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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▽†

Kanako Tatematsu,[1] Yasuhito Tanaka,[1]* Fuat Kurbanov,[1] Fuminaka Sugauchi,[2] Shuhei Mano,[3] Tatsuji Maeshiro,[4] Tomokuni Nakayoshi,[5] Moriaki Wakuta,[6] Yuzo Miyakawa,[7] and Masashi Mizokami[1,8]

Department of Clinical Molecular Informative Medicine[1] and Department of Gastroenterology and Metabolism,[2] Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan; Nagoya City University Graduate School of Natural Sciences, Nagoya, Japan[3]; Control and Prevention of Infectious Diseases, Department of Medicine and Therapeutics, Faculty of Medicine, University of the Ryukyus, Okinawa, Japan[4]; Heart Life Hospital, Okinawa, Japan[5]; Wakusan Clinic. Okinawa, Japan[6]; Miyakawa Memorial Research Foundation, Tokyo, Japan[7]; and Research Center for Hepatitis and Immunology, International Medical Center of Japan Kohnodai Hospital, Chiba, Japan[8]

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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-

* Corresponding author. Mailing address: Department of Clinical Molecular Informative Medicine, Nagoya, City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya 467-8601, Japan. Phone: (81) 52-853-8292. Fax: (81) 52-842-0021. E-mail: ytanaka@med.nagoya-cu.ac.jp.

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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

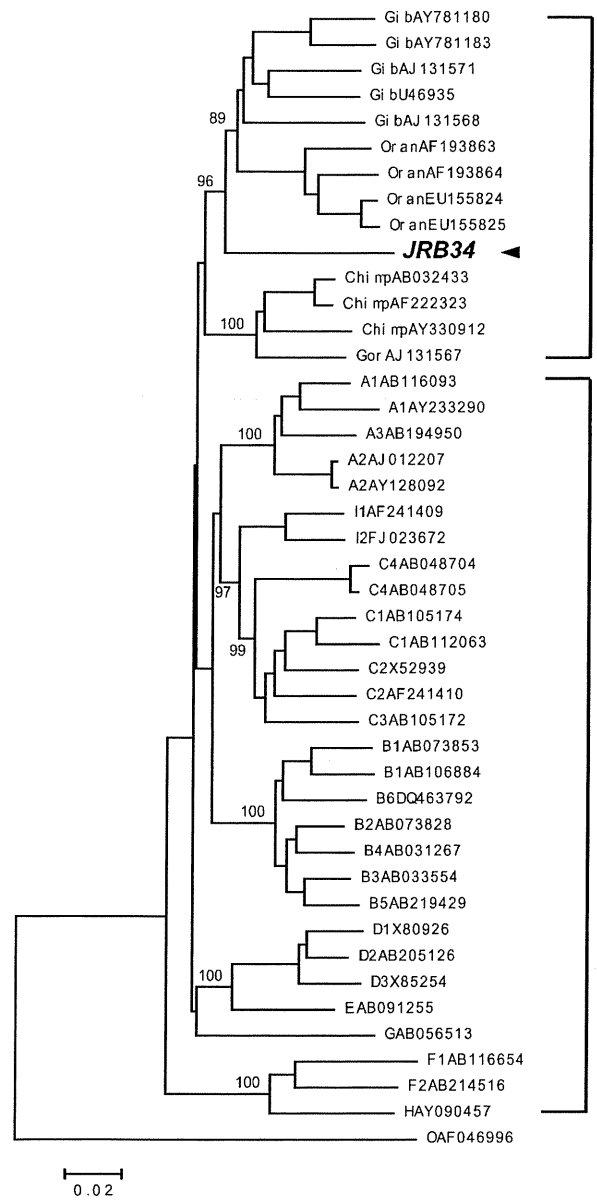

FIG. 1. Phylogenetic tree constructed on the entire genome sequences of 44 HBV isolates representing four ape and eight human genotypes. A woolly monkey HBV isolate serves as an outgroup. The HBV/J isolate (JRB34) is indicated by an arrowhead, and the genetic distance is indicated by a bar below.

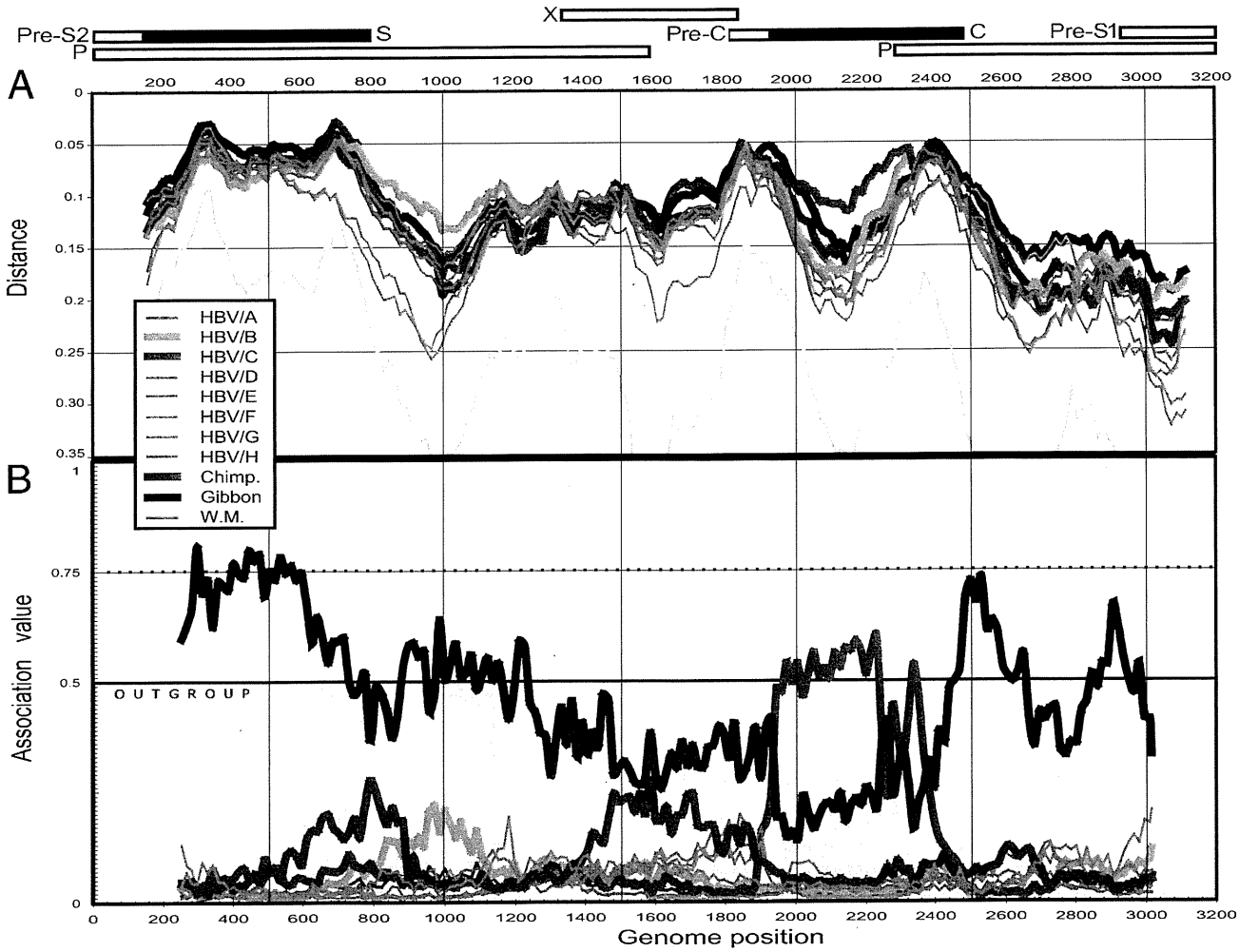

FIG. 2. Complete genome scanning carried by PHYLIP, the phylogeny inference package implemented in the Simmonic software, for the JRB34 strain versus 228 selected nonrecombinant HBV genotypes (HBV/Ba and HBV/I not included) reference strains grouped by genotype. Kimura two-parameter distance model (A) 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

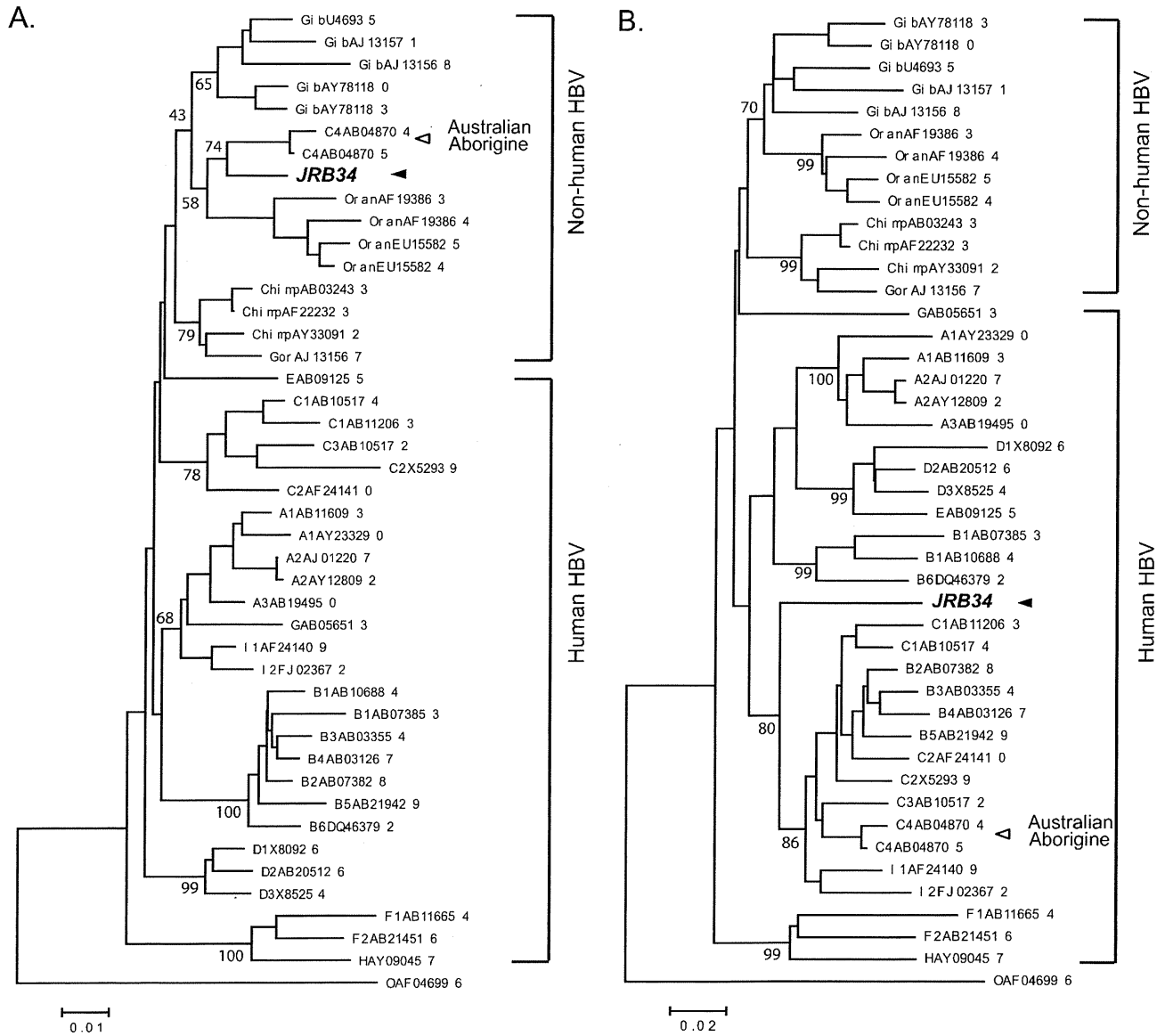

FIG. 3. Phylogenetic tree constructed on the preS/S gene (A) and C gene (B) sequences of 44 HBV isolates representing four ape and eight human genotypes. A woolly monkey HBV isolate serves as an outgroup. The HBV/J isolate (JRB34) is indicated by an arrowhead, and an HBVC4 isolate from Australian aborigine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

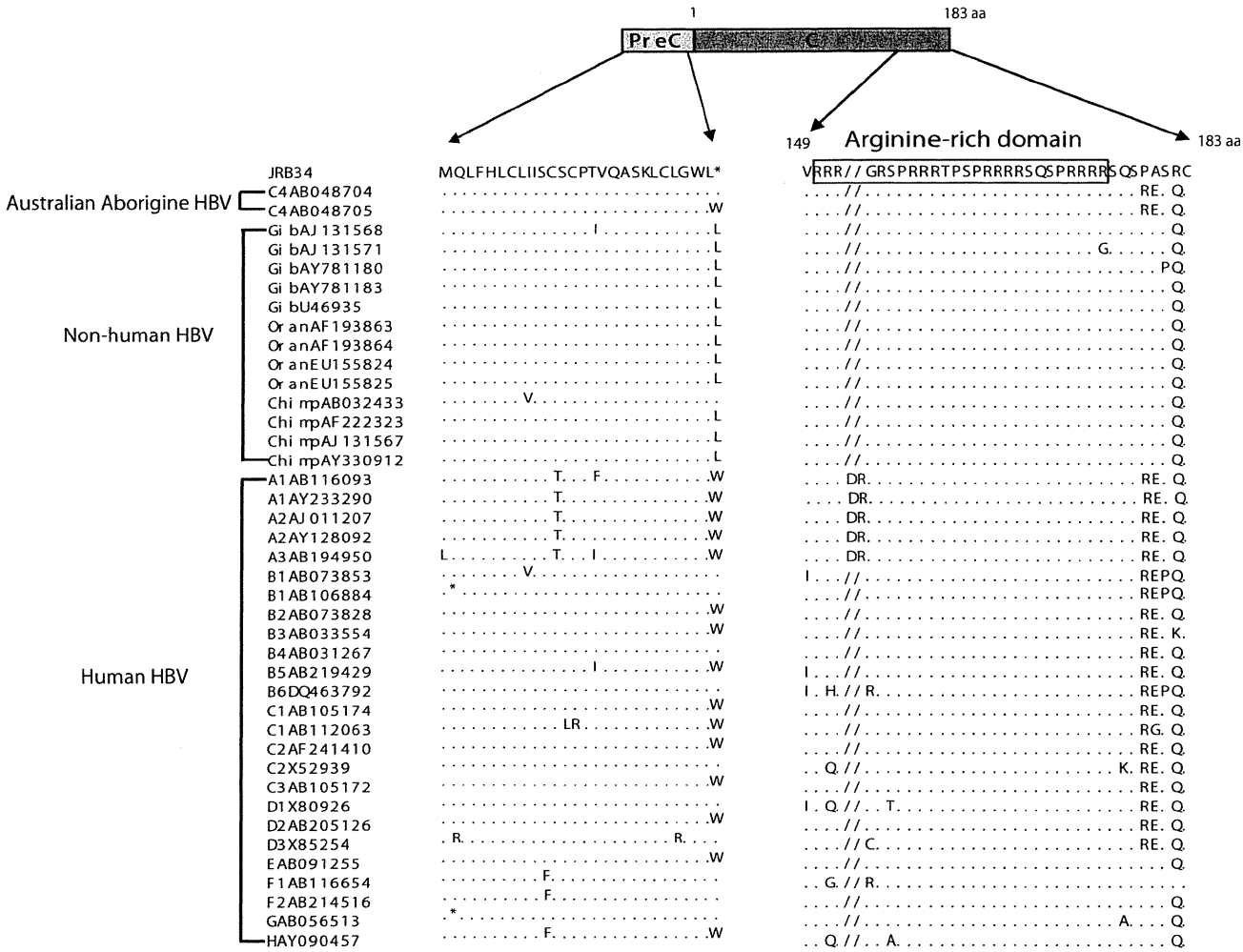

FIG. 4. Comparison of the amino acid sequence in the preC gene and carboxy-terminal amino acid sequences in the C gene of HBV isolates of various genotypes. The sequence of the HBV/J isolate (JRB34) is indicated at the top. Dots represent amino acids shared by JRB34, and a dash indicates the deletion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

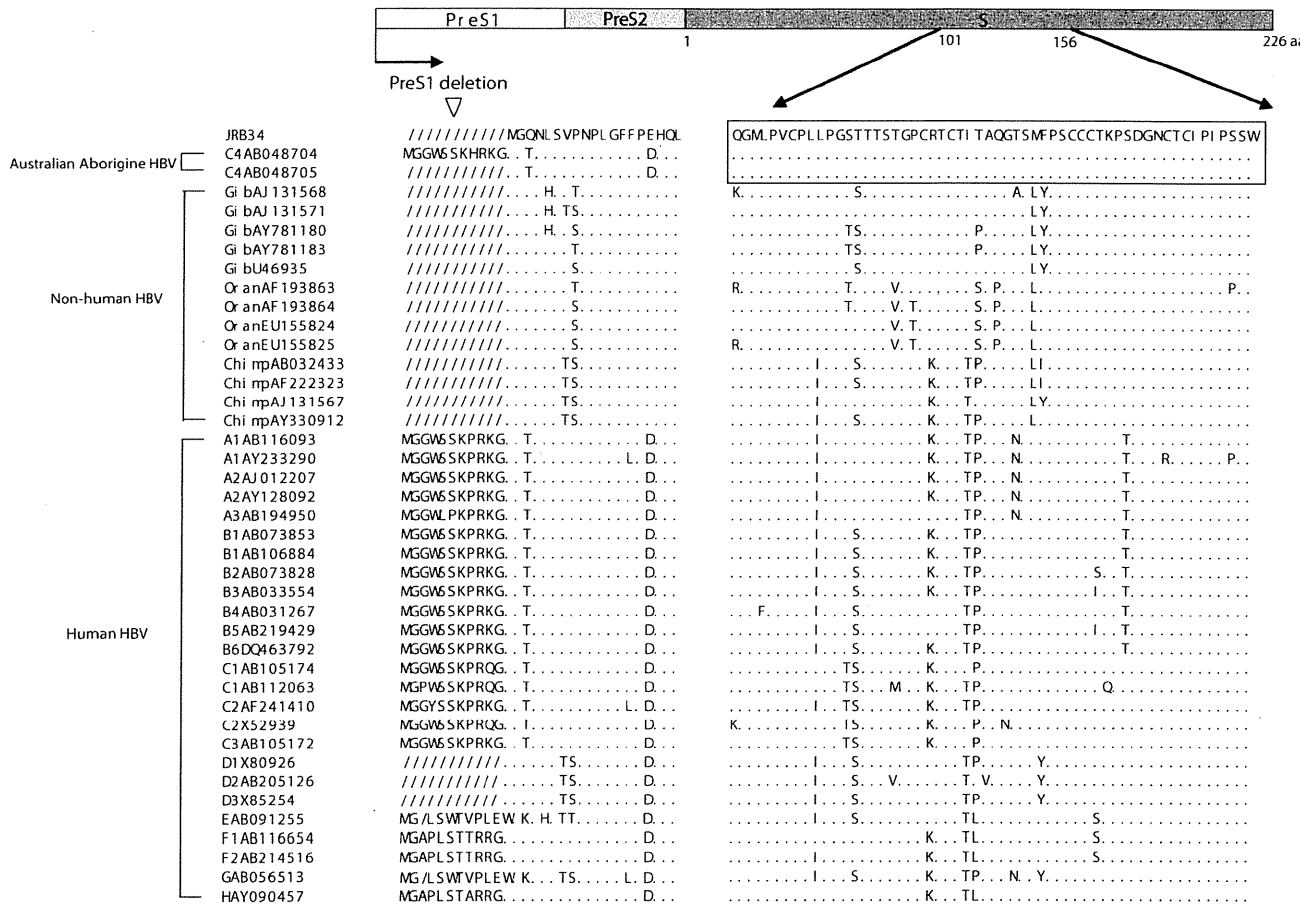

FIG. 5. Comparison of amino acid sequences of the preS/S gene among HBV isolates of various genotypes. The sequence of the HBV/J isolate (JRB34) is indicated at the top. Dots represent amino acids shared by JRB34, and a dash indicates the deletion of an amino acid. The sequence from positions 101 to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

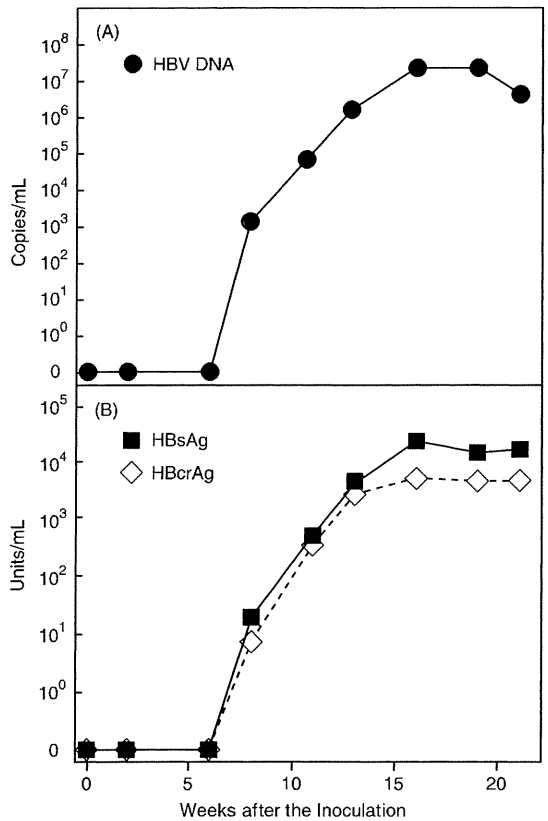

FIG. 6. Markers of HBV infection in two chimeric mice inoculated with the HBV/J isolate (JRB34). The levels of HBV DNA are illustrated in panel A, and those of HBsAg and HBcrAg are illustrated in panel B. Values represent the means for two mice.

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

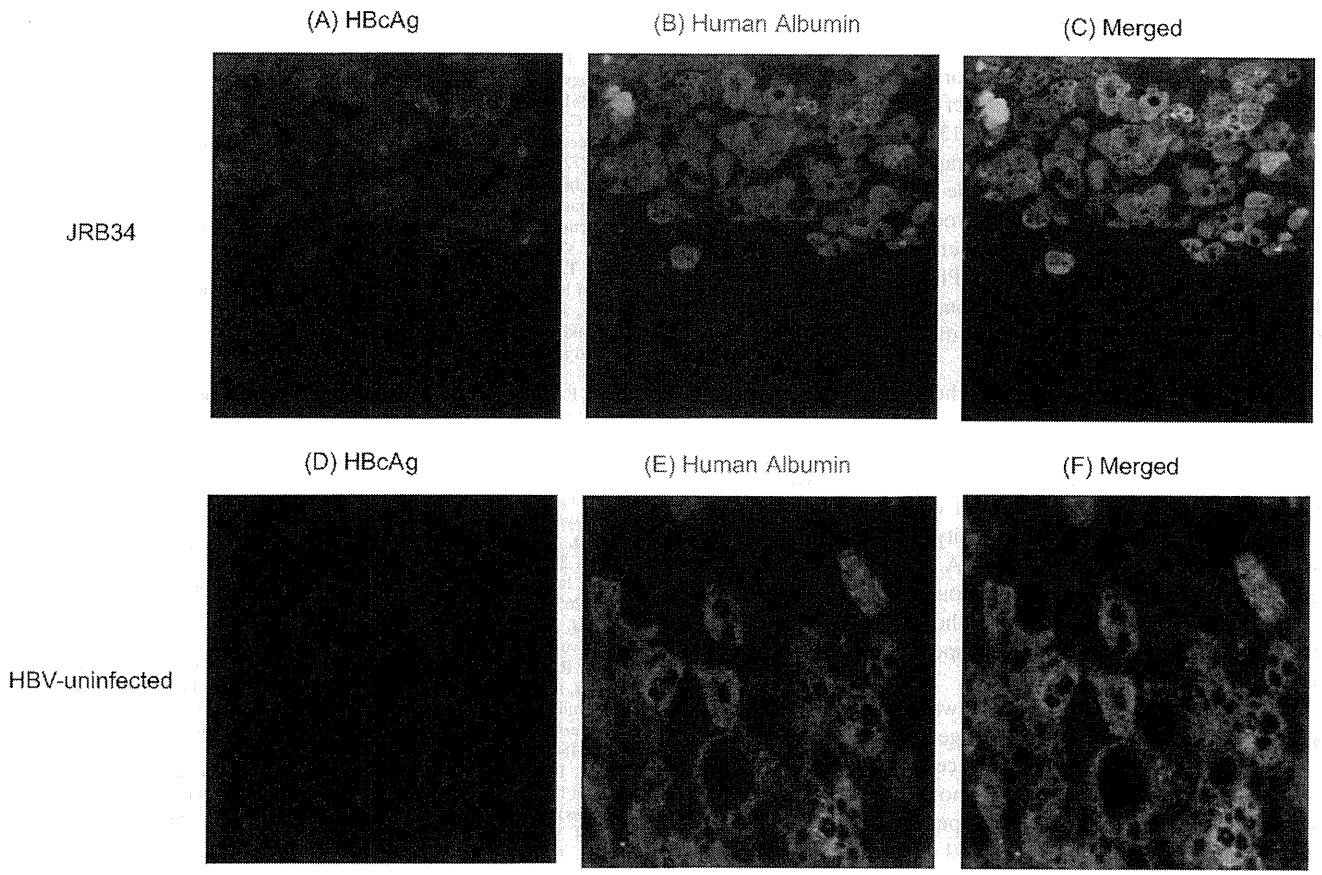

FIG. 7. (A and B) Immunofluorescent staining of a frozen liver section of a chimera mouse inoculated with the HBV/J isolate (JRB34). HBcAg is stained in panel A, and human albumin is stained in panel B. (C) Colocalization of HBcAg and human albumin is revealed by double staining. (D to F) HBV-uninfected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

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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Epidemic Spread of Hepatitis C Virus Genotype 3a and Relation to High Incidence of Hepatocellular Carcinoma in Pakistan

Anis Khan,[1] Yasuhito Tanaka,[1] Zahid Azam,[2] Zaigham Abbas,[3] Fuat Kurbanov,[1] Uzma Saleem,[2] Saeed Hamid,[2] Wasim Jafri,[2] and Masashi Mizokami[1]*

[1]*Department of Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya, Japan*
[2]*Department of Medicine, The Aga Khan University, Stadium Road, Karachi, Pakistan*
[3]*Department of Hepatogastroenterology Sind Institute of Urology and Transplantation, Karachi, Pakistan*

Studies conducted in different populations worldwide revealed an association between HCV genotype 1 and the development of hepatocellular carcinoma (HCC) than in infection with other HCV genotypes. There are reports which reveal the association of HCV genotype 3a (HCV-3a) with hepatic steatosis and fibrosis but its relation with the development of HCC has not been investigated. In Pakistan, where the incidence of HCC is increasing, 189 patients with chronic liver disease including 82 with HCC were enrolled. HCV genotypes were determined by phylogeny in the NS5B region and the epidemic history of HCV-3a was examined using coalescent theory based methods. HCV-3a was the predominant genotype (81.4%) in the cohort studied, followed by 3b (9.3%), 3k (2.3%), 1a (1.5%), 1c (1.5%), 1b (0.8%), and 2a (0.8%) where 76% of HCC and 86% of non-HCC were infected with HCV-3a. The significant factors associated with HCC were older age (mean ± SD) 55.8 (±9.9) ($P < 0.0001$), and male gender ($P < 0.001$). HCV RNA was significantly higher in patients with HCC and chronic hepatitis than in liver cirrhosis ($P < 0.0001$). Molecular evolutionary analysis revealed a distinct phylogenetic cluster of HCV-3a in Pakistan and an estimation of the effective number of HCV infections indicated the appearance of HCV-3a in this region around 1920s and a rapid exponential growth in the 1950s. This indicates that the epidemic spread of HCV-3a occurred earlier in Pakistan than in other countries in which this genotype has been reported. HCV-3a which spread earlier in Pakistan may be associated with an increasing incidence of HCC. ***J. Med. Virol. 81:1189–1197, 2009.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: HCV; genotype 3a; hepatocellular carcinoma; molecular evolutionary analysis; transmission; Pakistan

INTRODUCTION

Chronic infections with hepatitis C virus (HCV) or hepatitis B virus (HBV) are the most important causes of hepatocellular carcinoma (HCC). According to the World Health Organization (WHO), approximately 350 million people are infected chronically with HBV [2004] and 170 million with HCV [1999]. In the developing countries of Asia and Africa, although HBV infection is the commonest cause of chronic liver diseases, HCV is evolving rapidly and in most areas has become more important than HBV as a potential cause of substantial morbidity and mortality [Shepard et al., 2005]. But the relative importance of HBV and HCV infections in the etiology of HCC is known to vary greatly and can change overtime [Lu et al., 2006; Raza et al., 2007].

HCV has been classified into six major genotypes [Simmonds et al., 1993; Bukh et al., 1994; Robertson et al., 1998] and within each genotype there are many subtypes varying in geographical distribution and transmission patterns [Simmonds et al., 1993]. Subtypes 1a, 1b, 2a, 2b, and 3a are distributed globally and

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*Correspondence to: Prof. Masashi Mizokami, MD, PhD, Department of Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medical Sciences, Kawasumi 1, Mizuho, Nagoya 467-8601, Japan.
E-mail: mizokami@med.nagoya-cu.ac.jp

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account for the majority of HCV infections worldwide [Smith et al., 1997; Mondelli and Silini, 1999]. Genotypes 1, 2, and 4 appear to be endemic to the regions of West and Central Africa and the Middle East, whereas divergent endemic strains of genotypes 3 and 6 are found in Southeast Asia [Frank et al., 2000]. The risk factors cited most frequently as accounting for the majority of HCV transmission worldwide are blood transfusions from unscreened blood donors, injection drug use, unsafe therapeutic injections, and other healthcare-related procedures. Potential percutaneous exposure to blood also contribute to the transmission of HCV [Shepard et al., 2005].

Although HCV infection has both acute and chronic forms, most infections are asymptomatic initially and available assays do not distinguish acute from chronic or resolved infection. The time lag between HCV infection and the development of cancer takes more than 2–3 decades [Kiyosawa et al., 1990; Mansell and Locarnini, 1995; Shiratori et al., 1995], hence a major determinant of the future burden of the disease is the past and present incidence of infection. HCV infection has been implicated in the increasing incidence of HCC in several developed countries and many others have projected a steady increase in the incidence of HCV-related complications in future decades [Deuffic et al., 1998; el-Serag, 2001]. Using methods based on coalescent theory, the epidemic history of HCV in the population can be reconstructed from the observed genetic diversity of viral strains. The molecular clock theory has been applied successfully in previous studies to examine the population dynamics for HCV [Pybus et al., 2001, 2003; Tanaka et al., 2002, 2006] including the epidemic history of HCV infection in intravenous drug users [Pybus et al., 2005].

In the developing world the future burden of HCV infection is more difficult to predict because of the poor quality of the available epidemiological data. The HCV seroprevalence data in Pakistan ranges between 2.4% and 6.5% [Luby et al., 1997; Mujeeb et al., 2000; Khattak et al., 2002] among the general population. It is estimated that nearly 80% of HCCs in Pakistan have anti-HCV [Khokhar et al., 2003] principally male subjects who develop HCC in the 5th or 6th decade of life.

It is thought that genetic heterogenicity of HCV may account for some of the differences in the outcome of the disease and response to treatment. Several studies have evaluated specifically the role of HCV genotypes in the severity of the disease but many questions have not been answered [Silini et al., 1995; Zein and Persing, 1996; Zein et al., 1996a; Zein, 2000]. There is a lack of data on the correlation between HCV genotypes and the severity of liver disease in the Indian sub-continent particularly in Pakistan, which could have elucidated the factors behind the increasing incidence of HCC in Pakistan. The present study was conducted to investigate: (1) the etiology of chronic liver disease in Pakistan; (2) the relative contribution of HCV and HBV in the development of HCC; (3) Molecular epidemiology of HCV genotypes and the origin and worldwide spread of HCV genotype 3a (HCV-3a) using Coalescent-based approach based on principles both of population genetics and mathematical epidemiology.

MATERIALS AND METHODS

Serum Samples

One hundred eighty nine serum samples were collected from consecutive untreated patients with chronic liver disease in Pakistan during January 2006 to September, 2007. Informed consent was obtained at each centre or hospital from each patient for participating in the virology research. The study protocol conformed to the 1975 Declaration of Helsinki and was approved by the Ethnic Committees of each institution. Patients were divided into two groups, non-HCC and HCC. The non-HCC group consisted of patients with chronic hepatitis and liver cirrhosis, diagnosed on the basis of clinical, biochemical examination and ultrasonography (US). The patients with liver cirrhosis were diagnosed prinicipally by ultrasonographic findings such as coarse liver architecture, nodular liver surface, blunt liver edges and hypersplenism. Patients with HCC were diagnosed by two out of three criteria, that is, serum alfa-fetoprotein (AFP) >400 IU/ml, contrast enhancement by computer tomography (CT) or either magnetic resonance imaging (MRI) or liver biopsy. The base line clinical characteristics of the patients are shown in Table I.

Serological Tests

Patients were screened for HBsAg by enzyme immunoassay (EIA) (Abbott Laboratories, Abbott Park, IL) and anti-HCV at each centre using a third-generation EIA kit (Abbott Laboratories). All samples were retested for anti-HCV, HBsAg, anti-HBc, anti-HBs by chemiluminescence with commercial assay kits (Fujirebio, Inc., Tokyo, Japan).

Hepatitis D virus antibody (anti-HDV) was assessed using Abbott Anti-Delta EIA assay. Serum AFP was measured by RIA labelled kit (LBA AFP-L3, Waco Chem. Indus. Ltd., Waco, TX). All the samples were also tested for HIV co-infection Genedia HIV-1/2 (Fujirebio, Tokyo, Japan). Biochemical markers such as alanine-amino transferase (ALT), aspartate-amino-transferase (AST), alkaline phosphatase (ALP), bilurubin, albumin and prothrombin time (PT) were also measured in samples at local hospitals.

HCV Genotyping and RNA Quantitation

Total RNA were extracted from the serum samples using the SepaGene RV-R Nucleic acid extraction kit (Sanko Junyaku Co., Ltd, Tokyo, Japan) in accordance with the manufacturer's protocol. Viral RNA were reverse transcribed to complementary DNA using SuperScript II RNase H^- Reverse Transcriptase (Invitrogen Corp., Carlsbad, CA) and random hexamer primer (Takara Shuzo Co. Ltd, Tokyo, Japan) as described previously [Ohno et al., 1997]. Confirmation

of the presence of HCV-RNA in the samples was carried out by amplifying the highly conserved 5'UTR region and HCV genotypes were determined for both structural (E1/Core) and non-structural (NS5B) viral genes using either one or both genotyping polymerase chain reactions (PCR) [Hashimoto et al., 1988; Ohno et al., 1997] and/or direct sequencing with genotype universal primers [Tanaka et al., 2002].

HCV RNA in all HCV RNA-positive samples was quantified by real-time PCR as described previously [Takeuchi et al., 1999] with slight modifications in an ABI7500 FAST system. The detection limit of the assay was, as few as, 10 copies/ml.

Confirmation of HBV DNA and HDV RNA in Samples

HBV DNA was extracted by QIAamp DNA Blood Mini Kit (Qiagen, Inc., Hilden, Germany) from 100 ml of each HBsAg positive serum. Partial core and S regions were amplified in order to detect HBV DNA in the samples using the primers described previously [Sugauchi et al., 2001]. The detection limit for this study was 100 copies/ml [Tanaka et al., 2004b].

HDV RNA was extracted from anti-HDV positive samples and reverse transcribed into cDNA using a random hexamer primer as described for HCV [Ohno et al., 1997]. A part of the HDVAg coding region of HDV was amplified using specific primers described previously [Nakano et al., 2001].

Sequences and Phylogenetic Analysis

Amplicons obtained in the NS5B region (nucleotides from 8,278 to 8,618) were directly sequenced with Prism Big Dye (Applied Biosystems, Foster City, CA) in an ABI 3100 DNA automated sequencer. The sequences for phylogenetic analysis were retrieved from DDBJ/EMBL/GeneBank. Alignments were performed using CLUSTAL W (http://clustalw.ddbj.nig.ac.jp/top-e.html) and neighbor-joining trees were constructed with 6-Parametric method and bootstrapped 1,000 times to confirm the reliability of the phylogenetic tree [Shin et al., 2008].

The nucleotide sequence data reported in this paper appears in the DDBJ/EMBL/GenBank nucleotide sequence database with the accession number(s) AB444429 to AB444582.

Statistical Analysis

Statistical differences were evaluated by Fisher's exact probability test and Chi-square test with Yates' correction where appropriate, using the STATA software version 8.0 (Stata Corp. LP, College Station, TX). Differences were considered significant for P values smaller than 0.05.

Molecular Evolutionary Analyses

A reconstructed tree was built on the NS5B sequence of 336 nucleotides by a heuristic maximum-likelihood topology search with stepwise addition and the nearest-neighbor–interchange algorithms. Tree likelihood scores were calculated using the HKY85+G method with the molecular clock enforced, using Parsimony (PAUP) (Sinauer Associates, Inc., Publishers, Sunderland, MA) version 4.0b8. To confirm the reliability of the phylogenetic tree, either a bootstrap re-sampling test or an interior branch test for the neighbor joining tree was performed 1,000 times.

As estimates of the demographic history, a non-parametric function known also as the skyline plot was obtained by transforming coalescent intervals of an observed genealogy into a piecewise plot that represents an effective number of infections through time. A parametric for maximum-likelihood was estimated with the computer software Genie v3.5 (University of Oxford, Oxford, UK), in order to build a statistical framework to infer the demographic history of a population on phylogenies reconstructed on sampled DNA sequences. This model assumes a continuous epidemic process in which the viral transmission parameters remain constant through time. Model-fitting was evaluated by likelihood ratio tests of the parametric maximum-likelihood estimates.

RESULTS

Seroprevalence of HCV, HBV, and/or HDV Among Patient Groups

A total of 189 serum samples were collected from patients with chronic liver disease (non-HCC = 107, HCC = 82) in Pakistan. Male gender predominated in this study, with a male to female ratio of 1.2, and associated significantly with HCC ($P < 0.001$). The mean age of patients in HCC group was significantly higher than non-HCC (55.8 ± 9.9 vs. 41.3 ± 11.5, $P < 0.0001$). These estimations are summarized in Table I. Serum AFP was significantly higher in patients with HCC than in patients without HCC ($P < 0.0005$), while other biochemical markers did not reach statistical significance between both groups (Table I). HCV infection was found to be high in both groups (87% and 79.2%, respectively). HCV RNA was detectable in 76.3% of patients without HCC and 89.2% of patients with HCC. The seroprevalence of anti-HBc was 79.4% in patients without HCC and 74.3% in patients with HCC. Although the serum anti-HBs was relatively less detectable in the cohort studied but it was relatively high in the patients without HCC than in patients with HCC (42% and 32.9% respectively). A total of 23 patients (12.1%) were positive for HBsAg (11.2% in patients without HCC and 13.4% in patients with HCC). Serum HBV DNA was detectable in 58.3% of patients without HCC and 81.8% of patients with HCC among HBsAg-positive cases in each group. Overall 30.4% of the cases were positive for anti-HDV, with a higher incidence (50%) in patients without HCC than HCC (9%) among HBsAg positive cases, but not constituting a significant difference. HDV viremia was detectable in 3 of 6 (50%) patients without HCC but was undetectable in HCC (Table I). None of the samples in