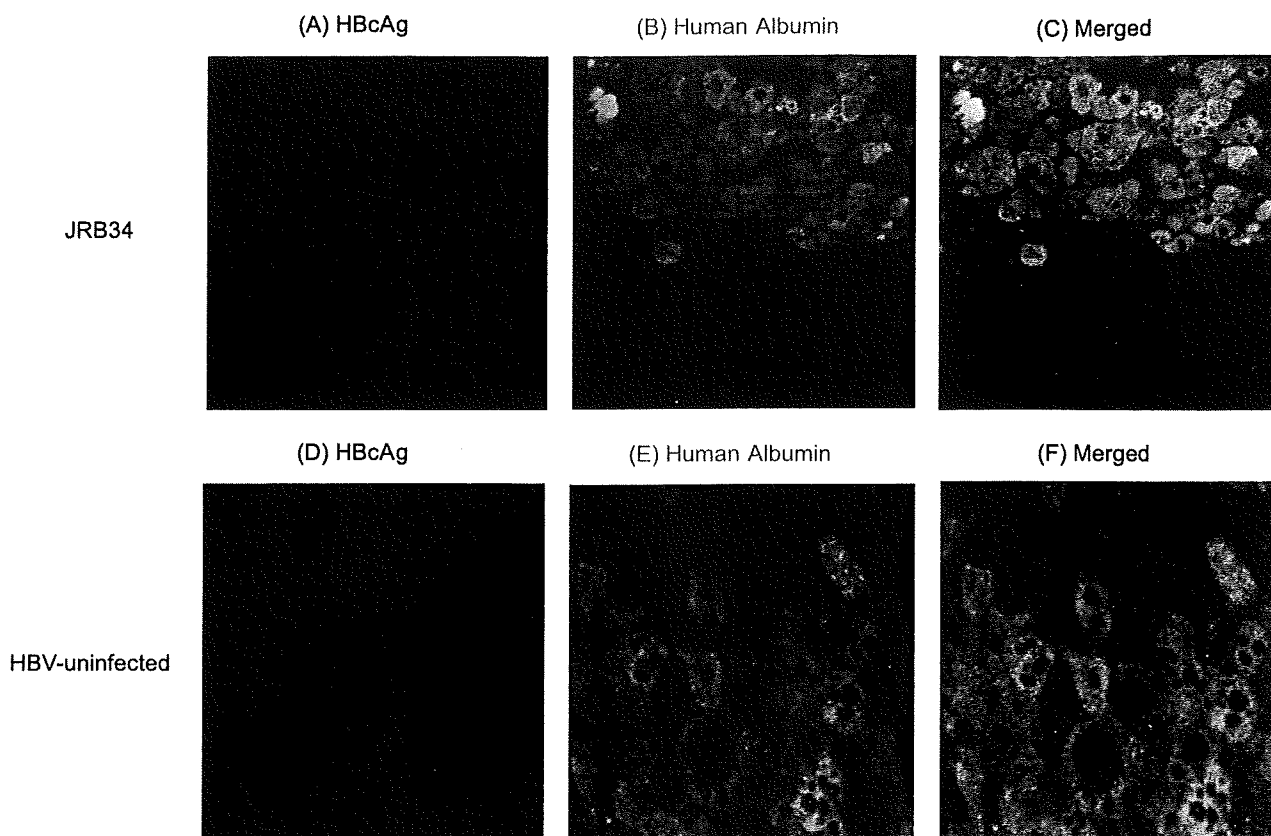

FIG. 7. (A and B) Immunofluorescent staining of a frozen liver section of a chimera mouse inoculated with the HBV/J isolate (JRB34). HBcAg is stained in panel A, and human albumin is stained in panel B. (C) Colocalization of HBcAg and human albumin is revealed by double staining. (D to F) HBV-uninfected mouse liver shows that only human albumin is stained.

outside genotype I strains (Fig. 4). Taken together, genotype J is phylogenetically close to gibbon/orangutan genotypes in the entire genome and to genotype C (C4 in particular) in the S and C genes. However, despite observed interchangeable relatedness with gibbon and genotype C/I strains, no strong evidence of recombination was confirmed in the JRB34.

In the sequence of C gene, carboxyl-terminal arginine-rich region, required for binding with HBV DNA, was preserved in JRB34. It had the G1896A stop codon in the precore region that aborts the translation of HBeAg (5, 30) and A1762T/G1764A double mutations in the core promoter that interfere with the transcription of HBeAg by downregulating preC mRNA (28, 45); they are compatible with the HBeAg$^-$ anti-HBe$^+$ phenotype of the patient from whom JRB34 was isolated. Since the double mutations are detected frequently in HBV DNA sequences from patients with HCC (17, 33), it could be implicated in hepatocarcinogenesis of the patient from whom JRB34 was isolated. It is not certain, however, if precore and core-promoter mutations had existed in HBV transmitted to the patient who is presumed to have been infected 60 years ago. Since amino acid sequences constituting antigenic loops of HBsAg (6) were the same as those of Australian aborigine isolates of C4, they would share antigenic epitopes of HBsAg. The amino acids at codons 122 and 160 were arginine (with G at nt 365) and lysine (with G at nt 479), respectively (27), in agreement with subtype *ayw* of HBsAg from this patient. Five domains (A to E) of DNA polymerase/reverse transcriptase in the P gene were preserved well in HBV/J, and it did not have mutations in the Tyr-Met-Asp-Asp motif in the domain C that determines the sensitivity to lamivudine (2).

How and when the patient contracted infection with HBV/J is not certain. It is very unlikely, however, that he acquired infection in Japan via perinatal or horizontal transmission. There are no wild primates in Okinawa, where the patient was originally from, and the prevalent human HBV genotypes are limited to B (60%), C (39%), and sporadic cases of A (1%) (32). Furthermore, HBV/J was not found among patient's family members who are currently alive (data not shown). The phylogenetic position within open reading frames of JRB34 in between gibbon/orangutan genotypes and human genotype C gives a clue where and when the patient had contracted HBV infection. He was drafted to Borneo during World War II (1939 to 1945); the island in the Southeast Asia is inhabited by gibbons and orangutans and has a local population mainly infected with genotypes B or C. Zoonotic infection of HBV has been previously reported (11, 46), and HBV of genotype E was recovered from a chimpanzee captured in West Africa where this genotype is common. There is a possibility that JRB34 of

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genotype J had been transmitted to the study patient in Borneo during the war (38).

The origin of genotype J in gibbon/orangutan or human inhabitants in Borneo is not certain but very likely. HBV DNA and/or HBsAg was detected in 26% (55/213) and 20% (58/297) of gibbons and orangutans, respectively, captured in Southeast Asia (38). HBV is also endemic in people living there, with a prevalence of HBsAg at 2 to 8%. There would be high chances for cross-species transmission of HBV where it prevails both in human beings and nonhuman primates. Phylogenetic analysis for close relationship between human and nonhuman HBV genotypes has indicated geographical influence rather than association with particular species (41).

It is remains to be determined whether genotype J and ape-derived strains originate from species-specific convergent evolution of distant strains or whether they have diverged from a single common ancestor sometime in the past and evolved independently thereafter. The validity of cross-species infection or species-specific evolution for genotype J would be verified by sequence analysis of HBV DNA from gibbons and humans living in Borneo. If they turn out to be the same, cross-species infection will be justified. Should genotype J be restricted to human beings, in converse, species-specific infection will be confirmed.

In conclusion, a novel HBV genotype was identified in the Ryukyu isolate and provisionally named genotype J. Phylogenetic analyses over the full-length sequence and open reading frames indicate a close relationship of genotype J with gibbon/orangutan genotypes and human genotype C. The index patient would have been infected with HBV/J while he resided in Borneo inhabited by gibbons and orangutans. Although only one HBV isolate of genotype J (JRB34) has been identified, this may be only the tip of an iceberg. It would be worthwhile to examine the genotype of HBV infecting people and gibbons, as well as orangutans, living in Borneo and neighboring countries for mapping the epidemiology of genotype J and finding any clinical relevance.

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REFERENCES

1. **Abe, A., K. Inoue, T. Tanaka, J. Kato, N. Kajiyama, R. Kawaguchi, S. Tanaka, M. Yoshiba, and M. Kohara.** 1999. Quantitation of hepatitis B virus genomic DNA by real-time detection PCR. J. Clin. Microbiol. **37:**2899–2903.
2. **Allen, M. I., M. Deslauriers, C. W. Andrews, G. A. Tipples, K. A. Walters, D. L. Tyrrell, N. Brown, L. D. Condreay, et al.** 1998. Identification and characterization of mutations in hepatitis B virus resistant to lamivudine. Hepatology **27:**1670–1677.
3. **Arauz-Ruiz, P., H. Norder, B. H. Robertson, and L. O. Magnius.** 2002. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. J. Gen. Virol. **83:**2059–2073.
4. **Bollyky, P. L., and E. C. Holmes.** 1999. Reconstructing the complex evolutionary history of hepatitis B virus. J. Mol. Evol. **49:**130–141.
5. **Carman, W. F., M. R. Jacyna, S. Hadziyannis, P. Karayiannis, M. J. McGarvey, A. Makris, and H. C. Thomas.** 1989. Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. Lancet **ii:**588–591.
6. **Carman, W. F., A. R. Zanetti, P. Karayiannis, J. Waters, G. Manzillo, E. Tanzi, A. J. Zuckerman, and H. C. Thomas.** 1990. Vaccine-induced escape mutant of hepatitis B virus. Lancet **336:**325–329.
7. **Fung, S. K., and A. S. Lok.** 2004. Hepatitis B virus genotypes: do they play a role in the outcome of HBV infection? Hepatology **40:**790–792.
8. **Ganem, D., and A. M. Prince.** 2004. Hepatitis B virus infection–natural history and clinical consequences. N. Engl. J. Med. **350:**1118–1129.
9. **Grethe, S., J. O. Heckel, W. Rietschel, and F. T. Hufert.** 2000. Molecular epidemiology of hepatitis B virus variants in nonhuman primates. J. Virol. **74:**5377–5381.
10. **Hannoun, C., H. Norder, and M. Lindh.** 2000. An aberrant genotype revealed in recombinant hepatitis B virus strains from Vietnam. J. Gen. Virol. **81:**2267–2272.
11. **Hu, X., A. Javadian, P. Gagneux, and B. H. Robertson.** 2001. Paired chimpanzee hepatitis B virus (ChHBV) and mtDNA sequences suggest different ChHBV genetic variants are found in geographically distinct chimpanzee subspecies. Virus Res. **79:**103–108.
12. **Huy, T. T. T., T. N. Trinh, and K. Abe.** 2008. New complex recombinant genotype of hepatitis B virus identified in Vietnam. J. Virol. **82:**5657–5663.
13. **Kimura, T., A. Rokuhara, Y. Sakamoto, S. Yagi, E. Tanaka, K. Kiyosawa, and N. Maki.** 2002. Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. J. Clin. Microbiol. **40:**439–445.
14. **Kurbanov, F., Y. Tanaka, A. Kramvis, P. Simmonds, and M. Mizokami.** 2008. When should "I" consider a new hepatitis B virus genotype? J. Virol. **82:**8241–8242.
15. **Lee, W. M.** 1997. Hepatitis B virus infection. N. Engl. J. Med. **337:**1733–1745.
16. **Lindh, M., A. S. Andersson, and A. Gusdal.** 1997. Genotypes, nt 1858 variants, and geographic origin of hepatitis B virus: large-scale analysis using a new genotyping method. J. Infect. Dis. **175:**1285–1293.
17. **Liu, C. J., B. F. Chen, P. J. Chen, M. Y. Lai, W. L. Huang, J. H. Kao, and D. S. Chen.** 2006. Role of hepatitis B viral load and basal core promoter mutation in hepatocellular carcinoma in hepatitis B carriers. J. Infect. Dis. **193:**1258–1265.
18. **Liu, C. J., J. H. Kao, and D. S. Chen.** 2005. Therapeutic implications of hepatitis B virus genotypes. Liver Int. **25:**1097–1107.
19. **Lole, K. S., R. C. Bollinger, R. S. Paranjape, D. Gadkari, S. S. Kulkarni, N. G. Novak, R. Ingersoll, H. W. Sheppard, and S. C. Ray.** 1999. Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. J. Virol. **73:**152–160.
20. **MacDonald, D. M., E. C. Holmes, J. C. Lewis, and P. Simmonds.** 2000. Detection of hepatitis B virus infection in wild-born chimpanzees (*Pan troglodytes verus*): phylogenetic relationships with human and other primate genotypes. J. Virol. **74:**4253–4257.
21. **Magiorkinis, E. N., G. N. Magiorkinis, D. N. Paraskevis, and A. E. Hatzakis.** 2005. Re-analysis of a human hepatitis B virus (HBV) isolate from an East African wild born *Pan troglodytes schweinfurthii*: evidence for interspecies recombination between HBV infecting chimpanzee and human. Gene **349:**165–171.
22. Reference deleted.
23. **Miyakawa, Y., and M. Mizokami.** 2003. Classifying hepatitis B virus genotypes. Intervirology **46:**329–338.
24. **Morozov, V., M. Pisareva, and M. Groudinin.** 2000. Homologous recombination between different genotypes of hepatitis B virus. Gene **260:**55–65.
25. **Norder, H., A. M. Courouce, P. Coursaget, J. M. Echevarria, S. D. Lee, I. K. Mushahwar, B. H. Robertson, S. Locarnini, and L. O. Magnius.** 2004. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. Intervirology **47:**289–309.
26. **Norder, H., A. M. Courouce, and L. O. Magnius.** 1994. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. Virology **198:**489–503.
27. **Okamoto, H., M. Imai, F. Tsuda, T. Tanaka, Y. Miyakawa, and M. Mayumi.** 1987. Point mutation in the S gene of hepatitis B virus for a *d/y* or *w/r* subtypic change in two blood donors carrying a surface antigen of compound subtype *adyr* or *adwr*. J. Virol. **61:**3030–3034.
28. **Okamoto, H., F. Tsuda, Y. Akahane, Y. Sugai, M. Yoshiba, K. Moriyama, T. Tanaka, Y. Miyakawa, and M. Mayumi.** 1994. Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. J. Virol. **68:**8102–8110.
29. **Okamoto, H., F. Tsuda, H. Sakugawa, R. I. Sastrosoewignjo, M. Imai, Y. Miyakawa, and M. Mayumi.** 1988. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. J. Gen. Virol. **69**(Pt. 10):2575–2583.
30. **Okamoto, H., S. Yotsumoto, Y. Akahane, T. Yamanaka, Y. Miyazaki, Y. Sugai, F. Tsuda, T. Tanaka, Y. Miyakawa, and M. Mayumi.** 1990. Hepatitis B viruses with precore region defects prevail in persistently infected hosts along with seroconversion to the antibody against e antigen. J. Virol. **64:**1298–1303.
31. **Olinger, C. M., P. Jutavijittum, J. M. Hubschen, A. Yousukh, B. Samountry, T. Thammavong, K. Toriyama, and C. P. Muller.** 2008. Possible new hepatitis B virus genotype, southeast Asia. Emerg. Infect. Dis. **14:**1777–1780.
32. **Orito, E., T. Ichida, H. Sakugawa, M. Sata, N. Horiike, K. Hino, K. Okita, T. Okanoue, S. Iino, E. Tanaka, K. Suzuki, H. Watanabe, S. Hige, and M. Mizokami.** 2001. Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. Hepatology **34:**590–594.

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33. **Orito, E., M. Mizokami, H. Sakugawa, K. Michitaka, K. Ishikawa, T. Ichida, T. Okanoue, H. Yotsuyanagi, and S. Iino.** 2001. A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. Hepatology **33:**218–223.
34. **Palumbo, E.** 2007. Hepatitis B genotypes and response to antiviral therapy: a review. Am. J. Ther. **14:**306–309.
35. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
36. **Sakamoto, T., Y. Tanaka, E. Orito, J. Co, J. Clavio, F. Sugauchi, K. Ito, A. Ozasa, A. Quino, R. Ueda, J. Sollano, and M. Mizokami.** 2006. Novel subtypes (subgenotypes) of hepatitis B virus genotypes B and C among chronic liver disease patients in the Philippines. J. Gen. Virol. **87:**1873–1882.
37. **Sall, A. A., S. Starkman, J. M. Reynes, S. Lay, T. Nhim, M. Hunt, N. Marx, and P. Simmonds.** 2005. Frequent infection of *Hylobates pileatus* (pileated gibbon) with species-associated variants of hepatitis B virus in Cambodia. J. Gen. Virol. **86:**333–337.
38. **Sa-nguanmoo, P., C. Thongmee, P. Ratanakorn, R. Pattanarangsan, R. Boonyarittichaikij, S. Chodapisitkul, A. Theamboonlers, P. Tangkijvanich, and Y. Poovorawan.** 2008. Prevalence, whole genome characterization and phylogenetic analysis of hepatitis B virus in captive orangutan and gibbon. J. Med. Primatol. **37:**277–289.
39. **Shin-I, T., Y. Tanaka, Y. Tateno, and M. Mizokami.** 2008. Development and public release of a comprehensive hepatitis virus database. Hepatol. Res. **38:**234–243.
40. **Simmonds, P., and S. Midgley.** 2005. Recombination in the genesis and evolution of hepatitis B virus genotypes. J. Virol. **79:**15467–15476.
41. **Starkman, S. E., D. M. MacDonald, J. C. Lewis, E. C. Holmes, and P. Simmonds.** 2003. Geographic and species association of hepatitis B virus genotypes in non-human primates. Virology **314:**381–393.
42. **Stuyver, L., S. De Gendt, C. Van Geyt, F. Zoulim, M. Fried, R. F. Schinazi, and R. Rossau.** 2000. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. J. Gen. Virol. **81:**67–74.
43. **Sugauchi, F., M. Mizokami, E. Orito, T. Ohno, H. Kato, S. Suzuki, Y. Kimura, R. Ueda, L. A. Butterworth, and W. G. Cooksley.** 2001. A novel variant genotype C of hepatitis B virus identified in isolates from Australian Aborigines: complete genome sequence and phylogenetic relatedness. J. Gen. Virol. **82:**883–892.
44. **Sugauchi, F., E. Orito, T. Ichida, H. Kato, H. Sakugawa, S. Kakumu, T. Ishida, A. Chutaputti, C. L. Lai, R. Ueda, Y. Miyakawa, and M. Mizokami.** 2002. Hepatitis B virus of genotype B with or without recombination with genotype C over the precore region plus the core gene. J. Virol. **76:**5985–5992.
45. **Takahashi, K., K. Aoyama, N. Ohno, K. Iwata, Y. Akahane, K. Baba, H. Yoshizawa, and S. Mishiro.** 1995. The precore/core promoter mutant (T1762A1764) of hepatitis B virus: clinical significance and an easy method for detection. J. Gen. Virol. **76**(Pt. 12):3159–3164.
46. **Takahashi, K., B. Brotman, S. Usuda, S. Mishiro, and A. M. Prince.** 2000. Full-genome sequence analyses of hepatitis B virus (HBV) strains recovered from chimpanzees infected in the wild: implications for an origin of HBV. Virology **267:**58–64.
47. **Tanaka, Y., and M. Mizokami.** 2007. Genetic diversity of hepatitis B virus as an important factor associated with differences in clinical outcomes. J. Infect. Dis. **195:**1–4.
48. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
49. **Tiollais, P., P. Charnay, and G. N. Vyas.** 1981. Biology of hepatitis B virus. Science **213:**406–411.
50. **Wang, Z., Z. Liu, G. Zeng, S. Wen, Y. Qi, S. Ma, N. V. Naoumov, and J. Hou.** 2005. A new intertype recombinant between genotypes C and D of hepatitis B virus identified in China. J. Gen. Virol. **86:**985–990.
51. **Wiegand, J., D. Hasenclever, and H. L. Tillmann.** 2008. Should treatment of hepatitis B depend on hepatitis B virus genotypes? A hypothesis generated from an explorative analysis of published evidence. Antivir. Ther. **13:**211–220.
52. **Wong, D. K., Y. Tanaka, C. L. Lai, M. Mizokami, J. Fung, and M. F. Yuen.** 2007. Hepatitis B virus core-related antigens as markers for monitoring chronic hepatitis B infection. J. Clin. Microbiol. **45:**3942–3947.

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Review Article

Geographical and genetic diversity of the human hepatitis B virus

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Hepatitis B virus (HBV) is one of the most widely distributed viruses that infect humankind. Distinct clinical and virological characteristics of the HBV-infection have been reported in different geographical parts of the world and are increasingly associated with genetic diversity of the infecting virus. HBV is classified into genotypes and subgenotypes that are associated with ethnicity and geography. The genetic diversity of HBV in its various aspects has been the subject of extensive investigations during the last few decades. Since molecular epidemiology research tools have become widely available, the number of new publications in this field has grown exponentially. This review summarises the recent publications on the geographical distribution of genetic variants of HBV, and proposes updated criteria for the identification of new genotypes and subgenotypes of the virus.

Key words: genotypes, hepatitis B virus, molecular epidemiology, recombination

INTRODUCTION

FOUR DECADES AGO, in 1965, the "Australian antigen" (now reffered to as the hepatitis B surface antigen, HBsAg) was detected in hemophilia patients[1] and was soon specifically associated with hepatits B virus (HBV).[2,3] Three decades ago, the HBV strains were divided into nine major serotypes based on antigenic determinants of HBsAg.[4] Two decades ago, the classification of the HBV by genome nucleotide sequence divergence was proposed.[5] A decade ago, a "unique phylogenetic cluster within genotype A strains was described, triggering consecutive investigations on HBV subgenotypes.[6]

Outlined are the most important key-events in a chain of findings that accumulated in the current image of the HBV diversity. The chain was tortuous before powerful tools such as PCR and nucleotide sequencing became available to researchers. These tools enabled the annual number of publications in this field to grow exponentially. The current review will discuss the most recently published observations on HBV diversity, particularly their geographical distribution, and taxonomical aspects.

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CURRENTLY KNOWN HBV GENOTYPES

A TOTAL OF 18 complete genome sequences were available for comparison when the first four genotypes of HBV (designated A to D, consecutively) were originally proposed and divergence exceeding 8% of the complete genome was indicated as a criterion for genotype identification.[5] Almost at the same time, genotyping based on the phylogenetic clustering was proposed.[7] Consecutively, by sequencing the HBsAg coding region, four new strains were designated as novel genotypes E and F based on both, percent evaluation of nucleotide divergence and phylogenetic analysis. This added new criteria for genotyping; 4% of nucleotide divergence in HBsAg coding sequence.[8] Shortly after, the genotype F was confirmed by an analysis of the full genome sequence.[9] Relatively recent reports identified the last two of the 8 currently known genotypes, genotype G[10] and H.[11]

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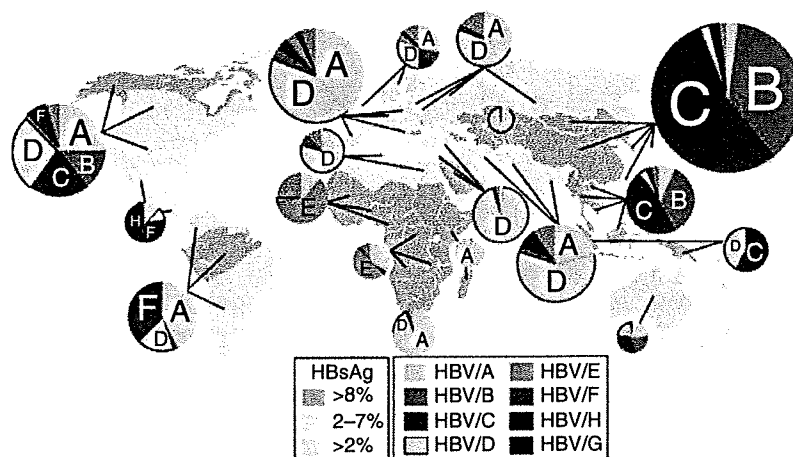

Figure 1 Hepatitis B virus (HBV) infection endemicity is based on the 2005 estimation of hepatitis B surface antigen (HBsAg) seroprevalence (Centers for Disease Control and Prevention Travelers' Health: Yellow Book Chapter 4 – Prevention of Specific Infectious Diseases: Hepatitis, Viral, Type B URL: http://wwwn.cdc.gov/travel/yellowbook/ch4/hep-b.aspx). Percentile distribution of genotypes is indicated for each geographic region.

DISTRIBUTION OF GENOTYPES IN THE WORLD

EPIDEMIOLOGICAL GEOGRAPHICAL DISTRIBUTION of HBV genotypes is being continuously investigated in different parts of the world. For this review we summarised epidemiological studies published within the last decade. A total of 256 papers were analysed. The results of the geographical distribution of genotypes are graphically summarised in Figure 1A. The detailed summary presented in Table 1 contains the number of examined HBV carriers in each particular subregion, and the corresponding references.

The total number of HBV-infected individuals genotyped during the last 10 years consist of approximately 45 000, with more than half of that number in Eastern Asia. From the data accumulated, it can be seen that a single genotype can only be predominately found in

Table 1 Prevalence of hepatitis B virus genotypes in different geographical regions

Geographical subregion	*n*	A	B	C	D	E	F	H	G	Mixed	UT	References
Eastern Africa	43	93.0			2.3						4.7	12–14
South Africa	404	74.3	0.7	1.5	19.3	1.2					3.0	15–17
Central Africa	126	31.0		3.2	3.2	49.2			1.6	11.9		18–20
Western Africa	759	11.3	0.3		1.6	59.2				2.9	24.8	21–29
Northern Africa	331	0.3	5.7	0.9	79.2					9.4	4.5	30–34
Western Asia	1652	0.9	0.2	0.5	94.8		0.1				3.5	35–58
Central Asia	118	11.0		0.8	88.1							59–61
Southern Asia	3023	21.5	0.9	8.9	58.7					3.9	6.1	25,62–79
East Europe	1674	30.5	0.9	0.7	50.4					6.0	11.5	25,80–86
European Union	4968	38.5	3.3	4.3	42.6	3.4	1.4	0.2	0.7	2.0	3.7	10,20,25,81,87–116
North Europe	442	28.3	10.9	10.6	30.8	5.0	1.4	0.2	0.2	2.0	10.6	117–122
North America	3412	25.1	14.3	20.8	27.7	0.2	7.3	0.1	0.9		3.6	10,25,108,123–131
Central America	225	11.6		0.4	11.6		36.0	35.1	3.6	1.3	0.4	132–135
South America	1393	42.6	0.5	1.9	17.4	0.1	35.9		0.1	0.6	0.9	25,136–157
Atlantic Island	84	54.8		1.2	23.8		2.4			17.9		110
Southeastern Asia	2024	6.7	35.2	47.3	4.1	0.7	0.4		0.9	3.4	1.2	20,25,108,158–170
Eastern Asia	23577	2.0	36.9	55.0	2.2					1.9	1.9	25,108,171–255
Pacific Islands	274			57.7	42.3							225,239,256,257
Australia	132	22.7	22.7	31.8	21.2				0.8		0.8	258–260
TOTAL	44661	13.1	22.9	34.5	19.9	1.6	2.1	0.2	0.2	2.1	3.3	

UT, untypeable

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Eastern Africa and Western Asia (A and D, respectively) where the prevalence of other genotypes was less than 5%. On the other hand, in countries with high levels of immigration, a variety of genotypes are being reported; all of the known genotypes can be found in the Europe and North America. In Australia, genotypes A, B, C and D were reported in equal prevalence. Two genotypes, A and D are prevalent in European Union (except for the Mediterranean where D predominates), and in Central/Southern Asia. Genotypes B and C are the major variants in South and South East Asia and the Pacific region, while genotypes A and F are the most common in South America or E and A in Central Africa. Genotype E is restricted to Central and West Africa however, its prevalence tends to increase in Europe. Genotype F is subdivided into 4 subgenotypes, and is prevalent in Central and South America and Alaska. In recent reports the subgenotypes of genotype F were further subdivided into clades (Table 2).

A recent study from Peru described a full genome analysis for three strains from Peru that belonged to subtype F1 and suggested they should be considered as clade 1c within subgenotype F1.[281] Genotype G was found in Europe and the United States. A few cases of genotype G infection have been reported from Asia,[227,283] and more recently from Brazil.[284] Despite the geographical dispersion of the reported G strains, they show a very low genetic diversity. Genotype H is frequent in and restricted to Central America where it was also reported in co-infection with genotype G.[133] The pattern of genotype distribution changes according to the pattern of global migration.

EVOLUTIONARY HISTORY OF THE GENOTYPES

THE FIRST ATTEMPT to date the evolutionary history of HBV was carried out by the phylogenetic analysis based on synonymous substitutions in the polymerase coding gene of hepadnavirus family strains isolated from the human, chimpanzee, woodchuck, ground squirrel and duck.[7] The substitution rate estimated in the study was 4.57×10^{-5} substitutions/site/year. This study concluded that the duck strain was the most divergent and shared the most recent common ancestor with other strains approximately 30 000 years ago, whereas different human HBV genotypes emerged about 3000 years ago.[7] However, the overlapping composition of Open Reading Frames (ORFs) in the HBV genome complicates an estimation of the synonymous substitutions, as the same mutation considered synonymous in one of the ORF may cause an aminoacid change in overlapping ORF.[285] The mutation rate of HBV estimated in the serial specimens collected at distant periods of time from genotype B infected carriers, was 7.9×10^{-5} substitutions/site/year.[271] Another study carried on genotype D strains representing localised epidemic in Western Japan, have set the mutation rate to 5.4×10^{-5}.[277] A study aiming to estimate the substitution rate using two independent data sets of non-overlapping ORF coding core protein, concluded that a reliable molecular clock does not exist.[286] Phylogenetic topology of the genotypes heavily depends on the genomic region and substitution model used in analysis, thus hinder any attempt to reconstruct the past spread of this virus.[286] In addition to the complex overlapping structure of the genome, a recombination of HBV severely hampers an assessment of its evolution.[287] New methodological approach is required to explore rules of the HBV evolution.

HBV RECOMBINATION

ONE OF THE most comprehensive analyses of occurrence and composition of HBV intergenotype recombinants indicated the existence of 24 phylogenetically independent recombinant forms of HBV involving all human genotypes as well as both chimpanzee and gibbon variants.[288] Further reports are constantly extending this number.[18,118,283,289] It has been shown that 60% of the intergenotype recombinants have the breakpoints within nucleotides 1640–1900.[289] It was also concluded that recombination sites often localise to gene boundaries.[288,289] Further, using a newly developed approach ("TreeOrder Scan") the authors could demonstrate that analysed in different parts of the HBV genome, genotypes are interchangeably shifting the relative phylogenetic topology. This consists with changes in the overall phylogenetic topology of the HBV genotypes that can be observed in trees reconstructed from different parts of the genome. Genotype G strains in particular demonstrate evidence of recombination with genotype A in the Small S fragment (nucleotides: 250–350) as well as genotype E with genotype D in the core gene (nucleotides: 1950–2500) and genotype H with genotype F within the *Small S* gene (nucleotides: 350–500).[288] It was hypothesised that some of the genotypes that are conventionally regarded as "nonrecombinant," demonstrate evidence of recombination, that is, during evolution in some cases, one or other of the ancestral HBV variants that might have been involved in recombination are virtually replaced by a more viable

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Table 2 Hepatitis B virus (HBV) subgenotypes

Genotype	Subgenotype	*n*	Complete genome Nucleotides diversity (complete genome)			Geography	Ref
			Clustering	Intra-subgenotype mean + SD (max)	Next closest negbour mean + SD (min)		
HBV/A							
	A1/Aa	78	yes	2.6 + 0.8 (5.5)	4.4 + 0.4 (3.3) for A4	Africa, Asia, South America	15,261,262
	A2/Ae	94	yes	1.7 + 0.9 (5.5)	4.7 + 0.7 (3.6) for A4	Europe, North America	15,261,262
	A3/Ac	8	yes	3.0 + 0.9 (4.1)	4.7 + 0.4 (3.8) for A1	Western Africa	19,21,22
	A4	3	no	2.9 + 0.9 (3.5)	3.8 + 0.2 (3.4) for A3	Western Africa	21,263
	A5	0	?	?	?	Western Africa	21
HBV/B							
	B1/Bj	38	yes	2.4 + 0.6 (4.1)	4.6 + 0.5 (3.6) for B2	Japan	264–266
	B2	173	yes	1.7 + 0.8 (4.0)	4.4 + 0.5 (2.9) for B4	China, Taiwan	190,200,264–268
	B3	5	yes	1.6 + 0.6 (2.7)	3.6 + 0.5 (2.9) for B5	Indonesia	269
	B4	21	yes	2.7 + 0.6 (4.4)	5.0 + 0.5 (4.3) for B3	Vietnam, Cambodia	269
	B5	7	yes	2.8 + 1.5 (4.5)	5.2 + 0.6 (4.0) for B2	the Philippines	166,167
	B6	27	yes	2.7 + 0.7 (4.2)	5.7 + 0.6 (4.6) for B3	Native populations in Arctic	270,271
	B7	2	no			Indonesia	161
HBV/C							
	C1/Cs	97	yes	2.4 + 0.7 (5.1)	4.4 + 0.5 (3.1) for C2	South and South East Asia	272–274
	C2/Ce	295	yes	2.5 + 0.6 (4.7)	4.9 + 0.5 (3.8) for C3	Eastern Asia (Korea, Japan) and North China	
	C3	3	yes	4.2 + 1.2 (5.2)	5.8 + 0.6 (4.6) for C1	Pacific	269
	C4	2	yes	0.9	6.6 + 0.6 (6.0) for C3	Australia	256
	C5	8	yes	2.0 + 1.0 (3.4)	6.2 + 0.5 (5.0) for C1	Philippines, Vietnam	167
HBV/D							
	D1	88	yes	2.3 + 0.8 (5.2)	3.1 + 0.6 (1.7) for D2	North Africa, Europe, Central Asia	84,269,275
	D2	53	yes	3.0 + 0.8 (5.8)	4.2 + 0.6 (2.6) for D3	North Europe, Russia, Japan (Ehime)	269,276–278
	D3	66	yes	2.9 + 1.1 (5.9)	4.1 + 0.7 (2.3) for D1	South Africa, Europe	
	D4	7	yes	2.6 + 1.2 (4.9)	4.6 + 0.6 (3.5) for D1	Australia	256
	D5	2	yes	2.4	5.2 + 0.5 (4.9) for D4	Eastern India	73
HBV/F							
	F1a	4		1.1 + 0.2 (1.4)	2.0 + 0.2 (1.6) for 1b	Central America: Costa Rica	279,280
	F1b	7		0.4 + 0.1 (0.6)	1.9 + 0.3 (1.5) for 1d	Venezuela, Argentina, Alaska	154,279,281,282
	F1d	2		2.2	2.8 + 0.3 (2.4) for 1a	Japan	279,281
	F2a	9		1.1 + 0.3 (1.4)	3.2 + 0.2 (2.8) for 2b	Brasil, Venezuella, Nicaragua	24,154
	F2b			0.5 + 0.1 (0.6)	4.1 + 0.9 (2.8) for F4		
	F3	23	yes	1.1 + 0.9 (4.2)	4.5 + 0.3 (3.9) for F2	Venezuella	
	F4	6		1.9 + 0.9 (3.7)	4.6 + 0.6 (3.8) for F3	Argentina, Bolivia	142

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product of the recombination. Discovery of "pure" genotypes E, G or H strains would confirm this hypothesis. Most of the studies that have found a high prevalence of both D and E were reported in Europe, in particular France.[92–95,103] The only country less affected by recent migration is Cameroon, where these two variants might have been endemic for a longer period of time. However, sequencing of a number of Cameroonian HBV/E strains to date did not reveal any evidence of the presence of a "pure" genotype E that is not "sharing" its core gene sequence with genotype D.[21,22] Genotypes G and H have the highest prevalence in Mexico,[133,134] a country where genotypes A and F are also prevalent.[132,290] Hence further molecular epidemiological studies in Cameroon and Mexico may reveal traces of "pure" non-recombinant ancestors of currently known genotypes.

GENOTYPES COINFECTION

AS MORE THAN one genotype is predominant in most of the geographic regions, coinfection between the predominating genotypes is not a rare finding; especially for B and C,[169,176,179,202,223,251,291] or A and D.[19,21,27,63,69,70,77,79,105,110,138,139] Co-infections with different genotypes of HBV are being reported with various frequencies. The frequency, however, seems to have a stronger association with the genotyping method rather than a geographic region or genotype endemic in a studied population. Most of the reported cases of co-infection with different genotypes were detected by using multiplex PCR or hybridisation assays and are rarely confirmed by conventional cloning and sequencing.[20,81,95,206] However, genotyping based on PCR with specific primers, probes, and/or restriction enzymes may produce misleading non-specific results due to single nucleotide polymorphisms. This is particularly important in case the PCR-based genotyping assays are applied when studying populations with only a few representing HBV sequences in the database, which means that the local variability of HBV strains was not considered when the assay was designed. In some reports, coinfections detected between genotypes not endemic in the studied population. A cross-sectional international population study using PCR-RFLP genotyping, reported 10/47 cases of genotype C in African cohorts and 6 of the 10 cases were found in coinfection (mainly with genotype G.)[20] The same study detected genotypes E to be more frequent in Asian cohorts compared to European and African ones.[20] However these findings are discrepant with previous reports on the geographical distribution of the genotypes therefore the result obtained by PCR-based genotyping assay requires confirmation by cloning and sequencing.

SUBGENOTYPES

GENETIC VARIABILITY WITHIN the genotypes is being extensively investigated since the concept of subgenotyping has evolved from studies on genotype A[15,261] and B.[266] All genotypes except for E, G and H are currently subdivided into subgenotypes (Table 2). Analysis of accumulated sequence data of HBV indicated that nucleotide sequence divergence exceeding 4% but less than 7.5% in the entire genome sequence should be used as criterion for identifying subgenotypes, whereas divisions within the subgenotypes showing less than 4% divergence should be referred to as "clades."[262] In this view, the recently proposed subgenotype A4[21] with a mean and minimal nucleotide divergence from subgenotype A3; 3.8% and 3.4%, respectively, is a clade rather than a subgenotype (Table 2). This can be further supported by a phylogenetic tree constructed on the complete genome of the strains, showing the "A4" strains to group along with the A3 strains (Fig. 2). Similar geographic distribution of the strains (West Africa) concurs that "A4" and A3 strains represent the same subgenotype. The small genetic distance and similar endemicity of the recently proposed subgenotypes B3, B5 and B7 can also suggest that these can be considered as clades representing the same subgenotype of genotype B (Table 2).

EVOLVING OF GENOTYPING CRITERIA

AN EXTENSIVE ANALYSIS of accumulated HBV genome sequence data indicated that the nucleotide diversity of genotype H strains is less than 8% from its closest neighbour; genotype F.[279] It was further proposed to use 7.5% of nucleotide divergence in the complete genome as a cut off for designation of new genotypes.[262] A new genotype "I" was recently reported to be circulating in Vietnam.[294] However, the conclusions of the paper on the new genotype and on the complex intergenotypic recombination did not correspond with existing genotyping criteria.[295] First of all, the complete genome genetic diversity of the strain was lower than 7.5% from the closest neighbour; genotype C (7.0%). Second, the genetic recombination with other genotypes was evident.[288,295,296] And finally, the epidemiological significance of the aberrant variant in terms of a new genotype was questionable as only three strains

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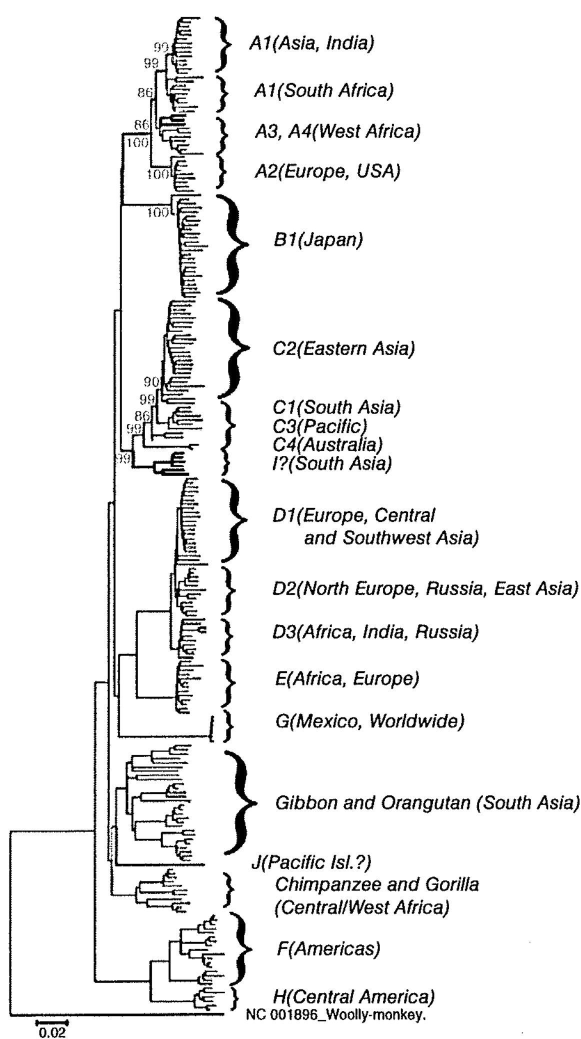

Figure 2 Phylogenetic tree constructed on selected complete genome strain references, which had no evidence of recombination. The neighbour joining tree was constructed using online aligning and tree drawing tools.[292,293]

have been reported during the 8 years since the first report describing this variant.[296] More recent study carried in Laos have revealed more strains that are genetically similar to the three Vietnamese isolates, providing evidence for the epidemiological value of the specific designation of the variant.[297] However, further studies are required to justify the classification of the variant into a separate genotype (HBV-I) or to consider it as a subgenotype of the existing genotype (HBV-C) (Fig. 2.) Another recent study from Japan, based on a strain isolated from an hepatocellular carcinoma patient who had a history of travelling to Borneo, revealed a novel genetic variant of HBV phylogenetically positioned between clusters of human and primate isolates.[298] The tentative genotype J strain show no evidence of recombination with any of known genotypes, and it is phylogenetically close to strains previously isolated from Gibbons and Orangutan (Fig. 2.) Epidemiological, virologic and clinical features of both provisional genotypes I and J require further studies to justify their classification.

Alternative approaches for the genotyping of HBV were recently proposed, suggesting that known variants of HBV can be grouped into 4 groups[299] or 3 groups,[300] however, the relevance of these classifications still needs to be substantiated from epidemiological and clinical points of view.

GENOTYPING CRITERIA

INTENSIVE INVESTIGATIONS HAVE indicated an uneven geographical distribution and epidemiology of distinct HBV genotypes and subgenotypes, however, many questions remain unanswered in terms of their virologic and clinical features. Further investigations in this field require standardised criteria for genotyping and subgenotyping, and these criteria need to be updated regularly in the context of new findings. Currently we propose the following check list of minimal requirements for defining genotypes and subgenotypes:

1. A complete genome sequence analysis is required to identify a new genotype or subgenotype.
2. Nucleotide divergence in a complete genome should exceed 7.5% to distinguish a genotype or 4% to distinguish a subgenotype. Variability below 4% confirmed by specific phylogenetic clustering can be used as a criterion to identify clades within subgenotypes.
3. Genotypes and subgenotypes should be identified by robust independent clustering on molecular evolutionary analysis based on complete HBV genomes.
4. Evidence of recombination with other known or unknown genotypes should be used as a criterion for identifying a subgenotype or clade of genotype involved in recombination rather than a new independent genotype.
5. Identification of a new genotype should be substantiated by its epidemiological, virological or clinical characteristics.

In conclusion, HBV demonstrates significant genetic and geographical divergence. Further studies are required to investigate genetic characteristics of the virus

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in less studied developing countries, especially those with a high endemicity. Updated unified criteria are required to resolve future issues in genotype assignment.

REFERENCES

1 Blumberg BS, Alter HJ, Visnich S. A "new" antigen in leukemia sera. *JAMA* 1965; 191: 541–6.
2 Okochi K, Murakami S. Observations on Australia antigen in Japanese. *Vox Sang* 1968; 15: 374–85.
3 Prince AM. An antigen detected in the blood during the incubation period of serum hepatitis. *Proc Natl Acad Sci USA* 1968; 60: 814–21.
4 Courouce-Pauty AM, Lemaire JM, Roux JF. New hepatitis B surface antigen subtypes inside the ad category. *Vox Sang* 1978; 35: 304–8.
5 Okamoto H, Tsuda F, Sakugawa H *et al.* Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J Gen Virol* 1988; 69 (Pt 10): 2575–83.
6 Bowyer SM, van Staden L, Kew MC, Sim JG. A unique segment of the hepatitis B virus group A genotype identified in isolates from South Africa. *J Gen Virol* 1997; 78 (Pt 7): 1719–29.
7 Orito E, Mizokami M, Ina Y *et al.* Host-independent evolution and a genetic classification of the hepadnavirus family based on nucleotide sequences. *Proc Natl Acad Sci USA* 1989; 86: 7059–62.
8 Norder H, Hammas B, Lofdahl S, Courouce AM, Magnius LO. Comparison of the amino acid sequences of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hepatitis B virus strains. *J Gen Virol* 1992; 73: 1201–8.
9 Naumann H, Schaefer S, Yoshida CF, Gaspar AM, Repp R, Gerlich WH. Identification of a new hepatitis B virus (HBV) genotype from Brazil that expresses HBV surface antigen subtype adw4. *J Gen Virol* 1993; 74: 1627–32.
10 Stuyver L, De Gendt S, Van Geyt C *et al.* A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J Gen Virol* 2000; 81: 67–74.
11 Arauz-Ruiz P, Norder H, Robertson BH, Magnius LO. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *J Gen Virol* 2002; 83: 2059–73.
12 Kramvis A, Restorp K, Norder H, Botha JF, Magnius LO, Kew MC. Full genome analysis of hepatitis B virus genotype E strains from South-Western Africa and Madagascar reveals low genetic variability. *J Med Virol* 2005; 77: 47–52.
13 Sugauchi F, Orito E, Kato H *et al.* Genotype, serotype, and phylogenetic characterization of the complete genome sequence of hepatitis B virus isolates from Malawian chronic carriers of the virus. *J Med Virol* 2003; 69: 33–40.
14 Hasegawa I, Tanaka Y, Kurbanov F *et al.* Molecular epidemiology of hepatitis B virus in the United Republic of Tanzania. *J Med Virol* 2006; 78: 1035–42.
15 Kimbi GC, Kramvis A, Kew MC. Distinctive sequence characteristics of subgenotype A1 isolates of hepatitis B virus from South Africa. *J Gen Virol* 2004; 85: 1211–20.
16 Kew MC, Kramvis A, Yu MC, Arakawa K, Hodkinson J. Increased hepatocarcinogenic potential of hepatitis B virus genotype A in Bantu-speaking sub-saharan Africans. *J Med Virol* 2005; 75: 513–21.
17 Hardie DR, Williamson C. Analysis of the preS1 gene of hepatitis B virus (HBV) to define epidemiologically linked and un-linked infections in South Africa. *Arch Virol* 1997; 142: 1829–41.
18 Bekondi C, Olinger CM, Boua N *et al.* Central African Republic is part of the West-African hepatitis B virus genotype E crescent. *J Clin Virol* 2007; 40: 31–7.
19 Makuwa M, Souquiere S, Telfer P *et al.* Identification of hepatitis B virus subgenotype A3 in rural Gabon. *J Med Virol* 2006; 78: 1175–84.
20 Toan NL, Song H, Kremsner PG *et al.* Impact of the hepatitis B virus genotype and genotype mixtures on the course of liver disease in Vietnam. *Hepatology* 2006; 43: 1375–84.
21 Olinger CM, Venard V, Njayou M *et al.* Phylogenetic analysis of the precore/core gene of hepatitis B virus genotypes E and A in West Africa: new subtypes, mixed infections and recombinations. *J Gen Virol* 2006; 87: 1163–73.
22 Kurbanov F, Tanaka Y, Fujiwara K *et al.* A new subtype (subgenotype) Ac (A3) of hepatitis B virus and recombination between genotypes A and E in Cameroon. *J Gen Virol* 2005; 86: 2047–56.
23 Suzuki S, Sugauchi F, Orito E *et al.* Distribution of hepatitis B virus (HBV) genotypes among HBV carriers in the Cote d'Ivoire: complete genome sequence and phylogenetic relatedness of HBV genotype E. *J Med Virol* 2003; 69: 459–65.
24 Huy TT, Ishikawa K, Ampofo W *et al.* Characteristics of hepatitis B virus in Ghana: full length genome sequences indicate the endemicity of genotype E in West Africa. *J Med Virol* 2006; 78: 178–84.
25 Huy TT, Ushijima H, Win KM *et al.* High prevalence of hepatitis B virus pre-s mutant in countries where it is endemic and its relationship with genotype and chronicity. *J Clin Microbiol* 2003; 41: 5449–55.
26 Candotti D, Danso K, Allain JP. Maternofetal transmission of hepatitis B virus genotype E in Ghana, West Africa. *J Gen Virol* 2007; 88: 2686–95.
27 Candotti D, Opare-Sem O, Rezvan H, Sarkodie F, Allain JP. Molecular and serological characterization of hepatitis B virus in deferred Ghanaian blood donors with and without elevated alanine aminotransferase. *J Viral Hepat* 2006; 13: 715–24.
28 Vray M, Debonne JM, Sire JM *et al.* Molecular epidemiology of hepatitis B virus in Dakar, Senegal. *J Med Virol* 2006; 78: 329–34.

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