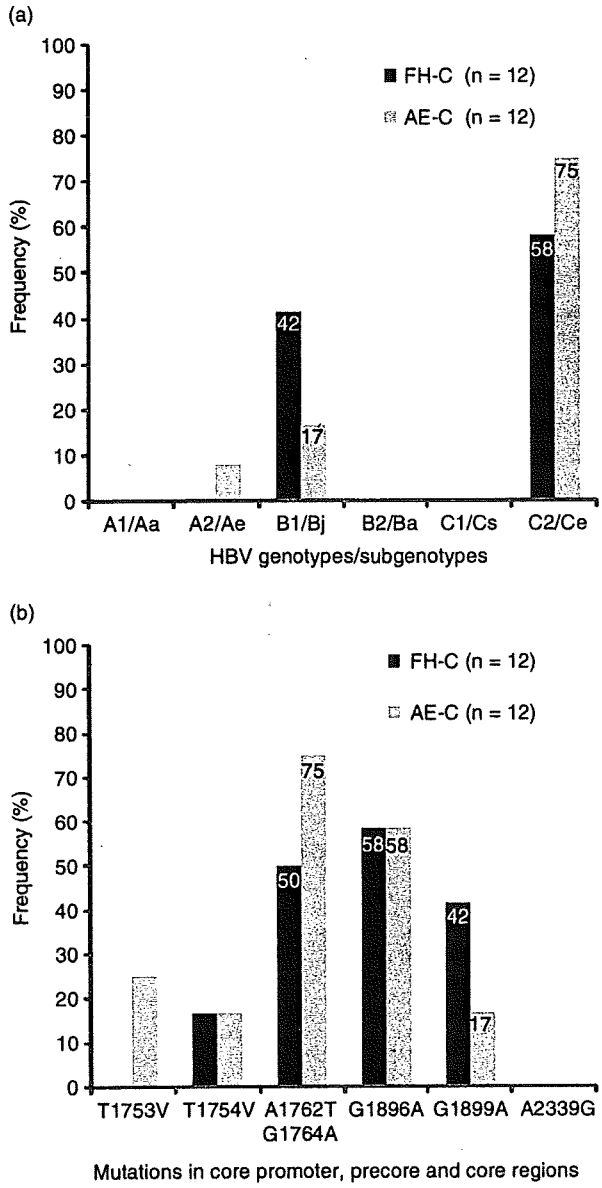

Figure 3 Genotypes/subgenotypes (a) and mutations in core promoter, pre-core and core regions (b) between the 12 transient hepatitis B virus infection (FH-T) and the 12 acute self-limited hepatitis B (AHB) patients.

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

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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Distribution of Hepatitis B Virus Genotypes among Patients with Chronic Infection in Japan Shifting toward an Increase of Genotype A▽

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Acute hepatitis B virus (HBV) infection has been increasing through promiscuous sexual contacts, and HBV genotype A (HBV/A) is frequent in patients with acute hepatitis B (AHB) in Japan. To compare the geographic distribution of HBV genotypes in patients with chronic hepatitis B (CHB) in Japan between 2005 and 2006 and between 2000 and 2001, with special attention to changes in the proportion of HBV/A, a cohort study was performed to survey changes in genotypes of CHB patients at 16 hospitals throughout Japan. Furthermore, we investigated the clinical characteristics of each genotype and examined the genomic characteristics of HBV/A isolates by molecular evolutionary analyses. Of the 1,271 patients, 3.5%, 14.1%, and 82.3% were infected with HBV/A, -B, and -C, respectively. In comparison with our previous survey during 2000 and 2001, HBV/A was twice as frequent (3.5% versus 1.7%; $P = 0.02$). The mean age was lower in the patients with HBV/A than in those with HBV/B or -C. Based on phylogenetic analyses of 11 full-length genomes and 29 pre-S2/S region sequences from patients, HBV/A isolates were imported from Europe and the United States, as well as the Philippines and India. They clustered with HBV/A from AHB patients and have spread throughout Japan. HBV/A has been increasing in CHB patients in Japan as a consequence of AHB spreading in the younger generation through promiscuous sexual contacts, aided by a tendency of HBV/A to induce chronic hepatitis. The spread of HBV/A infection in Japan should be prevented by universal vaccination programs.

Hepatitis B virus (HBV), a member of the *Hepadnaviridae*, is a circular, partially double-stranded DNA virus and is one of the major causes of chronic liver diseases, including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC).

The HBV genome is composed of approximately 3,200 nucleotides. HBV is classified into eight genotypes, designated A to H, based on an intergroup divergence of 8% or more in the complete nucleotide sequence (3, 23, 26, 37). They have distinct geographical distributions and are associated with differences in clinical and virological characteristics, such as severity of liver disease and response to antiviral therapies (7, 8, 12, 13, 22, 28). Furthermore, subgenotypes have been reported for HBV/A, -B, and -C and named A1 to -3 (17, 38), B1 to -6 (31, 32, 40), and C1 to -6 (20, 31, 45). Equally, other genotypes are classified into subgenotypes. There have been increasing lines of evidence to indicate influences of HBV subgenotypes on the outcome of liver disease and the response to antiviral therapies (1, 39, 44).

In 2001, we reported the geographic distribution of HBV genotypes in Japan (27). Of the 720 Japanese patients with chronic HBV infection (CHB), 12 (1.7%) harbored HBV/A, 88 (12.2%) HBV/B, 610 (84.7%) HBV/C, 3 (0.4%) HBV/D, and 7 (1.0%) mixed genotypes. HBV/C was detected in over 94%

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of patients on the Japanese mainland, while HBV/B was found in 64% of those in Okinawa, the southernmost islands, and 44% of those in the Tohoku area in the northern part of the mainland.

Recently, acute HBV infection (AHB) has been increasing in Japan, predominantly through promiscuous sexual contacts. In addition, it was reported that HBV/A was more frequent in patients with acute hepatitis than in those with chronic hepatitis (29, 41, 49). Recent studies suggest that the chances for progression to chronic disease may differ among patients acutely infected with HBV of distinct genotypes (21, 25); patients infected with HBV/A run an increased risk of becoming HBV carriers. Hence, it is of utmost concern whether chronic HBV/A infection is increasing in Japan.

In the present study, we compared the geographic distribution of HBV genotypes in Japan during 2005 and 2006 with 2000 and 2001, with special attention to changes in the proportion of HBV/A. Furthermore, we investigated the clinical characteristics of each genotype and examined the genomic characteristics of HBV/A isolates by molecular evolutionary analyses.

TABLE 1. Characteristics of 1,271 CHB patients

Parameter	Value
Characteristic	
Male gender [no. (%)]	766 (60.3)
Age (yr; mean ± SD)	51.4 ± 14.0
Diagnosis	
Inactive carrier state [no. (%)]	206 (16.2)
Chronic hepatitis [no. (%)]	786 (61.8)
Cirrhosis [no. (%)]	175 (13.8)
HCC [no. (%)]	104 (8.2)
Antiviral treatment [no. (%)]	577 (45.4)
Blood tests	
Platelets (10^4/mm^3)	21.4 ± 30.2
ALT (IU/liter)	59.8 ± 103.0
ALP (IU/liter)	270.4 ± 136.0
γ−GTP (IU/liter)	47.4 ± 66.1
HBV markers	
HBeAg [no. (%)]	399 (31.4)
HBV DNA (median [range] [log copies/ml])	4.2 (<2.6 to >7.6)

MATERIALS AND METHODS

Patients. From September 2005 to October 2006, sera were collected from 1,370 consecutive patients with CHB at 16 representative hospitals that were liver centers in their respective regions throughout Japan for the purpose of investigating the geographic distribution of HBV genotypes in Japan. All of the patients were diagnosed after they had been followed for at least 12 months. Patients diagnosed with AHB were excluded from the study; they had a sudden onset of clinical symptoms of hepatitis, along with high-titer antibody to HBV core antigen of the immunoglobulin M class in serum. Their sera were tested for alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (γ-GTP), and hepatitis B e antigen (HBeAg), as well as antibody to HBeAg (anti-HBe) (Dinabot, Tokyo, Japan). Four clinical diagnoses were established for them. The inactive carrier state was defined by the presence of HBV surface antigen (HBsAg) with normal ALT levels over 1 year (examined at least four times at 3-month intervals) and without evidence of portal hypertension. Chronic hepatitis was defined by elevated ALT levels (>1.5 times the upper limit of normal [35 IU/liter]) persisting over 6 months (with at least three bimonthly tests). Cirrhosis was diagnosed principally by ultrasonography (coarse liver architecture, nodular liver surface, blunt liver edges, and hypersplenism), platelet counts of <100,000/cm^3, or a combination thereof. Histological confirmation by fine-needle biopsy of the liver was performed as required. HCC was diagnosed by ultrasonography, computerized tomography, magnetic resonance imaging, angiography, tumor biopsy, or a combination thereof.

The study protocol conformed to the 1975 declaration of Helsinki and was approved by the ethics committees of the respective institutions. Every patient or his/her next of kin gave informed consent to the purpose of the study.

Genotypes and subgenotypes of HBV. The six HBV genotypes (A to F) were determined serologically by enzyme immunoassay (EIA) using commercial kits (HBV Genotype EIA; Institutes of Immunology Co., Ltd., Tokyo, Japan). The method depends on the combination of epitopes on pre-S2 region products detected by monoclonal antibodies that were specific for each of them (46, 47). Subgenotypes of HBV/A, designated A1 and A2, were determined by direct sequencing of the pre-S2/S gene, followed by a phylogenetic analysis.

Quantification of HBV DNA and sequencing. HBV DNA levels in sera were quantitated with a commercial kit (Amplicor HBV Monitor; Roche Diagnostics, Basel, Switzerland) with a detection range from 2.6 to 7.6 log copies/ml. Nucleic acids were extracted from 100 μl of serum using the Qiaamp DNA Blood Minikit (Qiagen GmbH, Hilden, Germany). Eleven complete HBV/A genomes and 29 pre-S2/S region sequences were amplified by PCR with appropriate primer sets, as described previously (40). The amplified HBV DNA fragments were directly sequenced using the ABI Prism Big Dye kit version 3.0 (Applied Biosystems, Foster City, CA) in an ABI 3100 automated DNA sequencer (Applied Biosystems). All sequences were analyzed in both forward and reverse directions. Complete and partial HBV genome sequences were aligned using GENETYX version 11.0 (Software Development Co., Ltd., Tokyo, Japan).

Molecular evolutionary analysis of HBV. Reference sequences were retrieved from the DDBJ/EMBL/GenBank databases with their accession numbers for identification. To investigate the relationship between HBV isolates from patients with chronic and acute hepatitis B in Japan, HBV/A isolates (AH1 to -10) were randomly retrieved from them and sequenced in our previous study (29). Nucleotide sequences of HBV DNA were aligned by the program CLUSTAL X, and genetic distance was estimated by the six-parameter method (10) in the Hepatitis Virus Database (36). Based on these values, phylogenetic trees were constructed by the neighbor-joining method (30) with the midpoint rooting option. To confirm the reliability of the phylogenetic trees, bootstrap resampling tests were performed 1,000 times.

Statistical analysis. Categorical variables were compared between groups by the χ^2 test or Fisher's exact test and noncategorical variables by the Mann-Whitney U test. A P value of less than 0.05 was considered significant.

Nucleotide sequence accession numbers. The DDBJ/EMBL/GenBank accession numbers of the complete genome sequences of HBV isolates JPN_CH1 to -11 are AB453979 to AB453989.

RESULTS

Distribution of HBV genotypes among patients with CHB. Of the 1,370 serum samples, the genotype could not be determined for 99 (7.2%) by EIA due to low HBsAg levels, leaving 1,271 for analysis in this study (Table 1). Of these, 206 (16.2%) were inactive carriers, 786 (61.8%) had chronic hepatitis, 175 (13.8%) cirrhosis, and 104 (8.2%) HCC. They had a mean age of 51.4 ± 14.0 years and included 766 (60.3%) men. They had a median HBV DNA level of 4.2 log copies/ml, and 399 (31.4%) of them were positive for HBeAg. Antiviral treatment had been given to 577 (45.4%) of them with interferon, lamivudine, adefovir pivoxil, or entecavir.

The genotypes were HBV/A in 44 (3.5%), HBV/B in 179 (14.1%), HBV/C in 1,046 (82.2%), and HBV/D in 2 (0.2%) (Table 2). In comparison with our previous report on the distribution of genotypes in Japan in 2001 (27), HBV/A was more frequent in this study (3.5% versus 1.7%; $P = 0.02$). Of the 16 hospitals in this study, 10 overlapped with those in our previous report from 2001. In these 10 hospitals, HBV/A was more frequent in the present than in the previous survey (3.6% versus 1.7%; $P = 0.04$).

The distribution of HBV genotypes in Japan differed by

TABLE 2. Distribution of HBV Genotypes

Genotype	No. (%)	
	2005–2006 (n = 1,271)	2000–2001[a] (n = 720)
A	44 (3.5[b])	12 (1.7)
B	179 (14.1)	88 (12.2)
C	1,046 (82.3)	610 (84.7)
D	2 (0.2)	3 (0.4)
Mixed	0 (0.0)	7 (1.0)

[a] From Orito et al. (27).
[b] P = 0.02.

geographic location (Fig. 1). HBV/C was the most prevalent in the majority of areas. In the Tohoku area, the northern part of the Japanese mainland (Honshu), HBV/B was more prevalent than in the other areas of the Japanese mainland. In Okinawa, the southernmost islands of Japan, HBV/B was predominant. Of note, HBV/A was more frequent in the Kanto area (9.5%), the metropolitan area, and Okinawa (9.1%) than in the other areas.

Clinical differences among HBV/A, -B, and -C. Clinical backgrounds were compared among the patients infected with HBV/A, -B, and -C (Table 3). HBeAg was significantly less prevalent in the patients infected with HBV/B than in those infected with HBV/A or -C ($P < 0.01$ for each). When the positivity of HBeAg was stratified by age, HBeAg was markedly less common in patients infected with HBV/B than in those infected with HBV/A or -C who were older than 40 years of age (7/157 [4.5%] versus 4/19 [21.1%] [$P < 0.05$] or 215/755 [28.5%] [$P < 0.01$]) (Fig. 2). There were no significant differences in HBV DNA levels among patients infected with the three genotypes. As antiviral treatments might have influenced the severity of liver disease, clinical states were compared among patients infected with HBV/A, -B, and -C who did and did not receive it; antiviral treatments did not affect the above-mentioned trends represented in Table 3 in age, diagnosis, and HBeAg, as well as ALT and HBV DNA levels (data not shown).

Additionally, we compared the distributions of age and liver diseases in patients infected with HBV/A, -B, and -C. In patients infected with HBV/C, the prevalence of cirrhosis and HCC increased in those older than 50 years of age compared to younger patients (Fig. 3), whereas in the patients infected with HBV/B, cirrhosis and HCC were rare in elderly patients. The proportion of patients younger than 40 years of age was higher in those infected with HBV/A than in those infected with HBV/B or -C (25/44 [56.8%] versus 22/179 [12.3%] or 288/1,046 [27.5%]; $P < 0.01$ for each), while cirrhosis and HCC were also found in those older than 50 years of age infected with HBV/A.

Coinfection with human immunodeficiency virus type 1 (HIV-1) was found in 6 of the 44 (13.6%) patients infected with HBV/A compared to only 3 of the 1,046 (0.3%) patients infected with HBV/C ($P < 0.0001$); it occurred in none of the 179 patients infected with HBV/B.

Phylogenetic analyses. Among the 44 HBV/A isolates, the complete genome was sequenced successfully in 11 (JPN_CH1 to -11). Seven of them were classified as HBV/A2 and four as HBV/A1. A phylogenetic tree was constructed based on the complete genome sequences of these 11 isolates, along with those from two patients with AHB and those from 40 HBV/A isolates retrieved from the database (Fig. 4). Of the seven HBV/A2 isolates, the four from patients with CHB in this study formed a cluster with the Japanese isolates retrieved from the database and two from patients with AHB. Of the other three isolates, JPN_CH5 clustered with French and U.S. isolates, JPN_CH6 with German isolates, and JPN_CH7 with

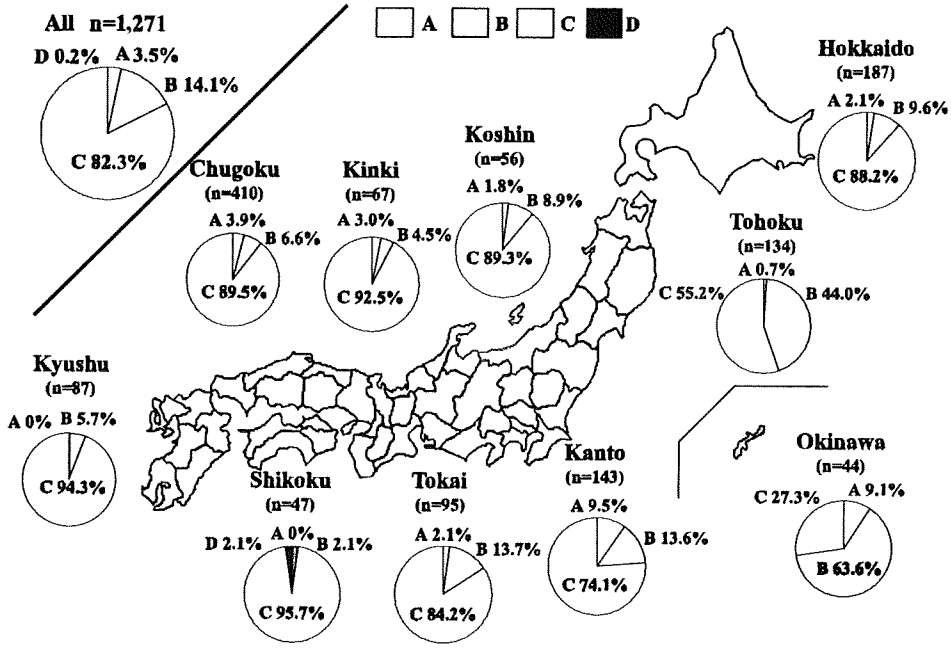

FIG. 1. Geographic distribution of HBV genotypes in patients with chronic HBV infection in Japan during 2005 and 2006.

TABLE 3. Clinical characteristics of individuals chronically infected with HBV of different genotypes

Parameter	Value for genotype:		
	A (n = 44)	B (n = 179)	C (n = 1,046)
Male gender [no. (%)]	32 (72.7)	112 (62.6)	621 (59.4)
Age (yr [mean ± SD])	41.3 ± 14.9[a]	55.8 ± 13.7[b]	48.8 ± 13.3
Diagnosis			
Inactive carrier state [no. (%)]	13 (29.5)[c]	63 (35.2)[b]	129 (12.3)
Chronic hepatitis [no. (%)]	26 (59)	103 (57.5)	656 (62.7)
Cirrhosis [no. (%)]	3 (6.8)	10 (5.6)[b]	162 (15.5)
HCC [no. (%)]	2 (4.5)	3 (1.7)[b]	99 (9.5)
Anti viral treatment [no. (%)]	13 (29.5)[d]	48 (26.8)[b]	516 (49.3)
Blood tests			
Platelet (10^4/mm^3)	23.3 ± 21.9	25.9 ± 35.9[e]	20.6 ± 29.5
ALT (IU/liter)	56.2 ± 83.8	42.2 ± 104.2[e]	63.0 ± 103.3
ALP (U/liter)	247.1 ± 123.0	255.5 ± 97.9	273.9 ± 141.9
γ-GTP (U/liter)	39.6 ± 34.6	49.3 ± 63.4	47.5 ± 67.6
HBV markers			
HBeAg [positive rate(%)]	15 (34.0)[f]	17 (9.5)[b]	367 (35.1)
HBV DNA (median [range]) (log copies/ml)	4.2 (<2.6–>7.6)	4.1 (<2.6–>7.6)	4.2 (<2.6–>7.6)

[a] $P < 0.01$, A versus B or C.
[b] $P < 0.01$, B versus C.
[c] $P < 0.01$, A versus C.
[d] $P < 0.05$, A versus C.
[e] $P < 0.05$, B versusC.
[f] $P < 0.01$, A versus B.

Spanish and Italian isolates. All four HBV/A1 isolates in this study formed a cluster with Philippine and Indian isolates.

In addition, the pre-S2/S region sequences of a total of 29 isolates were determined, including the 11 isolates whose complete genomes were sequenced. Of these, 21 (72%) were classified as HBV/A2 and the remaining 8 as HBV/A1. A phylogenetic tree was constructed based on the pre-S2/S region sequences from the 29 isolates, along with those from 10 patients with AHB infected with HBV/A and 47 HBV/A isolates retrieved from the database (Fig. 5). The 21 HBV/A2 isolates in the present study formed a cluster with Japanese, American, and European isolates retrieved from the database and those from patients with acute hepatitis. In addition, some of them were highly homologous with each other. Likewise, HBV/A1 isolates from eight patients with chronic hepatitis in this study were highly homologous with those from two patients with acute hepatitis and isolates from the Philippines and India. Based on the phylogenetic analyses, HBV/A isolates were imported from Europe and the United States, as well as the Philippines and India, and had infiltrated throughout Japan.

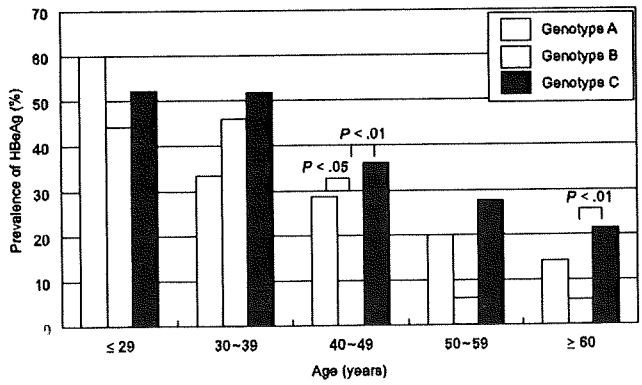

FIG. 2. Prevalence of HBeAg among patients infected with HBV of different genotypes stratified by the age.

DISCUSSION

Perinatal transmission from carrier mothers to their babies has been the principal route for establishing persistent HBV infection in Asian countries (19). In Japan, passive and active immunoprophylaxis with HBV immune globulin and vaccine has been mandated for babies born to HBeAg-positive carrier mothers since 1986; this was extended to HBeAg-negative carrier mothers in 1995. As a result, HBsAg has become rare in Japanese born after 1986; it was detected in only 0.2% of first-time blood donors younger than 19 years of age in 2000 (24). However, AHB has been increasing in Japan, predominantly through promiscuous sexual contacts.

In Japan, HBV/A is detected rarely among patients with CHB but is frequent in those with acute hepatitis (14, 25, 29, 41, 43). Yotsuyanagi et al. reported the distribution of genotypes in 145 Japanese patients with AHB and found HBV/A in 27 (19%), HBV/B in 8 (5%), and HBV/C in 109 (75%) (49). HBV/A is more frequent in metropolitan areas than other areas. The majority of patients with HBV/A infection in metropolitan areas have had extramarital sexual contacts with multiple irregular partners, through which they could have contracted infection. In support of this view, among men who have sex with men (MSM) who are coinfected with HBV and HIV-1 in Tokyo, most were infected with HBV/A (15, 35).

In Japan, AHB in adulthood becomes chronic in only ~1%

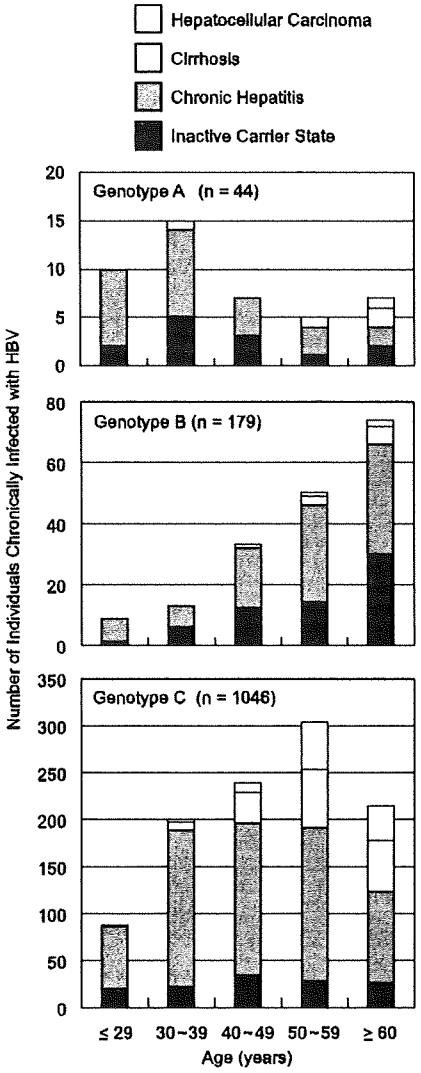

FIG. 3. Distribution of HCC, cirrhosis, chronic hepatitis, and inactive carrier state among the 1,271 patients infected with HBV of different genotypes stratified by the age.

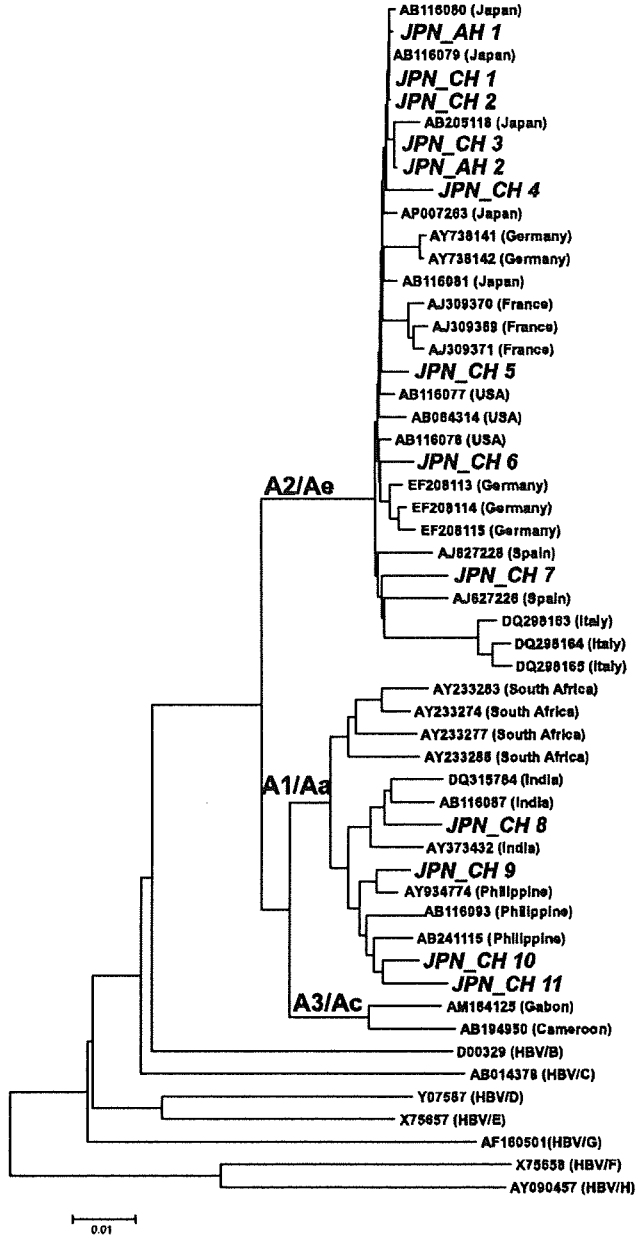

FIG. 4. Phylogenetic tree constructed based on the complete genome sequences of HBV/A isolates. Those from 11 patients with chronic infection in this study are shown in boldface italic (JPN_CH1 to -11), along with two isolates (JPN_AH1 and -2) from patients with acute hepatitis in Japan reported in our previous study (17). Representative isolates were retrieved from the DDBJ/EMBL/GenBank databases, including 21 HBV/Ae, 10 HBV/Aa, and 2 HBV/Ac isolates, along with 7 HBV isolates representative of the other seven genotypes. Isolates from the databases are identified by accession numbers, followed by the country of origin. The bar at the bottom spans 0.01 nucleotide substitutions per site.

of cases. This is much less than the progression to chronic disease (close to 10%) in Europe and the United States, where HBV/A prevails (34). Recent studies have suggested that the chances for persistence may differ among patients acutely infected with HBV of distinct genotypes (21, 25). In particular, acute infection with HBV/A may bring about an increased risk of progression to chronic disease. Therefore, an increase of acute infection with HBV/A would result in a surge of HBV/A among patients with CHB in Japan. In actuality, in comparison with our previous results during 2000 and 2001 (27), HBV/A was twice as frequent in this study (3.5% versus 1.7%; P = 0.02). HBV/A has been increasing in patients with CHB in the Kanto area, where HBV/A in patients with acute hepatitis is more frequent than in the other areas. In the islands of Okinawa, also HBV/A was found to be prevalent in this study. Of the four patients infected with HBV/A there, two were coinfected with HIV-1. They were both MSM, and they were suspected to have been infected with HIV through sexual contacts on the Japanese mainland. It has been reported that HIV infection increases the probability that AHBs will become chronic (2, 11, 33, 48). Because they share routes of transmission and the risk for HIV-1 and HBV infections, approximately

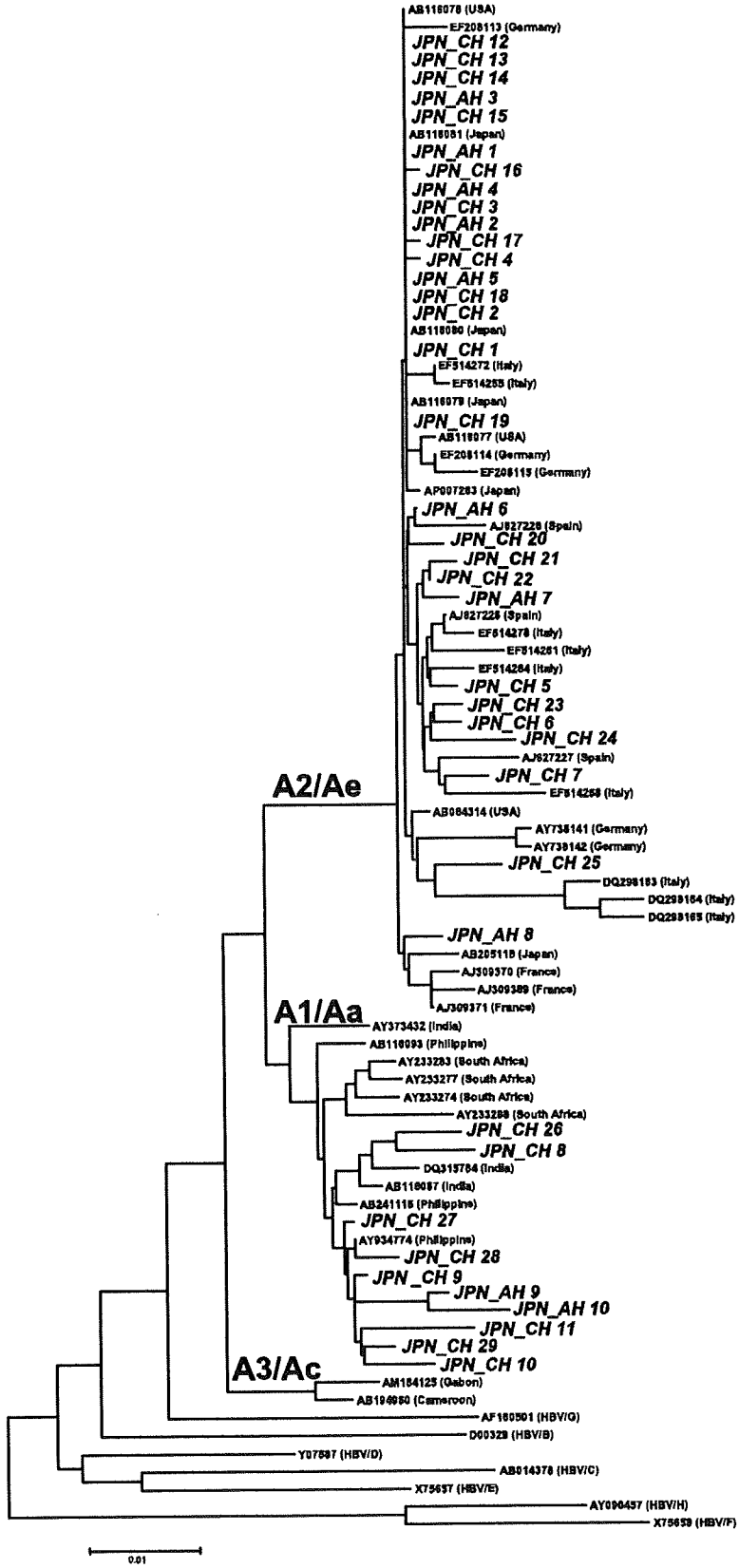

FIG. 5. Phylogenetic tree constructed based on pre-S2/S region sequences of HBV/A isolates. Those from 29 patients with chronic infection in this study are shown in boldface italic (JPN_CH1 to -29), along with 10 isolates (JPN_AH1 to -10) from patients with acute hepatitis in Japan reported in our previous study (17). Representative isolates were retrieved from the DDBJ/EMBL/GenBank databases, including 28 HBV/Ae, 10 HBV/Aa, and 2 HBV/Ac isolates and 7 HBV isolates representative of the other seven genotypes. Isolates from the databases are identified by accession numbers, followed by the country of origin. The bar at the bottom spans 0.01 nucleotide substitutions per site.

90% of patients with AIDS have markers of past or ongoing HBV infection (18). Thus, HBV carriers are more frequent in the HIV-1-positive than in the HIV-1-negative population (4, 9). Among patients with HIV infection in Japan, 6.3% are HBsAg positive, in particular, 8.3% of HIV-infected MSM (16). In this study, coinfection with HIV was found in 6 of the 44 (13.6%) patients infected with HBV/A. All of them were men. Their median age was 27.7 ± 4.1 years, and five patients were positive for HBeAg. Thus, there is a possibility that HIV-1 and HBV/A coinfections are increasing among young people in Japan, and the high rate of HBeAg positivity may be influenced by immune suppression due to HIV infection.

In the phylogenetic analysis, the HBV/A2 isolates recovered in this study were homologous to those from Europe and the United States, and some of them clustered with the Japanese isolates. On the other hand, there were HBV/A1 isolates that formed a cluster with those from the Philippines and India. Furthermore, some isolates from patients with acute hepatitis who were infected with HBV/A in Japan were highly homologous to HBV/A isolates from patients with chronic hepatitis. This invites speculation that some HBV/A isolates were introduced into Japan from foreign countries, while others have already settled down there and spread from patients with chronic infection to their contacts. HBV/A would have been infiltrating throughout Japan by these two different routes.

Clinical differences among patients infected with HBV/A, -B, and -C were observed. The mean age was lower in the patients infected with HBV/A than in those infected with HBV/B or -C. As mentioned above, AHB patients infected with HBV/A have been increasing in the younger generation in Japan, and around 10% of them would have progressed to chronic infection. This is one of the reasons why the patients infected with HBV/A are younger than those infected with HBV/B or -C. Most patients infected with HBV/B were negative for HBeAg, while a high proportion of the patients infected with HBV/A and -C had it. In particular, this difference was remarkable in the patients who were older than 40 years of age. Thus, the seroconversion rate for the loss of HBeAg among younger people may be higher in infection with HBV/B than in that with HBV/A or -C. Inactive carriers were commoner in HBV/A than in HBV/C infection, as well.

These lines of evidence indicate that the activity of hepatitis is lower in HBV/B than HBV/C infection, and patients with HBV/B seroconvert from HBeAg to anti-HBe at young ages. In addition, cirrhosis and HCC were less frequent in the patients infected with HBV/B than in those infected with HBV/C. Therefore, the prognosis would be better in the patients infected with HBV/B than in those infected with HBV/C. These results are in accord with previous reports (5, 13, 28, 42). There have been few reports on the clinical features of patients with chronic hepatitis infected with HBV/A in Japan. Chu et al. have reported the distribution of HBV genotypes with reference to clinical characteristics in the United States (6). They have shown that HBV/A and HBV/C infections are accompanied by a higher frequency of HBeAg than HBV/B infection, while HBV/B is associated with a lower rate of hepatic decompensation than HBV/A and -C. In our study, inactive carriers were commoner, while cirrhosis and HCC were found less often in HBV/A than in HBV/C infection. HBeAg was more prevalent in the patients infected with HBV/A than in those infected with HBV/B who were older than 40 years of age. Therefore, it can be said that the prognosis is better for patients infected with HBV/A than for those infected with HBV/C; it may be poorer than for those infected with HBV/B.

In conclusion, HBV/A has been increasing among CHB patients in Japan. On the basis of phylogenetic analyses, some HBV/A isolates appear to have been imported from foreign countries. They clustered with HBV/A from AHB patients and have infiltrated throughout Japan. It is very likely that acute and chronic infections with HBV/A have been increasing in Japan. Obviously, immunoprophylaxis of perinatal HBV infection, implemented since 1986 on a national basis, has been insufficient to prevent horizontal HBV/A infection diffusing among high-risk groups by transmission routes shared by HIV infection. The foreseeable spread of HBV/A infection in Japan should be prevented by universal vaccination programs extended to high-risk groups or the general population.

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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

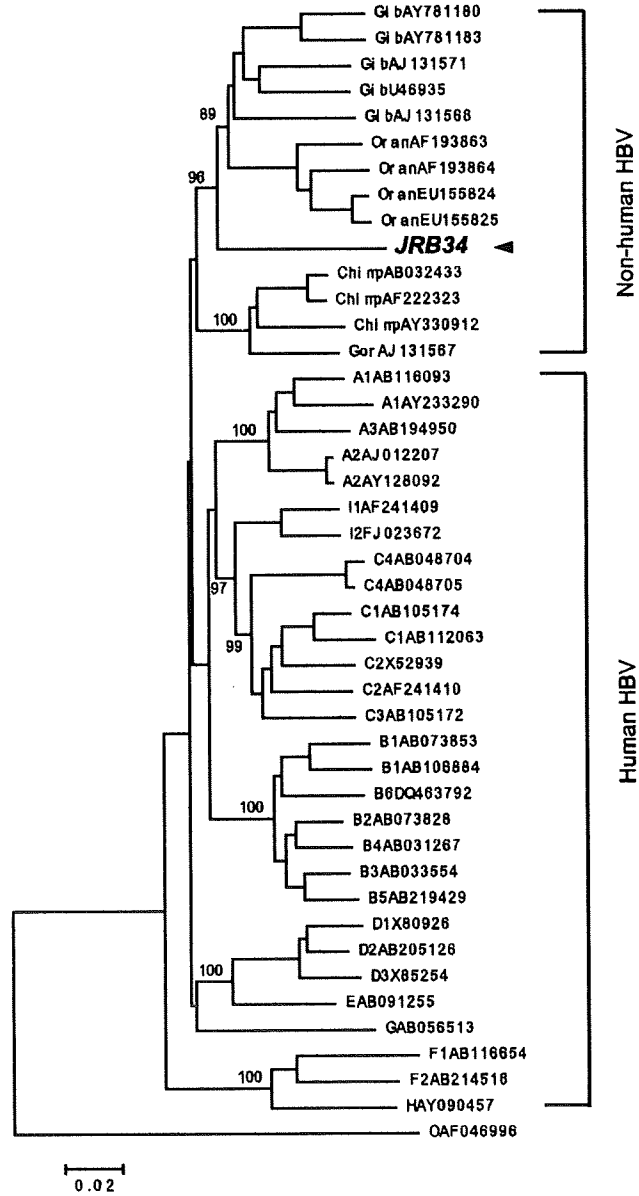

FIG. 1. Phylogenetic tree constructed on the entire genome sequences of 44 HBV isolates representing four ape and eight human genotypes. A woolly monkey HBV isolate serves as an outgroup. The HBV/J isolate (JRB34) is indicated by an arrowhead, and the genetic distance is indicated by a bar below.

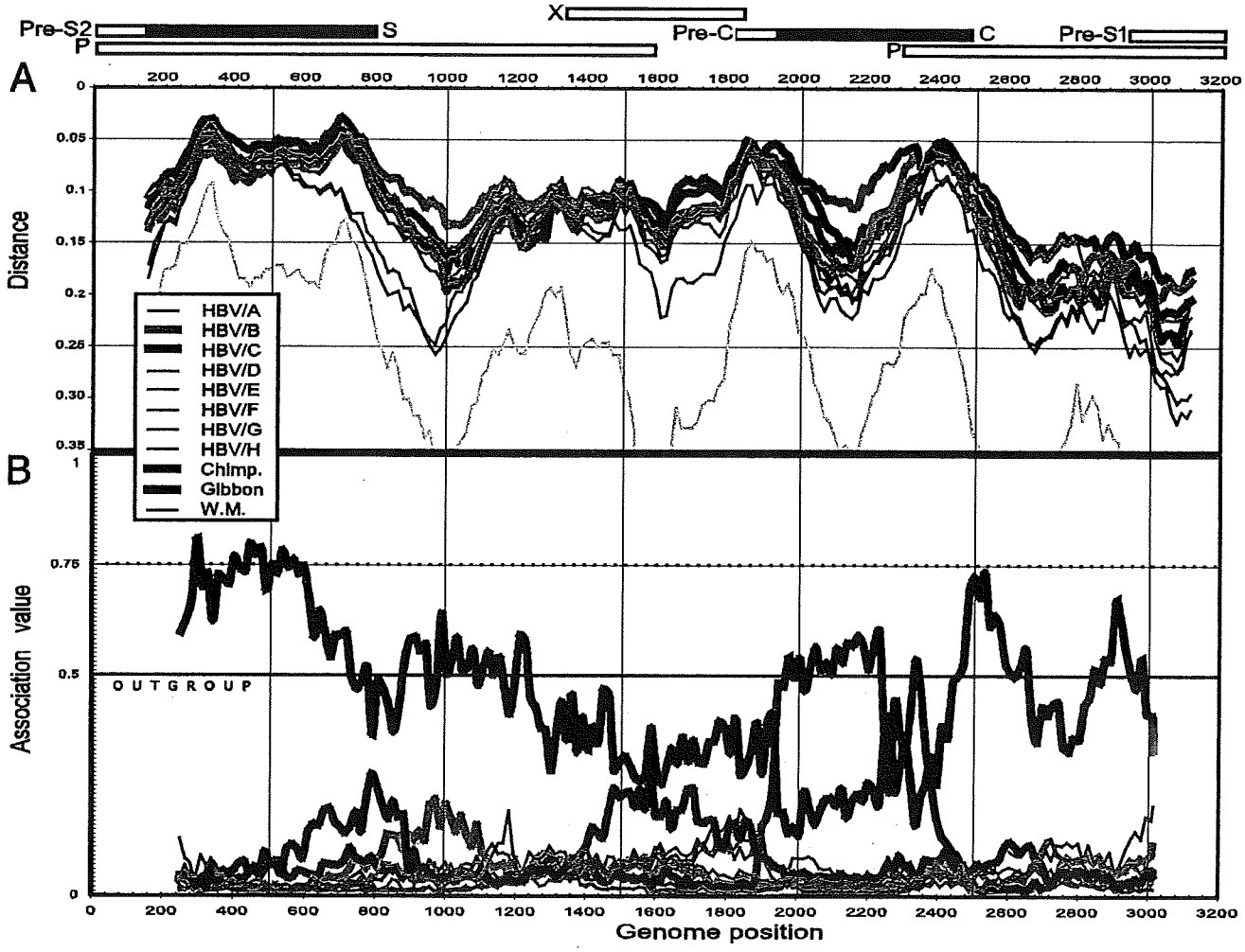

FIG. 2. Complete genome scanning carried by PHYLIP, the phylogeny inference package implemented in the Simmonic software, for the JRB34 strain versus 228 selected nonrecombinant HBV genotypes (HBV/Ba and HBV/I not included) reference strains grouped by genotype. Kimura two-parameter distance model (A)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

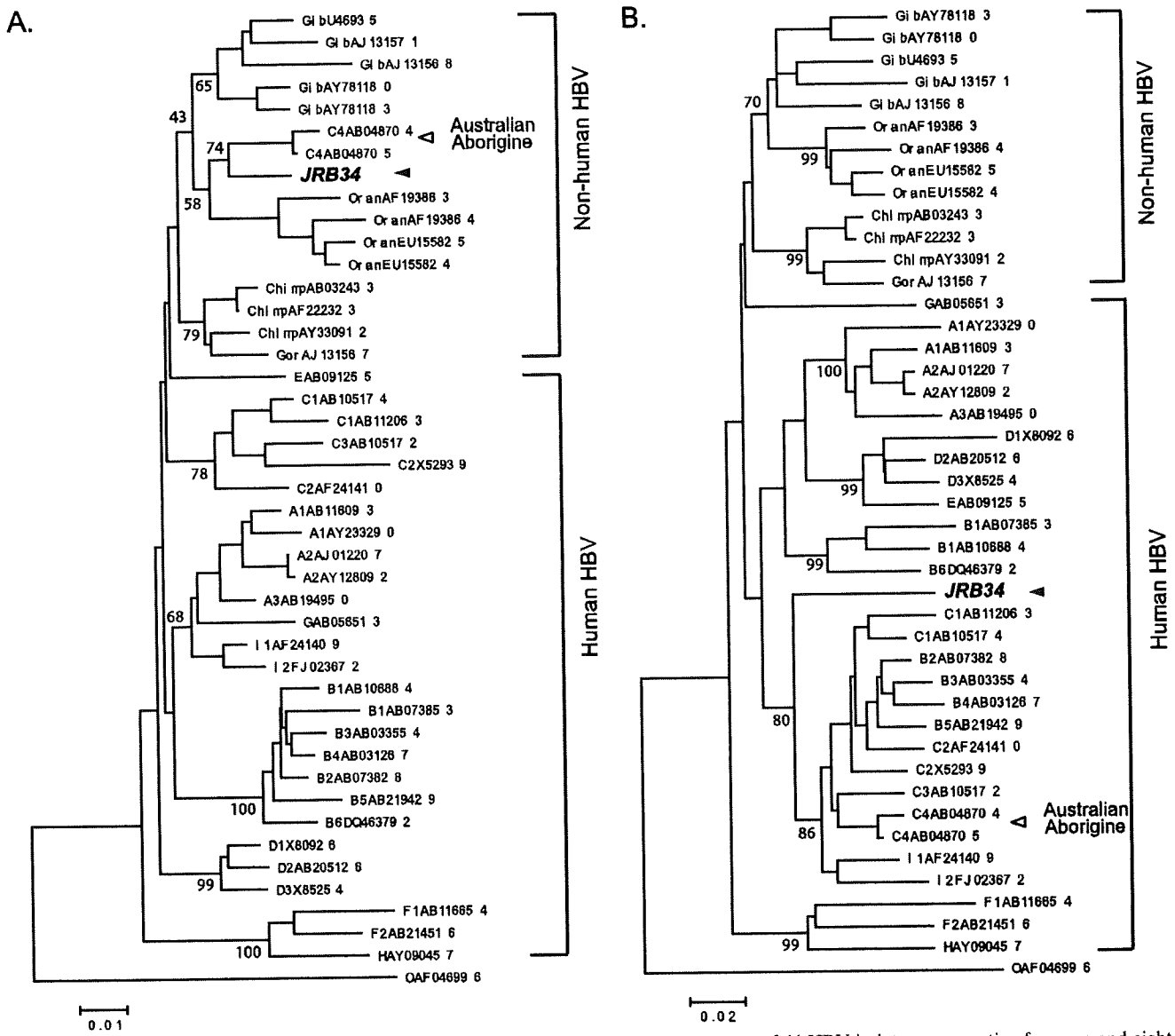

FIG. 3. Phylogenetic tree constructed on the preS/S gene (A) and C gene (B) sequences of 44 HBV isolates representing four ape and eight human genotypes. A woolly monkey HBV isolate serves as an outgroup. The HBV/J isolate (JRB34) is indicated by an arrowhead, and an HBVC4 isolate from Australian aborigine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

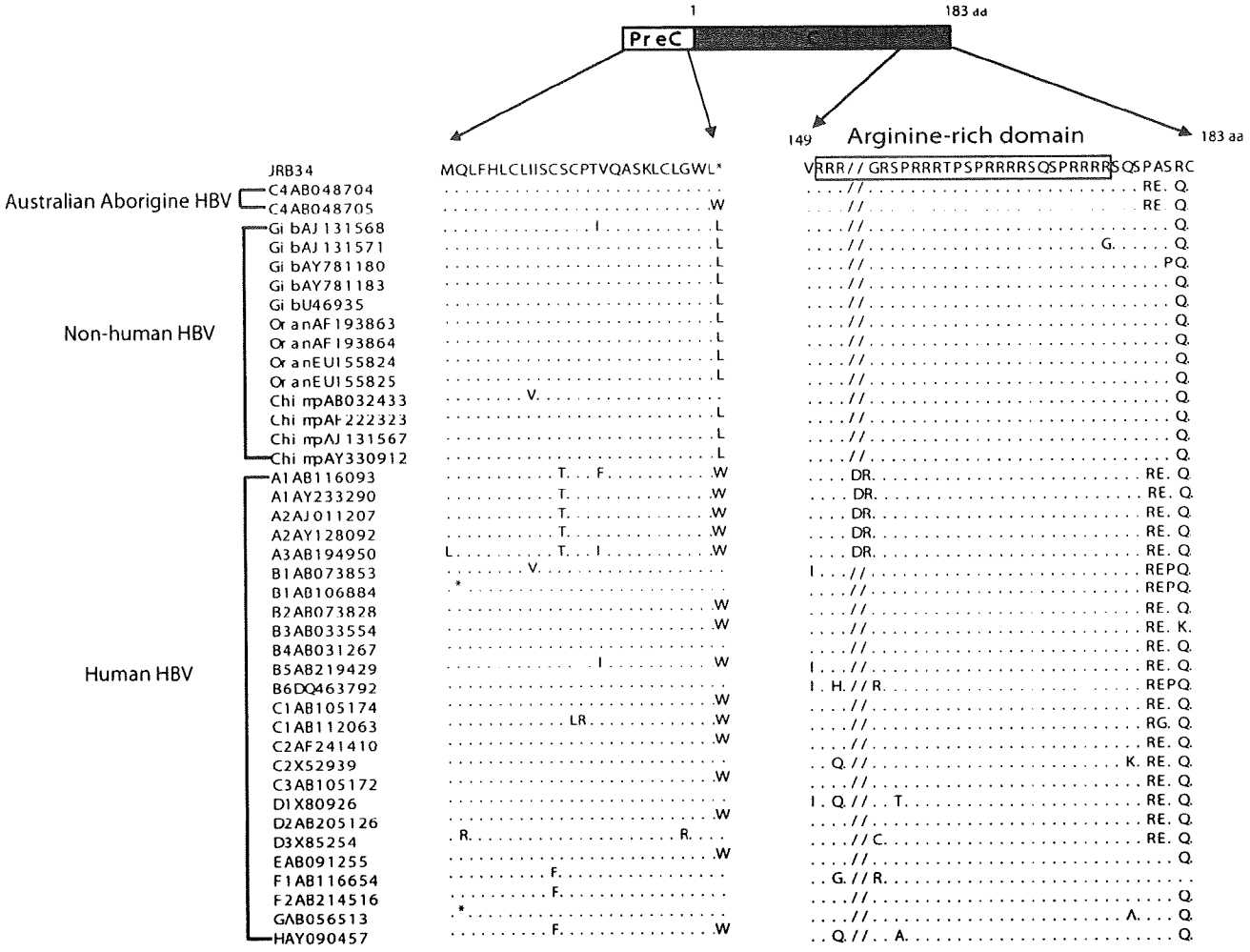

FIG. 4. Comparison of the amino acid sequence in the preC gene and carboxy-terminal amino acid sequences in the C gene of HBV isolates of various genotypes. The sequence of the HBV/J isolate (JRB34) is indicated at the top. Dots represent amino acids shared by JRB34, and a dash indicates the deletion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

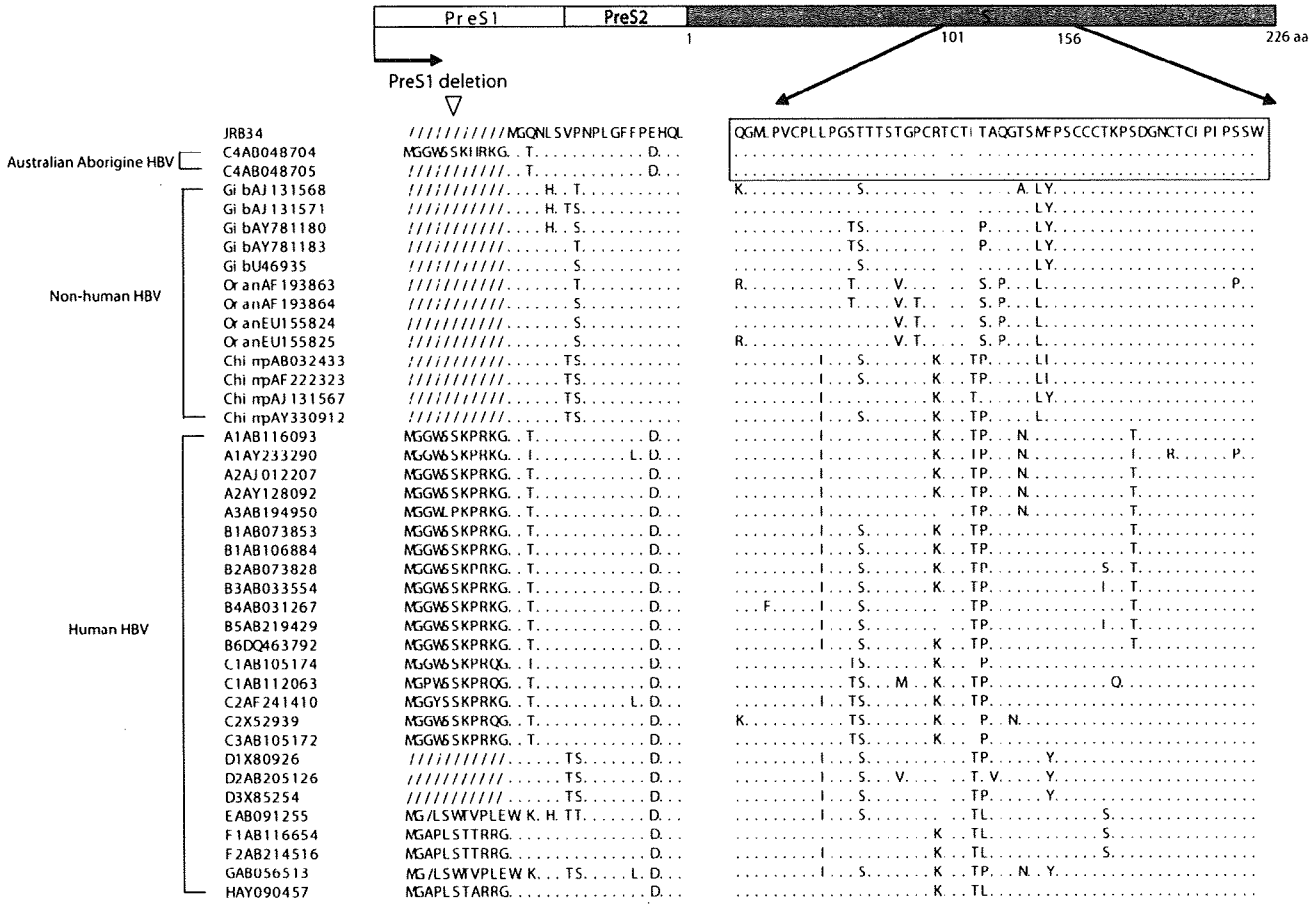

FIG. 5. Comparison of amino acid sequences of the preS/S gene among HBV isolates of various genotypes. The sequence of the HBV/J isolate (JRB34) is indicated at the top. Dots represent amino acids shared by JRB34, and a dash indicates the deletion of an amino acid. The sequence from positions 101 to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

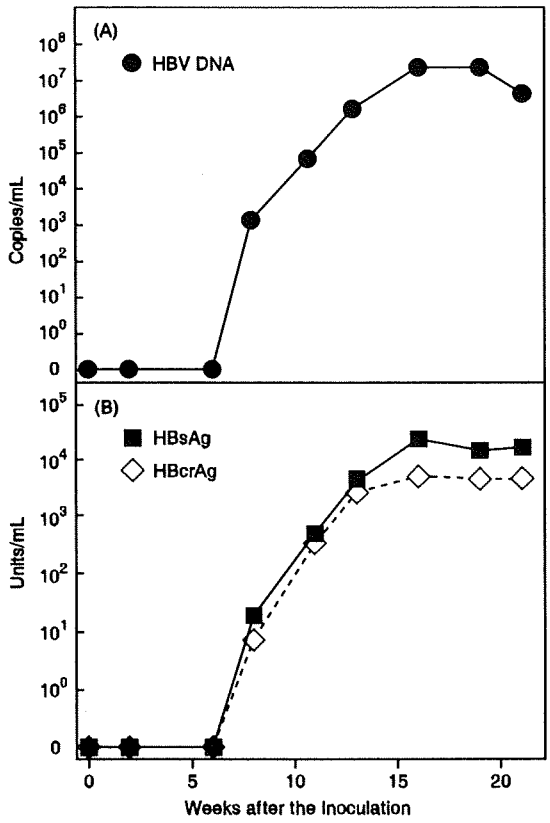

FIG. 6. Markers of HBV infection in two chimeric mice inoculated with the HBV/J isolate (JRB34). The levels of HBV DNA are illustrated in panel A, and those of HBsAg and HBcrAg are illustrated in panel B. Values represent the means for two mice.

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

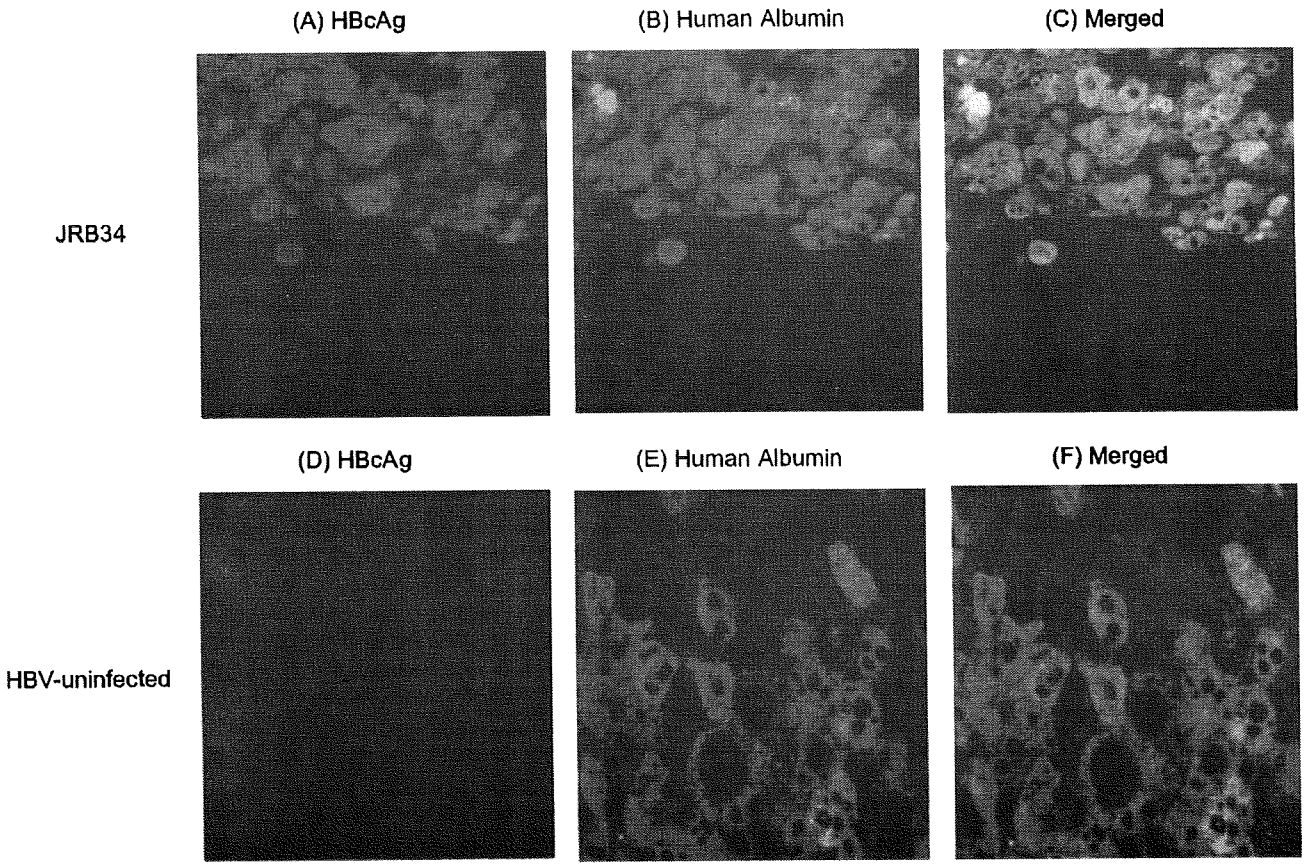

FIG. 7. (A and B) Immunofluorescent staining of a frozen liver section of a chimera mouse inoculated with the HBV/J isolate (JRB34). HBcAg is stained in panel A, and human albumin is stained in panel B. (C) Colocalization of HBcAg and human albumin is revealed by double staining. (D to F) HBV-uninfected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