Table 1. Inoculum Profiles on HBV Isolates of Distinct Genotypes/Subgenotypes

Genotype (Subgenotype)	Isolates	Mice (n)	Accession No.	Precore (1896)	HBeAg
A (A2/Ae)	A2_US	4	AB246337	Wild	+
	A2_JPN1	3	AB246338	Wild	+
	A2_JPN2	3	AB362931	Wild	+
C (C2/Ce)	C2_JPN22	4	AB246344	Wild	+
	C2_JPNAT	4	AB246345	Wild	+
	C2_JPN31	3	AB362932	Wild	+
B (B1/Bj_wild)	B1_JPN35w	4	AB246341	Wild	+
	B1_JPN56w	3	AB246342	Wild	+
	B1_JPN58w	4	AB362933	Wild	+
B (B1/Bj_PCm)*	B1_JPN35m	3	[a]	Mutant	−
	B1_JPN56m	3	[a]	Mutant	−
	B1_JPN58m	3	[a]	Mutant	−

[a]Accession numbers are not shown because these 3 clones identical to the above described HBV/B clones were constructed with G1896A point mutation.

of the host and environmental factors complicates evaluation of the veritable virologic differences between genetic variants of HBV in a clinical study. Therefore, a model that eliminates these factors and allows a direct comparison of early dynamics of HBV genotypes is essential for such investigation.

Recently engineered severe combined immunodeficient mice transgenic for urokinase-type plasminogen activator received human hepatocyte transplants (hereafter referred to as *chimeric mice*)[16-18] and are suitable for the experiments with hepatitis viruses in vivo,[19,20] and offer a rare opportunity in modeling the early kinetics of the HBV replication.[21]

In the present study, infecting human hepatocytes in chimeric mice, we show that different HBV genotypes and even particular mutations within the same genotype have distinct virologic characteristics that may have contributed to the distinct histologic outcomes.

Materials and Methods

Inoculation of Chimeric Mice With the Liver Repopulated for Human Hepatocytes

The chimeric mice were purchased from Phoenix Bio Co, Ltd (Hiroshima, Japan). Human hepatocytes were imported from BD Biosciences (San Jose, CA). The human serum albumin was measured by enzyme-linked immunosorbent assay using commercial kits (Eiken Chemical Co Ltd, Tokyo, Japan). The serum levels of the human albumins and the body weight were required to be identical among all of the mice to provide reliable comparison. All mice were infected successfully with HBV recovered from preinfected-mice sera or sera of patients as described in our previous report.[21] Briefly, a mixture of immature virions can be present in supernatants of cell culture transfected with plasmids expressing HBV[22,23]; therefore, to avoid direct use of the supernatants in experimental mice, the preinfected mice were infected instead, using the culture media, and then were used as a source of HBV inoculums for the experimental mice. Three clones for each HBV/A2, C2, B1_wild, or B1_PC mutant (precore stop-codon mutation [PCm]) were used in this study (Table 1), and each clone was inoculated to 3 or 4 mice.

Patients

Sera were obtained from 6 patients, 3 of whom had acute hepatitis B and the remaining 3 had fulminant hepatitis B. All sera were subjected to HBV extraction and direct sequencing, which determined genotype B (subgenotype Bj/B1) in all of them. HBV genome sequence analysis of the HBV clones isolated from 3 patients with fulminant hepatitis revealed both the presence of the PC mutation (G1896A) and the absence of any other featured mutations such as core promoter or tyrosine methionine aspartate aspartate mutations (Table 2). HBV strains isolated from the 3 acute hepatitis patients were wild type without core

Table 2. Characteristics of Patients From Whom HBV Isolates of Distinct Genotypes/Subgenotypes Were Recovered

Genotype/subgenotype	Isolates	Precore (1896)	Diseases	HBeAg	HBV (LGE[a]/mL)
B1/Bj_wild	B1_JPN1	Wild	AHB	+	6.8
	B1_JPN2	Wild	AHB	+	7.0
	B1_JPN3	Wild	AHB	+	6.7
B1/Bj_PCm	B1_JPN4	Mutant	FHB	−	8.7
	B1_JPN5	Mutant	FHB	−	8.0
	B1_JPN6	Mutant	FHB	−	8.6

AHB, acute hepatitis B; FHB, fulminant hepatitis B.
[a]Log genome equivalents.

promoter, precore, and tyrosine methionine aspartate aspartate mutations. The study design conformed to the 1975 Declaration of Helsinki, and was approved by the Ethic Committees of the participating institutions. Written informed consent was obtained from each patient.

Histopathologic Examination

Liver tissues were fixed in buffered formalin, embedded in paraffin, and stained with H&E, Masson's trichrome (MT), or orcein staining. To detect α-smooth muscle actin (α-SMA) and human nuclei, polyclonal antibodies against anti-α-SMA (Lab Vision Corp, Fremont, CA) and monoclonal antibody against anti-human nuclei (Chemicon International, Inc, Temecula, CA) were used as primary antibodies, respectively. The fibrosis stage was evaluated by an expert pathologist who was blinded to the nature of inocula (S.T.).

Dihydroethidium Labeling of Reactive Oxygen Species in Liver Tissue

In situ reactive oxygen species (ROS) production was evaluated by staining with dihydroethidium (Invitrogen, Carlsbad, CA) as previously reported with minor modification.[24] Briefly, in the presence of ROS, dihydroethidium is oxidized to ethidium bromide and stains nuclei bright red by intercalating with the DNA. The fluorescence was detected with laser scanning confocal microscopy. The relative stained area was quantified using National Institutes of Health image analysis for 5 randomly selected areas of digital images in each specimen.

Detection of 8-Hydroxydeoxyguanosine in Liver Tissue

Immunohistochemical detection of 8-hydroxydeoxyguanosine (8-OHdG) was performed as previously reported with minor modification.[25] The detailed protocol is shown in the Supplementary Materials and Methods section (see Supplementary material online at www.gastrojournal.org).

Results

Differences of Replication Efficiency Among HBV Genotypes

The inoculums, each containing approximately 10^5 copies of any 1 of the 4 clones: HBV/A2, C2, B1_wild, and B1_PC mutant (PCm), were inoculated to 3 or 4 mice. HBV DNA was quantified in murine sera weekly. One week after inoculation, HBV DNA was detected in both the HBV/A2 and C2 groups. The titer increased approximately by 2 logs within the next 2 weeks, and continued to increase until 7–12 weeks before reaching a plateau. HBV-DNA levels were 2 logs higher in the mice inoculated with HBV/C2 than HBV/A2 at 6–8 weeks postinoculation ($P < .05$) (Figure 1*A*).

To assess the role of the PC mutation, 2 variants of HBV/B1 were included in the comparison between the genotypes: the HBV/B1_wild and HBV/B1_PCm. Differently from HBV/A2 and HBV/C2, both of the HBV/B1 variants had shown a so-called *window* period; characterized by the HBV-DNA levels remaining undetectable until weeks 4–5 after the inoculation. However, after the window period, the HBV-DNA level of the B1_PCm detected at week 5 had rapidly increased in titer, reaching the levels of HBV/C2 and A2 by week 11 (Figure 1*A*). Interestingly, HBV-DNA levels of B1_wild did not show this rapid increment during the whole follow-up period (until week 25). HBV-DNA titer was 3 logs lower in mice inoculated with HBV/B1_wild compared with those with the other genotypes ($P < .01$). To evaluate the replication dynamics of the different genotypes, the time required for a 10-fold increment of the viral load (*log time*) was estimated. When the window periods of HBV/B1_PCm were excluded from the comparison, the log time was similar between the HBV/C2 and B1_PCm, ranging from 7.3 to 8.4 days, whereas HBV/A2 had a longer index (12.9 days), suggesting slower replication. However, the lowest replication efficiency was observed for HBV/B1_wild, with a log time of 27.7 days.

Distinct Characteristics on Antigen Production Among HBV Genotypes

The expression of hepatitis B surface antigen (HBsAg) and hepatitis B core-related antigen (HBcrAg) (depicted in Figure 1*B*) did not correspond with that of HBV DNA (Figure 1*A*) for HBV/B1_PCm, which had rapidly increased antigen expression in the early phase, and then decreased sharply. HBeAg of HBV/B1_PCm was undetectable as expected to confirm the function of the stop codon mutation. In contrast, dynamics of HBcrAg and HBeAg expression by HBV/C2 and HBV/A2 resembled those of HBV DNA. The HBcrAg levels of HBV/B1_PCm without HBeAg expression revealed lower levels than those of HBV/A2 or C2. To detect core protein alone without detecting HBeAg, only hepatitis B core antigen (HBcAg) was assessed in each mice group at the peak point of HBcrAg by enzyme-linked immunosorbent assay. The value of HBV/B1_PCm shown was equal to that of HBV/C2, and higher than that of HBV/A2 (data not shown). HBV antigens of HBV/B1_wild group were detectable, although they had extremely low levels, suggesting a very low replication level for this group. Core protein levels in liver tissue, with adjustment for human albumin levels, showed a similar trend to that of sera (data not shown).

Confirmation of HBV/B1_Wild Infectivity by Using Human Sera

Virus titer of the HBV/B1_wild group was very low and the log time was long in the present study. To further confirm these findings, we used 6 sera: 3 from

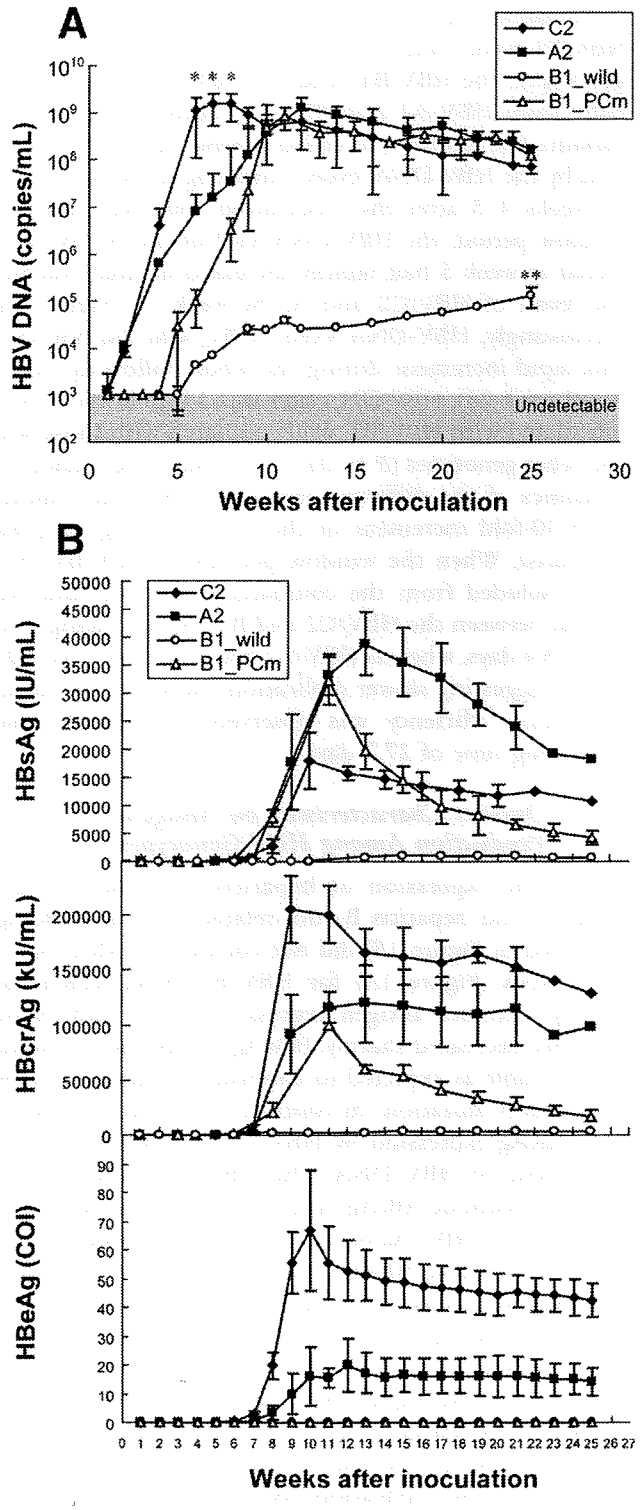

Figure 1. Comparative dynamic profile of HBV-DNA and antigen levels in sera of mice inoculated with preinfected-mice sera recovered from culture media transfecting HBV construct. (*A*) Levels of HBV DNA in sera of the chimeric mice inoculated with HBV/A2, C2, B1_wild, or B1_PCm. Shaded in *gray* is an area below the detection limit (<10^3 copies/mL) of the real-time detection PCR assay. *Statistical differences with a *P* value of less than .05. **Statistical differences with a *P* value of less than .01. (*B*) Dynamic profiles of HBV antigen expression, as revealed by quantification of HBsAg, HBeAg, and HBcrAg in sera of the chimeric mice (see Supplementary Materials and Methods section). For each group, mean values observed in 9–11 chimeric mice are depicted with the standard deviation bars.

acute hepatitis B patients harboring precore wild-type HBV/B1 and the other 3 from fulminant hepatitis B patients harboring precore nonsense-mutation B1 (B1_PCm) (Table 2). Three mice were inoculated with each one of the 6 serum specimens adjusted to contain approximately 10^6 copies of HBV DNA (Figure 2*A* and *B*). Serum HBV-DNA levels increased immediately after inoculation of HBV/B1_PCm and continued to increase until they reached a plateau at week 6 (Figure 2*A*), showing extremely high replication efficiency. The window period was shortened to 2 weeks in the acute hepatitis B serum group with HBV/B1_wild; however, the peak of mean HBV-DNA levels still was low (5×10^5 copies/mL), which was similar to the results by inoculation of preinfected-mice sera (Figure 1*A*). Neither serum levels of the human albumin nor the body weight differed among the mice groups. Based on direct sequencing, no mutations were detected in the HBV complete genomes from any mice 25 weeks after inoculation in comparison with those of inoculated strains.

HBV antigen expression levels of the groups inoculated with human serum samples were compared with those of the groups inoculated with the preinfected-mice sera (Figure 2*B*). HBV antigens of HBV/B1_PCm waxed and waned in profiles similar to that of the groups inoculated with the mice sera in the early phase.

Liver Pathology of Chimeric Mice Infected With Each Genotype

Figure 3 shows the histology of liver in representative chimeric mice infected with HBV/A2, C2, B1_wild, or B1_PCm during weeks 22–25. The immunofluorescence staining was performed using anti-HBcAg and anti-human albumin polyclonal antibody to confirm the location of HBV infection (Supplementary Figure 1; see Supplementary material online at www.gastrojournal.org). Colocalization of HBcAg and human hepatocytes was shown by double staining for HBcAg and human albumin. Almost all of the mice did not reveal apparent steatosis of hepatocytes with H&E stain. The majority of HBV/C2- or B1_PCm-infected human hepatocytes had a ground-glass appearance on H&E stain, fibrosis of stage 2 with MT stain, as well as neutrophil or monocyte invitation. In contrast, the mice infected with HBV/A2 or B1_wild had neither a ground-glass appearance nor fibrosis. To confirm the ground-glass appearance, these specimens were stained by orcein staining. The orcein staining clearly showed cytoplasmic positivity of human hepatocytes infected with HBV/B1_PCm or C2, but not the other group, including control mice.

Immunostaining Analysis on Expression of α-SMA

Active hepatic stellate cells (HSCs) express α-SMA in the early phase of fibrogenesis. To estimate the activation of stellate cells, we performed immunostaining

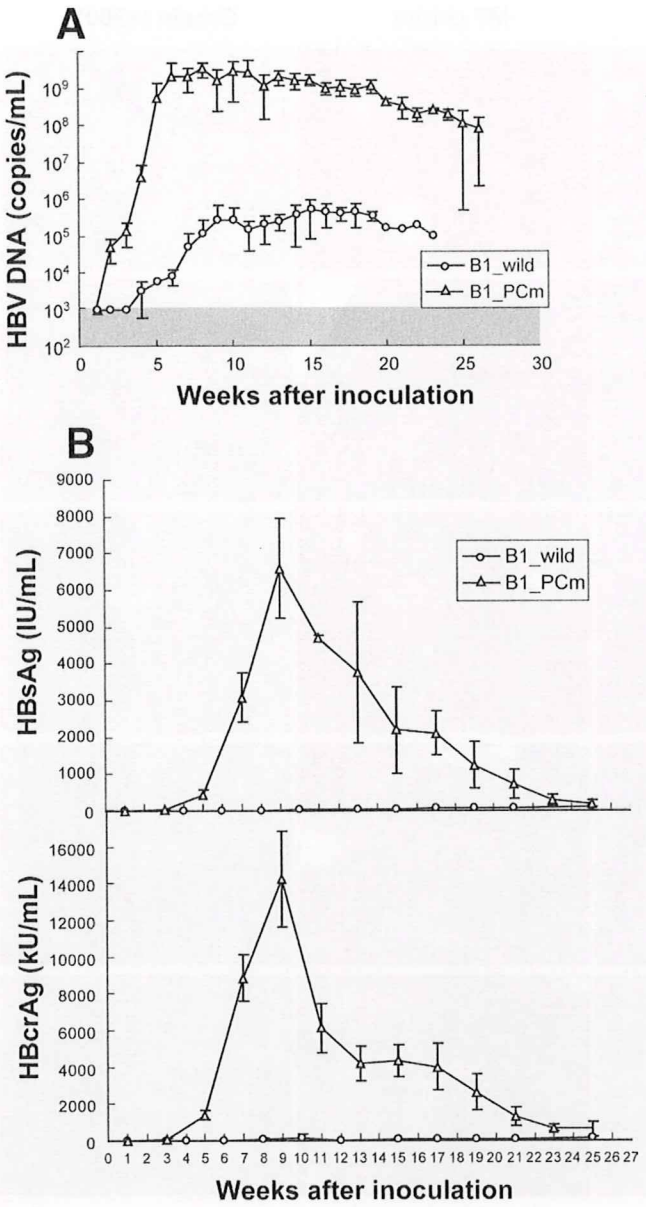

Figure 2. Comparative dynamic profile of HBV-DNA antigen levels in sera of mice inoculated with patient sera from HBV/B1_wild (PC wild-type) and HBV/B1_PCm (PC mutant). (*A*) Mice inoculated with sera from HBV/B1_wild–infected carriers developed acute hepatitis B or from HBV/B1_PCm-infected carriers developed fulminant hepatitis B and were assessed for levels of HBV DNA in mice sera with real-time detection PCR weekly. The area below the detection limit ($<10^3$ copies/mL) is shaded in *gray*. (*B*) Dynamic profiles of HBV antigens including HBsAg and HBcrAg in mice corresponding to panel *A*. For each genotype, mean values observed in 9–11 chimeric mice are depicted with the standard deviation bars.

using anti-α-SMA antibody. Immunostaining analysis showed strong staining of α-SMA around fibrosis, which was found by MT staining (Figure 4*A*). These results indicated that liver fibrosis of HBV/C2 and B1_PCm occurred via profibrotic cytokines from the activated HSCs but not artifacts. The specimen was double-stained for human nuclei and α-SMA to distinguish between human and mouse cells. As shown in Figure 4*B*, α-SMA and human nuclei did not stain in the same cells, suggesting that the active HSCs were of mouse origin.

Increased Oxidized State in Liver by HBV Infection

In the fibrosis process, current knowledge establishes that the production of ROS plays a critical role in HSC activation involving transforming growth factor-β1 (TGF-β1) signaling.[26] Because α-SMA expressed by HSCs was detected in chimeric mice liver, we next investigated ROS production in mice liver. The ROS production was confirmed by dihydroethidium staining (Figure 5*A*). The level of ROS production was increased statistically when mice were infected with HBV/B1_PC or C2 compared with HBV/A2 or B1_wild ($P < .01$) (Figure 5*B*). Figure 5*C* shows representative immunohistochemical staining for 8-OHdG, which is a marker of oxidative DNA damage, in liver; 8-OHdG–positive cells were recognized in both HBV/C2 and B1_PCm groups, whereas few 8-OHdG–positive cells were detected in the other groups. These data were consistent with those of ROS production.

Gene Expression of Fibrosis Markers in the Mice Liver

As for the change of factors associated with TGF-β1 signaling in the mice, serum alanine aminotransferase (ALT) and TGF-β1 levels were increased in the fibrosis group (B1_PC and C2) as compared with the nonfibrosis group (A2 and B1_wild) (Figure 6*A* and *B*). The TGF-β1 levels in the fibrosis group showed significant difference ($P < .01$). To determine whether the representative fibrosis-related genes were of human or mouse origin, we established species-specific primer sets. Polymerase chain reaction using the species-specific primers gave bands of specific size showing reliable specificity (Figure 6*C*) and dissociation curves (data not shown) (the detailed protocol is provided in the Supplementary Materials and Methods section). Gene expression levels of tissue inhibitor of metalloproteinase 1, matrix metalloproteinase 2, and collagen type 1α2 were quantified by real-time detection reverse-transcription PCR analyses. Specifically, gene expression of human tissue inhibitor of metalloproteinase 1 and mouse collagen type 1α2 represented significantly higher expression in the fibrosis group than that of the nonfibrosis or control groups ($P < .001$). Matrix metalloproteinase 2 and collagen type 1α2 messenger RNA (mRNA) of human origin were undetectable because these genes are produced predominantly in mesenchymal cells.[27]

Discussion

In the present study, the severe combined immunodeficient mice transgenic for urokinase-type plasminogen activator mouse with human hepatocytes was applied to evaluate genotype-dependent differences in the

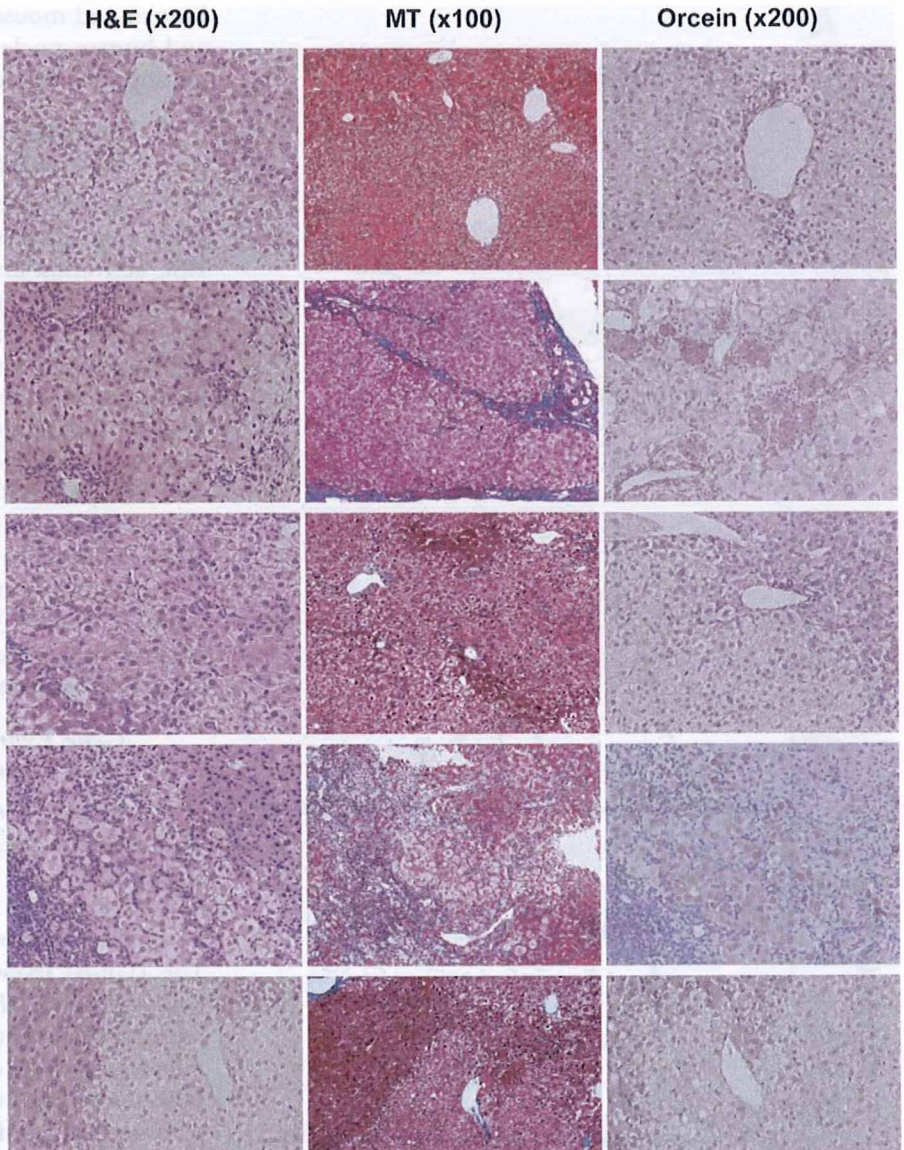

Figure 3. Immunohistochemical analysis of liver tissue. Comparison of liver histology in mice long-term (25 weeks) infected with HBV/A2, C2, B1_wild, B1_PCm, and noninfected control. Liver sections stained with H&E, MT, or orcein are shown. After deparaffinization, tissue slides were stained according to each method. Representative staining of C2 and B1_PCm showed a ground-glass appearance, fibrosis, and cytoplasmic positivity of human hepatocytes by orcein staining (brown), whereas these were absent in A2, B1_wild, and control mice. Original magnifications: H&E and orcein, 200×; MT, 100×.

expression of HBV DNA and antigens. This has allowed for an assessment of the direct cytopathic potential of different HBV genotypes (ie, particular subgenotypes) to be investigated without the host-related bias, under conditions of the absence of immune pressure. In addition, this may represent a novel mouse model for human liver fibrosis associated with ROS production leading to the activity of TGF-β by viral infection but not chemical trigger. The study thereby has shown that infection with HBV/C2 in contrast to HBV/A2 or B1_wild has induced an abundant ground-glass appearance of the human hepatocytes along with an increased fibrosis in the humanized liver of the chimeric mice in an immunosuppressive condition. A strong staining of α-SMA observed around areas of fibrosis indicated activation of HSCs in cases of HBV/C2 and B1_PCm, but not in A2 and B1_wild. In the chimeric mice, therefore, ROS production could play a critical role in HSC activation. In connection with this study, we have evaluated the liver damages in chimeric mice killed at 3 months postinfection (early phase dynamics). The viral dynamics and ROS production of HBV/C2 or B1_PCm evaluated in the early phase indicated levels of alterations similar to those observed after long-term infection (Supplementary Figure 2; see Supplementary material online at www.gastrojournal.org). Fibrosis stage and orcein staining levels (ground-glass appearance), however, were expressed in lesser levels than in the long-term infected mice, suggesting that the liver damage can be detected even in the early stage of the infection, but its level correlates with the duration of exposure to oxidative stress.

Our previous report showed that the intracellular virion retention and endoplasmic reticulum stress were the highest for HBV/C2.[21] Our data obtained in vitro and

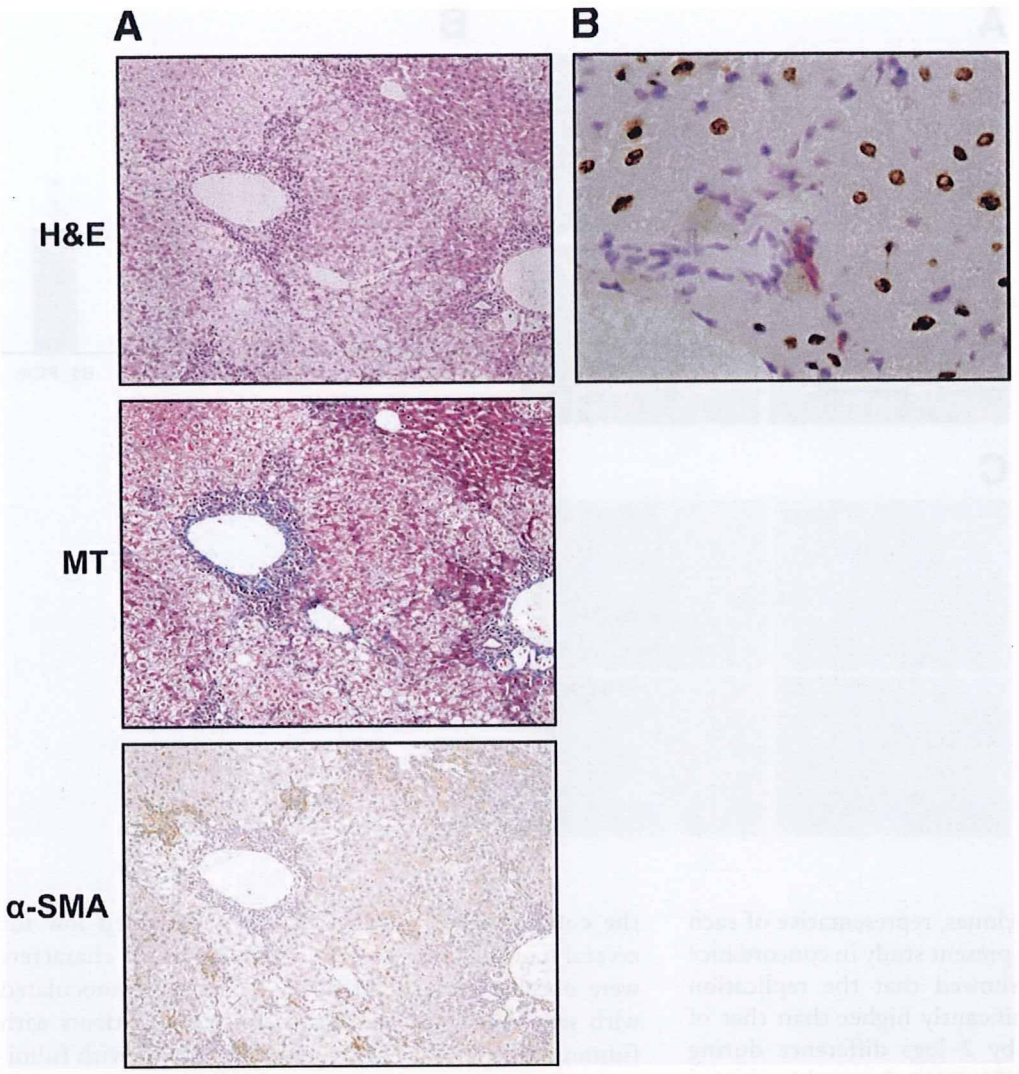

Figure 4. Confirmation of liver fibrosis by immunostaining using anti–α-SMA antibody. (*A*) Liver sections stained with H&E, MT, or immunostaining using anti–α-SMA antibody (as described in the Materials and Methods section). (*B*) Nuclei stained *brown* with the antibodies indicate human origin, and α-SMA is stained in *red*, located in the cytoplasm without a stained nucleus. Shown are representative staining of images expressing fibrosis. Original magnification, 200×.

in vivo may explain in part previous results accumulated from clinical studies indicating that HCC more often was associated with HBV/C and the mean age of patients with HCC is younger in the HBV/C-infected group compared with the HBV/B1-infected group.[28,29] On the other hand, the low replicative capacity and hepatic injury of HBV/A2 may contribute to the ability of the subgenotype to evade the immune response and chronically persist in up to 10% of acutely infected adults (which is exceptionally rarely observed with HBV/C or HBV/B).[11,30–32] High levels of HBsAg secretion for HBV/A2 are in contrast with its low replicative activity, and this may be an important mechanism for the immune escape. However, some cautions must be exercised when extrapolating the results of in vivo models to patients because immune responses are not taken into account.

The hepatic injury during acute and chronic HBV infection genuinely is considered to be caused by the host's immune response against the infected hepatocytes.[33] However, in some immunosuppressed chronic HBV patients, high viremia and liver fibrosis may occur.[34,35] Previous reports have shown that HBV genotypes E or G cause intracellular changes and hepatocellular damage in human hepatocytes in severe combined immunodeficient mice transgenic for urokinase-type plasminogen activator.[2,3] We showed here that activation of oxidative stress led to TGF-β1 production in chimeric mice as reported in previous studies.[26] Accumulation of oxidative damage, 8-OHdG, might enhance the possibility of carcinogenesis as observed in HCC patients. These findings suggest that hepatic injuries could arise in the absence of a mature immune system and the difference of genotype would affect the cytopathic potential of the virus.

Chimeric mice were infected with HBV recovered from serum or culture medium containing virion from Huh7 cells transfected with HBV construct.[2,20,21,36,37] In our previous study, by using a single clone corresponding to HBV/A or C, we showed 2 logs difference during weeks 4–7 in the serum levels of HBV DNA between the cohort of mice inoculated with HBV/C and HBV/A.[21] In the present study, we extended the examination of the geno-

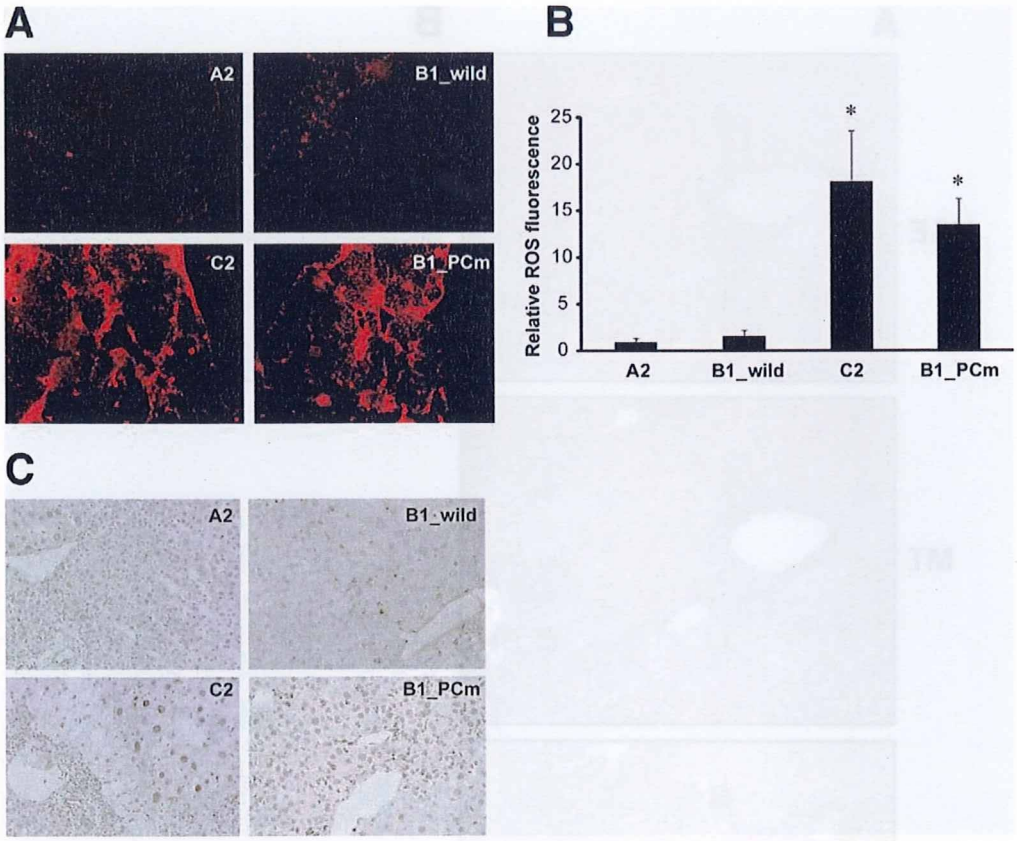

Figure 5. Differences in production of oxidative damage among HBV genotypes. (*A*) Frozen liver sections of mice inoculated with different HBV genotypes were stained by dihydroethidium. Fluorescence was detected with a laser scanning microscope. (*B*) Fluorescence intensities in randomly selected areas of digital images were quantified by National Institutes of Health image analysis software. *$P < .01$: A2 or B1_wild vs C2 or B1_PCm. (*C*) Oxidative damages in liver tissue were evaluated by staining of 8-OHdG–positive nuclei. Original magnifications, 200×.

type differences by using 3 clones, representative of each genotype. The results of the present study in concordance with our previous study showed that the replication efficiency of HBV/C is significantly higher than that of HBV/A, as was indicated by 2 logs difference during weeks 6–8 in the levels of HBV DNA detected in murine sera ($P < .05$). The ability of HBV/A to express more HBsAg, and that of HBV/C to produce more HBcrAg revealed in our previous in vitro study,[21] were both thereby confirmed by the present in vivo replication model using the chimeric mice.

Previous clinical observations on HBV/B1[11,28] prompted a deeper investigation on the impact of the PC mutation on the virologic characteristics of the genotype. The unique characteristic of HBV/B1_wild stood out among genotypes harboring no major mutations. The HBV/B1_wild group revealed low replication efficiency with window periods and low antigen expression. The lower replicative activity and hepatic injuries of HBV/A2 and B1_wild may partially explain why carriers with either HBV/A2 or HBV/B1 often are asymptomatic in contrast to those with HBV/C infection.[28,38,39] In our study, the PC mutation was the only difference between HBV/B1_PCm and HBV/B1_wild clone, and the former showed higher replication efficiency and severe damage in liver tissue. The antigen levels of the HBV/B1_PCm increased rapidly and decreased earlier than those of the HBV/A2 or C2 clone, whereas HBV/B1_wild showed that the concentrations of HBV antigens remained low for several months postinfection. These particular characters were observed for the HBV/B1_PCm group inoculated with sera from both preinfected mice and patients with fulminant hepatitis. The majority of patients with fulminant hepatitis and fatal acute exacerbation have been found to have the G1896A mutation.[11,40,41] A greater incidence of fulminant hepatitis might be associated with the high replication and protein production in the early phase, as was shown on the HBV/B1_PCm clone in this study. The defect of immunologic tolerance as a result of the absence of HBeAg may play an important role in the fulminant course of precore mutation in HBV infection.[42] This would concur with a previous report by Bocharov et al which proposed that enhanced HBV replication would efficiently stimulate immune responses, represented by the cytotoxic T-lymphocyte response,[43] suggesting that enhanced replication by HBV/B1 with G1896A mutation might lead to an extremely high cytotoxic T-lymphocyte response, resulting in fulminant hepatitis. But in this study, HBV/B1_PCm showed similar responses to HBV/C2 infection because chimeric mice did not have an immune system that was strong enough to invite strong cytotoxic T-lymphocyte response against viral infection. To uncover these unique characteristics of PC mutant, further study would be needed by using the infection model but not gene transfer.

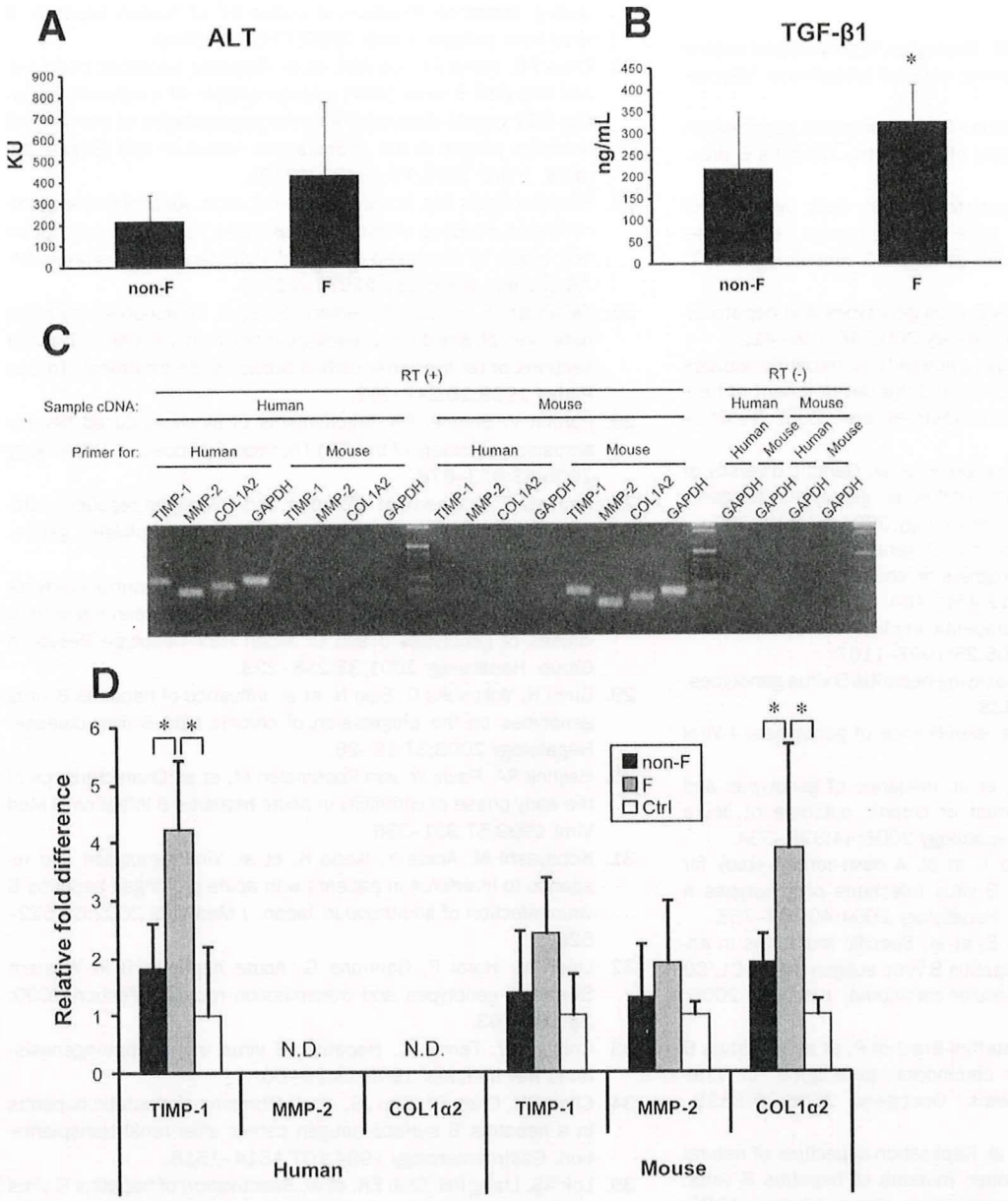

Figure 6. Differences in the expression levels of fibrosis-related genes among HBV genotypes. Quantification of (*A*) ALT and (*B*) TGF-β1 levels in mouse sera with enzyme-linked immunosorbent assay (see Supplementary Materials and Methods section. non-F, no fibrosis group (A2 and B1_wild); F, fibrosis group (C2 and B1_PCm). *$P < .01$: non-F vs F. (*C*) The specificity of each PCR using species-specific primer sets. The species-specific primer sets were established to determine whether mRNA of fibrosis-related genes were of human or mouse origin. Liver tissue of a HCC patient or a mouse without transplantation of human hepatocytes was used to check the primer sets for real-time detection PCR. The PCR products were run on 2% agarose gels to confirm the molecular sizes as well as species-specific amplifications. (*D*) Quantification of mRNA expression on fibrosis-related genes in each group by real-time reverse-transcription PCR. non-F group, n = 15; F group, n = 22; control, n = 8; ND, not detected; *$P < .001$.

Finally, the discrepancy between in vitro[21] and in vivo (present study) observations on HBV/B1_wild might have been caused by differences in the cells used for transfection (Huh7 cells) and infection (human hepatocytes from Caucasoid donors), respectively. Nonrecombinant type HBV/B strains (B1 and B6) have been detected in limited areas including Japan[44] and Alaska,[45] which were settled mainly by Mongoloid people. The existence of a window period on HBV/B1 might indicate a possibility that a receptor or co-receptor used by HBV/B1 is not equal to one adopted by other genotypes as shown in the human herpes virus.[46] Further studies using human hepatocytes from Mongoloid people would be required.

In conclusion, using an in vivo experimental system, we show that different HBV genotypes and even particular mutations are associated with different virologic and histopathologic characteristics. Infection with HBV/C2 as well as PC mutant of the HBV/B1 in immunosuppressive conditions can induce a direct cytopathic effect in the humanized part of the murine liver. This mouse model appears to be useful in the evaluation and prediction of pathogenic effects of various genotypes of HBV and certain HBV mutations.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2008.10.048.

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Characteristics of hepatitis B virus genotype G coinfected with genotype H in chimeric mice carrying human hepatocytes☆

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ABSTRACT

Accumulated evidence indicated that hepatitis B virus genotype G (HBV/G) is present exclusively in coinfection with other HBV genotypes. In Mexico, HBV/G from 6 men who had sex with men were coinfected with HBV/H. Phylogenetically complete genomes of the 6 Mexican HBV/G strains were closely related to previous ones from the US/Europe. Using uPA/SCID mice with human hepatocytes, monoinfection with HBV/G did not result in detectable HBV DNA in serum, whereas superinfection with HBV/G at week 10 inoculated HBV/H when HBV/H DNA was elevated to $>10^7$ copies/mL has enhanced the replication of HBV/G. The HBV/G was enhanced in another 3 inoculated with a serum passage containing HBV/G with a trace of HBV/H. Coinfection of mice with HBV/G and H induced fibrosis in the liver. In conclusion, the replication of HBV/G can be enhanced remarkably when it is coinfected with HBV/H. Coinfection with HBV/G may be directly cytopathic in immunosuppressive conditions.

Introduction

On the basis of the nucleotide sequence divergence exceeding 8% throughout the complete genome sequence, hepatitis B virus (HBV) has been classified into eight genotypes designated by capital letters A (HBV/A) through H (HBV/H) (Arauz-Ruiz et al., 2002; Norder et al., 1994; Okamoto et al., 1988; Stuyver et al., 2000). The genotypes have different geographical distributions, virological characteristics and clinical manifestations (Magnius and Norder, 1995; Miyakawa and Mizokami, 2003).

One of the less studied genotypes is the HBV/G. It was first described in 2000, among inhabitants of France and Georgia, USA (Stuyver et al., 2000). The isolated strains had 36 base-pairs' insertion in the core gene and two stop codons in the precore region depriving ability of the virus to translate HBeAg. Nevertheless, some of the carriers were HBeAg positive (Stuyver et al., 2000) that was shortly after explained by the coexistence of the "HBeAg-potent" HBV/A strains in coinfection (Kato et al., 2002a,b). Further studies reported circulation of the genotype in Thailand (Suwannakarn et al., 2005), Japan (Ozasa et al., 2006) and Mexico (Sanchez et al., 2007) indicating global distribution and association of the infection with specific risk groups, such as injection drug users (IDU) and men who had sex with men (MSM). The studies also demonstrated that throughout the world HBV/G strains possess unprecedented genetic homology and are mainly presented in coinfection with another endemic genotype. However, little is known about peculiarities of interaction of the HBV/G with various genotypes as well as about virological and clinical concerns of the coinfection.

Produced by genetic engineering, a mouse with severe combined immunodeficiency, carrying urokinase-type plasminogen activator transgenes controlled by albumin promoter (uPA/SCID) with transplanted human hepatocytes (Heckel et al., 1990; Rhim et al., 1994) was recently shown as an appropriate animal model for studying HBV (Dandri et al., 2001; Tsuge et al., 2005). Using this model it was demonstrated that during monoinfection, HBV/G might be able to replicate in hepatocytes at low level; but its replication was

Abbreviations: HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBcrAG, antigens related to HBV core; uPA/SCID, severe combined immunodeficiency transgenic with urokinase-type plasminogen activator.

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significantly enhanced by coinfection with HBV/A or C (Sugiyama et al., 2007).

Our purpose is to determine the complete sequences of HBV/G coinfected with HBV/H in sera obtained from MSM in Mexico and to elucidate the characteristics of HBV/G in coinfection with HBV/H using recently developed *in vivo* model.

Results

Phylogenetic relatedness of HBV complete genome sequences in Mexico

Six HBV/G strains in coinfection with HBV/H among MSM in Mexico were described in our previous study (Sanchez et al., 2007). In the present study, the complete genome sequences of not only 6 HBV/G strains but also 6 HBV/H strains from the same patients were determined by each specific PCR. The phylogenetic analyses indicated that the 6 HBV/G strains were close to those previously reported from the United States (US), France and Germany, and the 6 HBV/H strains were related to the previously reported ones in the US (Fig. 1). All 6 coinfected patients were positive for HBeAg, and asymptomatic carriers of chronic HBV infection at the time of sample collection (the details unknown).

Characteristics of HBV/G strains in Mexico

Sequence analyses revealed unique insertion of 36 nt in the core gene, two stop codons in the precore region and double mutation in the core promoter (CP) in all Mexican HBV/G strains, whereas no related mutations were found in the corresponding HBV/H strains (Fig. 2a and b). These data suggest that the HBeAg detected in serum of those patients had been produced by HBV/H. Additionally, several mutations, which might have affected the replication of the virus genome and amino acid substitutions of HBx, were found in the first half of the CP region, including the above double mutation (nt 1701–1765) (Fig. 2a).

Examining the genetic diversity and recombination of HBV/G

Comparing the complete sequences, both overall genetic distance among the HBV/G strains (0.0037±0.0005 per site) and percent nucleotide homology (0.30±0.24%) were much lower than those among the other intra-genotype groups. In consideration of previously reported recombination between HBV/A and HBV/G strains (i.e. AB056516) (Kato et al., 2002a), we have examined the Mexican HBV/G strains for possible event of intergenotypic recombination. Complete sequences of 3 to 5 clones isolated from each of the 6 HBV/G carriers revealed; no evidence of recombination by similarity and bootstrap scan (data not shown).

Intracellular expression of HBV DNA and antigens

Huh7 cells were transfected with a pUC19 vector carrying 1.24-fold the HBV genome. Three days post-transfection, they were harvested, lysed with NP-40 and tested for HBV DNA and antigens. The density of single-stranded (ss) HBV DNA was compared between HBV/G and H by Southern blotting. The expression of HBV DNA was higher for HBV/H than G, indicating that HBV/G had very low replication *in vitro* (Fig. 3a). As well, HBsAg, HBeAg and HBcrAg levels were much higher in HBV/H (Fig. 3b).

Superinfection with HBV/G on mice infected with HBV/H

Chimeric mice were infected with HBV/G and H particles propagated in Huh7 cells in order to confirm the infective efficiency. Monoinfection with HBV/G from the Huh7 cells culture medium did not result in detectable HBV DNA in mice serum (data not shown).

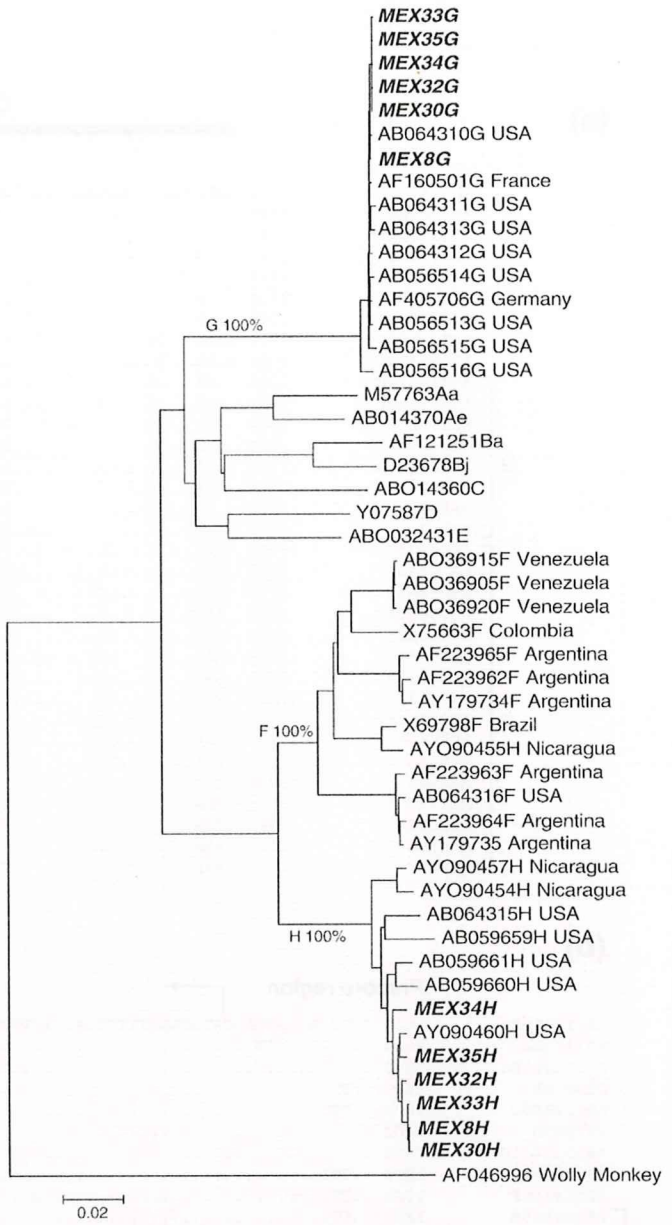

Fig. 1. A phylogenetic tree constructed using the complete nucleotide sequences of 50 HBV strains. The 6 HBV/G strains coinfected with HBV/H in Mexico (MEX), shown in bold italic, were compared with reference sequences recruited from GenBank/DDBJ/EMBL databases; 10 HBV/G, 7 HBV/H, 13 HBV/F, 7 representing genotypes A–E and 1 outgroup (Wolly Monkey). Aa and Ae are subgenotypes of HBV/A (Sugauchi et al., 2004). Ba and Bj are subgenotypes of HBV/B (Sugauchi et al., 2002). The country of origin is indicated after the accession number for each HBV/F, HBV/H and HBV/G strain. Bootstrap values are shown at the nodes of the main branches.

Then, according to our previous method (Sugiyama et al., 2007), the dynamics of HBV DNA, HBsAg and HBeAg assessed in 3 chimeric mice (ChiM_H1–H3) with HBV/G on H superinfection, are shown in Fig. 4 (a–c). Initially each of the mice received inoculation of around 10^5 copies of HBV/H recovered from the Huh7 cells culture supernatants, and the dynamics of HBV/H DNA indicated approximately 2 logs elevation within the following 5 weeks. At week 10 when HBV/H DNA level exceeded concentration of $>10^7$ copies/mL, the chimeric mice were superinfected by inoculation of HBV/G. The HBV/G DNA level increased within 5 weeks after the superinfection and plateaued around 10^7 copies/mL. Two HBV antigens (HBsAg and HBeAg) waxed and waned in profiles similar to that of HBV DNA.

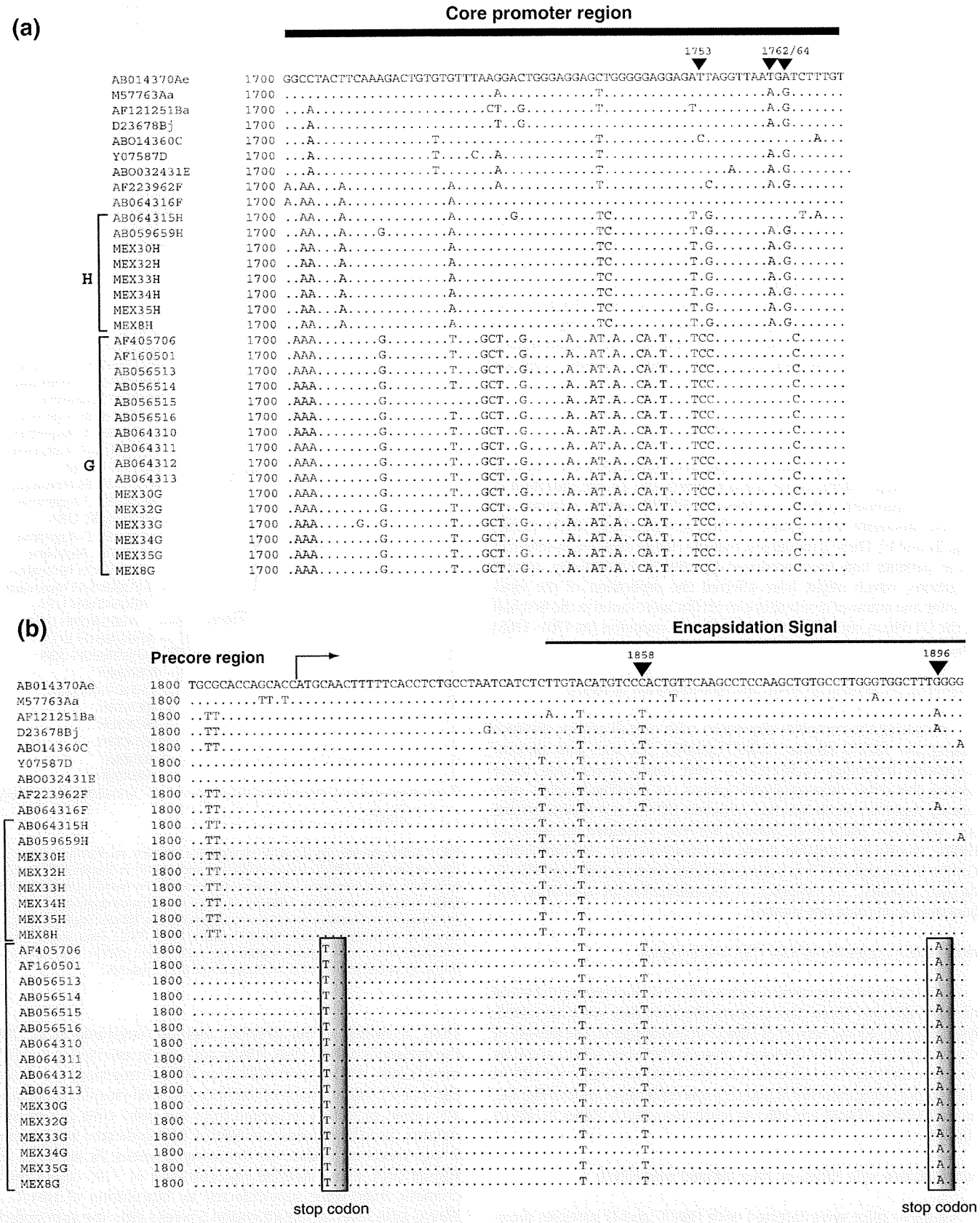

Fig. 2. Nucleotide sequences spanning (a) core promoter and (b) precore gene in HBV genotypes A–H. The reference sequence is shown for the AB014370Ae. The precore region includes pregenome encapsidation signal. Positions of the double mutation (A1762T and G1764A), T1753C, and C1858T making a pair with G1896A are indicated by inverted triangles. Two stop codons in the precore region are shaded.

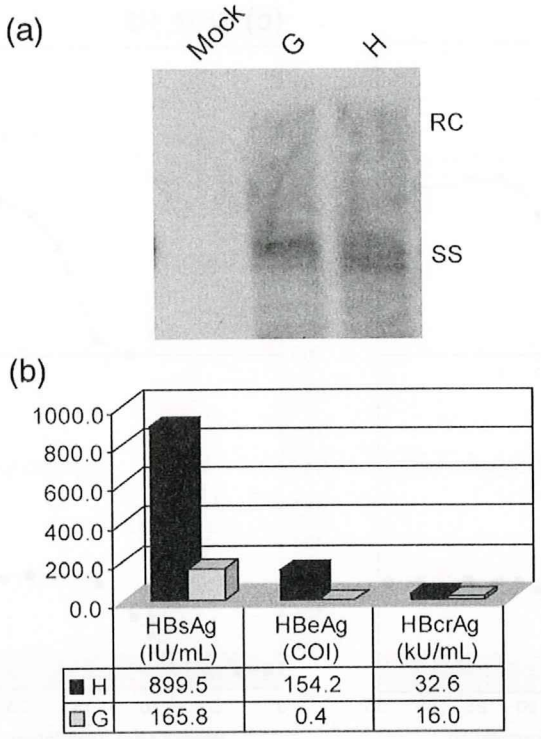

Fig. 3. Intracellular expression of HBV DNA and antigens. (a) The density of single-stranded (ss) HBV DNA was compared between HBV/G and H by Southern blotting. RC; relaxed circular double stranded DNA forms. (b) HBsAg, HBeAg and HBcrAg levels were also compared.

Coinfection of mice with HBV/H and G by inoculation with a mouse passage of G-on-H superinfection

Another 3 chimeric mice (ChiM202-17, ChiM212-22 and ChiM314-12) received serum from sacrificed ChiM_H2 with G-on-H superinfection taken at week 34 when the HBV/G and H DNA was around 5×10^6, 10^8 copies/mL, respectively (Fig. 4b). Profiles of HBV/H and G, after inoculation with 10^6 copies of HBV DNA, were similar among the 3 chimeric mice. Despite receiving the inoculation with a mouse passage supposedly containing HBV/G strain, the HBV/G DNA was not detectable until week 4 after the passage. At the week 4 when HBV/H DNA level exceeded concentration of $>10^7$ copies/mL, HBV/G started to increase and plateaued around 10^8 copies/mL at week 16 (Fig. 4d).

Cloning and sequencing HBV DNA in chimeric mice coinfected with HBV/H and G

HBV DNA clones from sera of the ChiM_H1 and ChiM_H2 sampled at 26 and 34 weeks, respectively (Fig. 4a and b) included those of HBV/H and G invariably. At least 5 clones were propagated and completely sequenced in each serum; but no mutation was observed when the clones were compared to the original inoculum of either genotype. No evidence of recombinations was detected between HBV/H and G on the basis of complete genome analyses.

Pathology in the liver of chimeric mouse infected with HBV/G and H

Fig. 5a shows histology of liver of a chimeric mouse 26 weeks after superinfection with HBV/G on H (ChiM_H1). The mouse coinfected with HBV/G and H revealed fibrosis of stage 1 (F1) and inflammation of grade 2 (A2) with Hematoxlin–Eosin and Masson's trichrome stain (Fig. 5a), whereas the mouse monoinfected with HBV/H had no fibrosis (Fig. 5b). ChiM_H2 also had F1A1 at week 34, but ChiM_H3 was not available for histological examination due to sudden death. Interestingly, a chimeric mouse (ChiM202-17) received serum from ChiM_H2 with G-on-H superinfection revealed F2A2 at week 24 (Fig. 5c). It might be difficult to evaluate its statistical significance due to small number.

Discussion

The HBV/G was initially reported in the US, Canada and Europe in coinfection with HBV/A (Kato et al., 2002a,b; Osiowy and Giles, 2003; Stuyver et al., 2000), which is the one of the prevalent genotypes in both of the regions. Recently, coinfection of HBV/G with H as well as G/C recombinant were reported in Mexico and Thailand, respectively (Sanchez et al., 2002, 2007; Suwannakarn et al., 2005), and accumulated data indicated trend to preferential occurrence of the HBV/G coinfection in population of MSM and IDU, possibly via particular infectious routes of transmission. A phylogenetic analysis of the complete genome of HBV/G strains isolated in the present study indicated their close relationship with those previously reported, despite the fact that they were all isolated from HBV carriers infected with different genotypes that were prevalent in the respective area. These suggest that the HBV/G is genetically homologous and has no specific preference for HBV genotype to be coinfected. Thus, the HBV/G may be found among HBV carriers in any part of the world regardless of the prevalent genotype.

Invariably coinfected with other genotypes, HBV/G was once deemed a defective virus that cannot replicate by itself. Recently, however, Chudy and his associates reported plasmapheresis and platelet donor who was infected with HBV/G alone and had transmitted it to two recipients in look-back studies (Chudy et al., 2006). Since the donor and two recipients were not coinfected with HBV of the other genotypes by the reversed hybridization assay (Hussain et al., 2003), capable of detecting HBV infection of any genotype in low titers, the authors concluded that HBV/G can monoinfect human beings without help from coinfecting HBV of other genotypes (Chudy et al., 2006).

The uPA/SCID mouse with the liver replaced for human hepatocytes (Heckel et al., 1990) is a very useful model for studies on hepatitis virus infection and replication *in vivo* (Dandri et al., 2001; Tsuge et al., 2005). Using this model we have previously demonstrated that despite of apparent dependence on other genotype strain (HBV/A or HBV/C) in replication, the viral load of the HBV/G eventually outgrows and takes over the coinfecting strain in the chimeric mice (Sugiyama et al., 2007).

In the present study, the chimeric mice had undetectable HBV DNA in the serum when monoinfected with HBV/G, whereas superinfection on HBV/H enhanced replication of the HBV/G. This observation is concordant with previous experimental study which indicated a rapid takeover in mice initially infected with HBV/A or C and superinfected with HBV/G (Sugiyama et al., 2007). The takeover was significantly enhanced in mice infected with HBV/G on HBV/A when compared to HBV/G on HBV/C; with the mean time required for a 10-fold increase (log time) in the HBV/DNA level 1.6 vs. 3.3 weeks, respectively (Sugiyama et al., 2007). Interestingly, the present study demonstrates that pattern of the genotypic interaction between HBV/G and H was similar to that described for HBV/G on C; with the HBV DNA log time 3.0 weeks, suggesting that HBV/G is better sustained in human population by its association with HBV/A than with other HBV genotypes. These experimental results were also supported by clinical observations of the takeover which was demonstrated in a patient coinfected with HBV/G and A by cloning and sequencing (Kato et al., 2002a). However, such a heavy dependence of HBV/G on other genotype strain does not seem to require intergenotypic recombination between them, as no recombination event was observed in chimeric mice coinfected with HBV/G and any other genotype strain. The mechanism how HBV/G depends on other genotypes for replication had been pursued in cotransfection experiments in Huh7 cells; our previous *in vitro* study showed that trans-complementation with core protein of HBV/A would be required for HBV/G to replicate

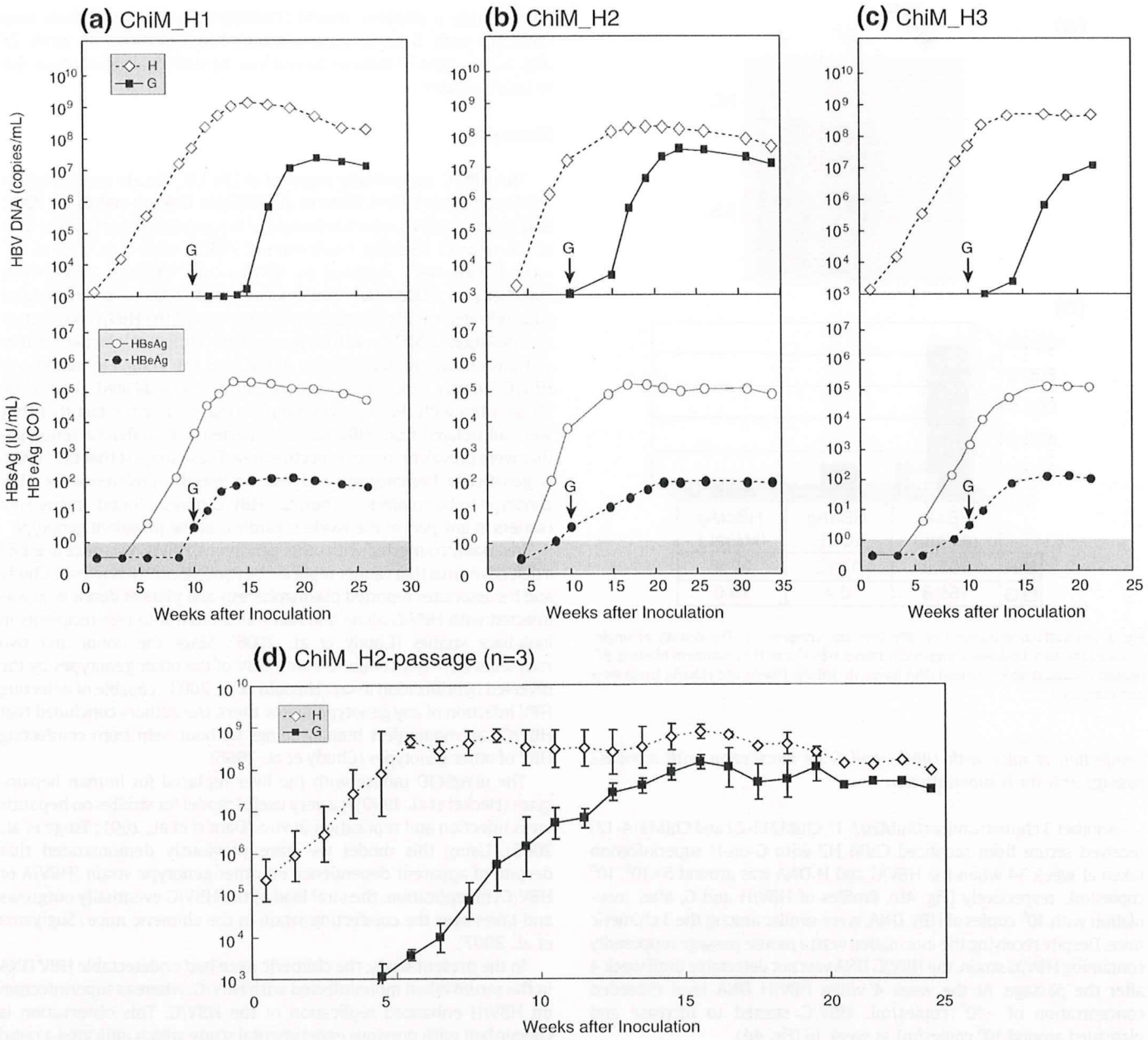

Fig. 4. Superinfection with HBV/G on chimeric mice infected with HBV/H [(a) ChiM_H1, (b) ChiM_H2, (c) ChiM_H3]. Inoculation with genotype G is indicated by large arrows. The dynamics of HBV DNA, HBsAg and HBeAg assessed superinfection of 3 chimeric mice (ChiM_H1–H3) are shown in panels a–c. Another 3 chimeric mice received serum from ChiM_H2 with G-on-H superinfection taken at week 34 when HBV/G DNA plateaued around 10^7 copies/mL (Fig. 4b). Profiles of HBV/H and G, after inoculation with 10^6 copies of HBV DNA, were similar among the 3 chimeric mice. Mean HBV DNA and standard deviation (bar) were shown in panel d.

actively (Sugiyama et al., 2007), suggesting that the replication of HBV/G might be also enhanced by core protein of HBV/H. Two other functional analyses of HBV/G had been already reported. Kremsdorf et al. have proposed the involvement of polymerase encoded by HBV/G in active replication (Kremsdorf et al., 1996) and Li et al. showed that lack of HBeAg expression rather than a replication defect could be the primary determinant for the rare occurrence of HBV/G monoinfection (Li et al., 2007). Hence, possibility remains for other viral elements beyond core protein from coinfecting genotypes to enhance the replication of HBV/G. Further studies are needed to elucidate the mechanism of HBV/G replication by cotransfection of other proteins beyond core protein of HBV/H or construction of domain-switch experiments between the genome of HBV/G and H.

Considering that coinfection with HBV/G may be associated with pathological manifestations, liver histology was investigated in tissue obtained from the mice used in this study. The chimeric mouse co-infected with HBV/G and H had developed fibrosis and inflammation (F1–2, A1–2) in the liver that was not observed in mice with HBV/G or H monoinfection. This might be supported by clinical data in Mexico; Mexican patients infected with the most prevalent HBV/H have milder liver damage when it is in monoinfection rather than in coinfection with other genotypes (personal communication). Our recent study also showed fibrosis of F1–F2 stage in the majority of the mice superinfected with HBV/G on A or C (Sugiyama et al., 2007). Clinically, Lacombe and her colleagues reported more severe fibrosis in human immunodeficiency type-1 (HIV)-positive French patients who were infected with HBV/G than the others (Lacombe et al., 2006). Taken together, clinical and experimental observations indicate that in immunodefficient conditions HBV/G possesses stronger disease-inducing capacity when it is coinfected with other genotype. Unfortunately, studied patients did not have exact diagnosis due to neither histopathological examination nor abdominal ultrasonography, although they were asymptomatic.

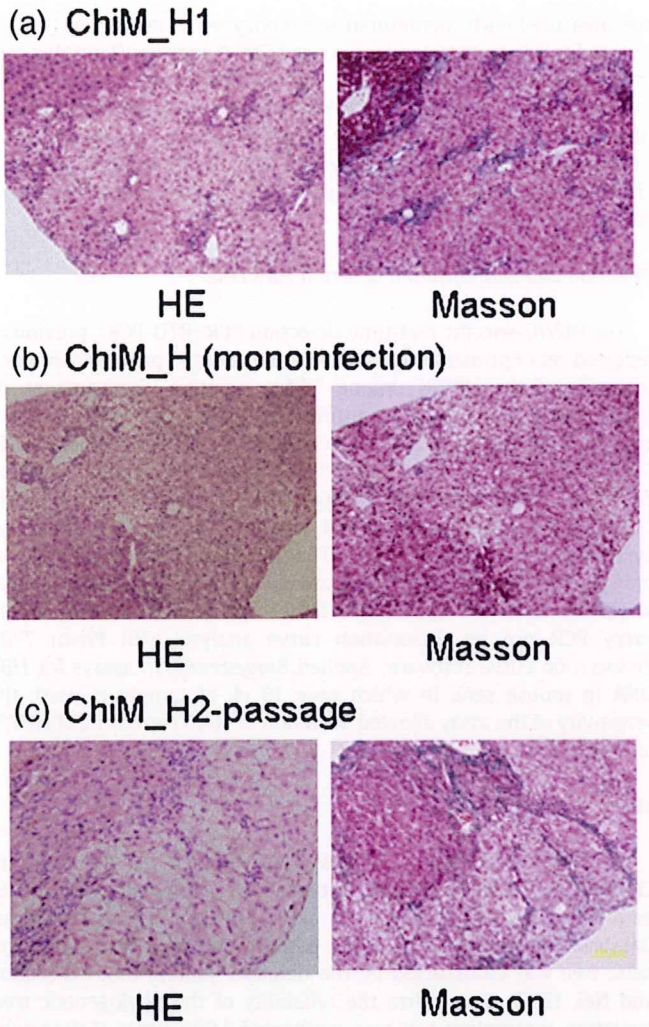

Fig. 5. Liver histology in chimeric mice superinfected with HBV/G on H. (a) A chimeric mouse 26 weeks after superinfection with HBV/G on H (ChiM_H1) revealed F1A2 with Hematoxlin–Eosin (HE) and Masson's trichrome stain. (b) A chimeric mouse 24 weeks after monoinfection with HBV/H had no fibrosis. The peak of HBV DNA was around 10^8 copies/mL. (c) Another chimeric mouse (ChiM202-17) received serum from ChiM_H2 with G-on-H superinfection revealed F2A2 at week 24.

Further prospected studies are required to investigate whether HBV DNA levels (viral replication) and/or other factors would affect liver fibrosis in immunosuppressive conditions.

Two remarkable viral genomic characteristics of the HBV/G have been established by previous reports; one of them is the unique insertion of 36 bp in the core gene, which is shared by all HBV/G strains studied herein and previously (Fig. 2). Although screening of the entire DNA Genome Bank (BLAST search) did not reveal any homologous to the insertion parts within other sequences including those of human, we took the advantage of these peculiar insertion sequences for designing the type-specific primers to be used for screening (Kato et al., 2001) and quantification of the genotype (Sugiyama et al., 2007). In respect to the function of this insertion, it may induce modifications of both the encapsidation signal sequence and the core protein structure, as previously proposed (Junker-Niepmann et al., 1990). Furthermore, HBV/G strains include two stop codons in the precore region at positions 2 and 28, both of which prohibit the translation of the HBeAg precursor (Carman et al., 1989; Okamoto et al., 1990), assumably resulting in HBeAg-negative phenotype of all HBV/G strains. In this study all patients infected with HBV/G, however, were positive for HBeAg. The coinfection with HBV/H would explain the presence of HBeAg in individuals infected with HBV/G, which would be consistent with a previous report (Kato et al., 2002a).

In conclusion, the replication of HBV/G can be enhanced remarkably when it is coinfected with HBV/H prevalent in Mexico. Coinfection with HBV/G may be directly cytopathic in immunosuppressive conditions. Further epidemiological, clinical and *in vitro* studies are required to confirm the clinical manifestation of the HBV/G coinfection with various genotypes and evaluate its genotypic peculiarities.

Methods

Serum samples

Nineteen HBsAg-positive sera were obtained from previously described MSM cohort (age range 22–30 years) in Mexico (Sanchez et al., 2007). HBeAg was detected with chemiluminescent enzyme immunoassay (Ortho Clinical Diagnostics, Tokyo, Japan).

Determination of HBV genotypes and the complete sequences of HBV

Initially, HBV genotypes were determined by EIA with monoclonal antibodies directed to the preS2 epitopes (Usuda et al., 1999, 2000), with use of commercial kits (HBV GENOTYPE EIA, Institute of Immunology Co., Ltd., Tokyo, Japan), allowing the determination of HBV/A–F and HBV/H. HBV/G was confirmed by the G-specific PCR with one of the primers deduced from the sequence of 36 nt. insertion in the core gene (Kato et al., 2001). The complete genomes were further determined. In brief, two partially overlapping fragments were amplified by nested PCR using two sets of primers with LA Taq (TaKaRa Bio Inc., Tokyo, Japan) (Table 1). Similarly, the complete genomes of HBV/H strains were also amplified as two overlapping fragments using different set of primers shown in the Table 2. Thereafter, the PCR products were cloned in a plasmid pGEM-T easy vector (Promega Corp., Madison, WI, US) and sequenced using sequencing primers (Tables 1 and 2) with Prism Big Dye (Applied Biosystems, Foster City, CA, US) on the ABI 3100 DNA automated sequencer. Reference sequences for comparative analyses were retrieved from the DDBJ/EMBL/GenBank database.

Plasmid constructs of HBV DNA

HBV DNA from a Mexican patient (MEX33) was extracted from 100 μL of serum using the QIAamp DNA blood kit (QIAGEN, GmbH, Hilden, Germany). Two overlapping fragments, fragment A and fragment B approximately 1700 bp long, covering the entire genome

Table 1
HBV DNA oligonucleotide primers for complete genomes of genotype G

Primer	Nucleotide sequence (5′–3′)	Position*	Polarity
1) Fragment A			
HBVG3157F	CCTCCTGCCTCCACCAATCG	3157–3176	Sense
HBVG1917R	AGCCAAAAAGGCCATATGGCA	1937–1917	Antisense
HBVG3187F	AGGCAGCCTACTCCCATCTC	3187–3206	Sense
HBVG1797R	CATGGTGCTGGTGCGCAGAC	1816–1797	Antisense
2) Fragment B			
HBVG1601F	ACGTTACATGGAAACCGCCA	1601–1620	Sense
HBVG103R	GATTGACGAGATGTGAGAGGCA	124–103	Antisense
HBVG1630F	CTCATCATCTGCCAAGGCAGT	1630–1650	Sense
HBVG56R	GAACTGGAGCCACCAGCAGG	75–56	Antisense
3) Sequencing primers			
HBSF2	CTTCATCCTGCTGCTATGCCT	407–426	Sense
HBVG894F	AAGTTGGGGTACTTTGCCAC	894–913	Sense
HBVG1013R	TGGGTAAAAGGAGCAGCGAAAC	1034–1013	Antisense
HBVG2052F	GGGAATCCTTAGAGTCCTCTG	2052–2072	Sense
HBVG2491F	TTCCTTGGACTCACAAGGTG2	2491–2510	Sense

* Nucleotide position of reference sequence (AB056513).

Table 2
HBV DNA oligonucleotide primers for complete genomes of genotype H

Primer	Nucleotide sequence (5′–3′)	Position	Polarity
1) Fragment A			
HBVH55F	TCCTGCTGGTGGCTCC	55–70	Sense
HBVH1801R	GTTGCATGGTGCTGGTGAAC	1820–1801	Antisense
HB6R	AACAGACCAATTTATGCCTA	1803–1784	Antisense
2) Fragment B			
HBVH1611F	GAGACCACCGTGAACGCCC	1611–1629	Sense
HBVH285R	GCCAGGACACCCGGGTGGTA	304–285	Antisense
HBVH229R	CGAGTCTAGACTCTGTGGTATTGTGAGG	256–229	Antisense
3) Sequencing primers			
HB2F	TGCTGCTATGCCTCATCTTC	414–433	Sense
HBVH760F	GCCAAATCTGTGCAGCATCTTGAG	760–783	Sense
HB5F	CTCTGCCGATCCATACTGCGGAA	1256–1278	Sense
HBVH1859F	ACTGTTCAAGCCTCCAAGCTGT	1859–1880	Sense
HBVH2415F	GTCGCAGAAGATCTCAATCTC	2415–2435	Sense
HBVH2814F	GGGTCACCATATTCCTGGGAA	2814–2834	Sense

*Nucleotide position of reference sequence (AB059659).

of HBV, were amplified by nested PCR (primers sequences shown in Table 2). Primers used for fragment A were HBVH55F and HBVH1801R for 1st PCR and HBVH55F and HB6R for 2nd PCR. Primers used for fragment B were HBVH1611F and HBVH285R for 1st PCR and HBVH1611F and HBVH229R for 2nd PCR. Then these fragments were ligated into pGEM-T vector (Promega, Madison, WI) and cloned in DH5α cells. Ten clones each (pGEM-fragA-1 to 10, pGEM-fragB-1 to 10) were obtained and the nucleotide sequences were determined. As reported previously (Fujiwara et al., 2005; Sugiyama et al., 2006), these fragments were constructed into the pUC19 vector deprived of promoters (Invitrogen Corp., Carlsbad, CA) by digestion with HindIII and EcoRI, resulting in 1.24-fold the HBV genome, just enough to transcribe over-sized pregenome and precore mRNA. Cloned HBV DNA sequences were determined with Prism Big Dye (Applied Biosystems, Foster City, CA) in the ABI 3100 automated sequencer. Based on our previous report (Sugiyama et al., 2007), a plasmid of HBV/G with 1.24-fold the HBV genome was also constructed in this study.

Cell culture and transfection

Huh7 cells were transfected with plasmids equivalent to 5 μg of HBV DNA constructs with use of the Fugene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN), and harvested after 3 days in culture. Transfection efficiency was monitored by enzymatic activity of secreted alkaline phosphatase (SEAP) in the supernatant of culture with addition of 0.5 μg of reporter plasmids expressing the SEAP.

Determination of HBV markers

HBsAg and HBeAg were determined by chemiluminescent enzyme immunoassay (CLEIA) with commercial kits in a fully automated Lumipulse CLEIA analyzer (Fujirebio Inc., Tokyo, Japan). HB core-related antigen (HBcrAg) was measured in serum using the CLEIA described previously (Kimura et al., 2002). Briefly, 150 μL of serum was incubated with 150 μL pretreatment solution containing 15% sodium dodecylsulfate at 60 °C for 30 min. The pretreated serum was added to a well coated with three monoclonal antibodies against denatured HBcAg and HBeAg. After washing, two other alkaline phosphatase-labelled monoclonal antibodies against denatured HBcAg and HBeAg were added as secondary antibodies. 200 μL substrate (AMPPD:(3-(2′-spiroadamantan)-4-methoxy-4- (3′-phosph oryloxy) phenyl-1, 2-dioxetane disodium salt) (Applied Biosystems, Bedford, MA, USA) solution was added and the assay tube was incubated for 5 min at 37 °C. HBcrAg assay with the relative chemiluminescence intensity was measured with chemiluminescent enzyme immunoassay (CLEIA) system for fully automated Lumipulse f CLEIA analyzer (Fujirebio Inc., Tokyo, Japan), and the HBcrAg concentration was estimated by comparison to a standard curve generated using recombinant HBeAg. In the present study, the cutoff value was tentatively set at 3.0 log U/mlmL. Sera containing over 7.0 log U/ml mL of HBcrAg were diluted 10- or 100-fold in normal human serum and re-tested to obtain the end titer.

Detection and quantification of serum HBV DNA

For HBV/G-specific real-time detection PCR (RTD-PCR), previously reported set of primers was used, where one of the primers contained sequence of the HBV/G unique 36-bp insertion (Sugiyama et al., 2007). For HBV/H DNA quantification, following primers were applied; HBVH29F: 5′-GTT CCA CCA AGC ACT GTT GG-′3, HBV229R: 5′-CGA GTC TAG ACT CTG TGG TAT TGT GAG G-′3. Amplification and detection were performed using SYBR Green PCR Master Mix (Applied Biosystems) on the ABI Prism 7700 Sequence Detection System (Applied Biosystems). The quantification standard was prepared by serial dilution of a known amount of the cloned plasmid of HBV/G or H. The specificity of these primers was confirmed in every PCR run by dissociation curve analysis (ABI Prism 7700 dissociation curve software; Applied Biosystems). In assays for HBV DNA in mouse sera, in which only 10 μL of sample is used, the sensitivity of the assay allowed detection of 1000 copies/mL of HBV/G or HBV/H DNA.

Molecular evolutionary analysis

Nucleotide sequences of HBV were aligned by the program CLUSTAL X, and the genetic distance was estimated with the 6-parameter method (Gojobori et al., 1982) in the Hepatitis Virus Database (Robertson et al., 1998). Based on these values, a phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987). To confirm the reliability of the phylogenetic tree, bootstrap resampling test was performed 1,000 times (Felsenstein, 1985).

Examination for recombination

Intergenotypic recombination was searched for using the method of Robertson et al. (Robertson et al., 1995) with use of the SimPlot program and bootscanning analysis (Lole et al., 1999). The mean genetic distances were calculated with a window size of 200 bp and a step size of 50 bp in this study.

Southern blot hybridizations

Southern blot hybridizations were performed with a full-length probe of HBV/G or H by previous methods (Fujiwara et al., 2005). No significant differences were observed in the detection between internal control HBV DNA and each probe.

Inoculation of chimeric mice with the liver repopulated for human hepatocytes

uPA$^{+/+}$/SCID$^{+/+}$ mice with the liver repopulated for human hepatocytes (chimeric mice) were purchased from Phoenix Bio Co., Ltd. (Hiroshima, Japan). The human hepatocytes were obtained from a single donor (female, 6 years, African American). Human serum albumin was measured by ELISA with commercial assay kits (Eiken Chemical Co.Ltd, Tokyo, Japan). They were inoculated with HBV recovered from culture supernatants of Huh7 cells transfected with plasmids containing 1.24-fold HBV genome constructs of the HBV/G or H (Sugiyama et al., 2006, 2007).

Histopathological examination

Liver tissues were fixed in formaldehyde, embedded in paraffin and stained with hematoxylin–eosin (H–E) or Masson's trichrome. The fibrosis stage and inflammation grade were evaluated by independent expert pathologists.

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Evaluating the performance of Affymetrix SNP Array 6.0 platform with 400 Japanese individuals

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Abstract

Background: With improvements in genotyping technologies, genome-wide association studies with hundreds of thousands of SNPs allow the identification of candidate genetic loci for multifactorial diseases in different populations. However, genotyping errors caused by genotyping platforms or genotype calling algorithms may lead to inflation of false associations between markers and phenotypes. In addition, the number of SNPs available for genome-wide association studies in the Japanese population has been investigated using only 45 samples in the HapMap project, which could lead to an inaccurate estimation of the number of SNPs with low minor allele frequencies. We genotyped 400 Japanese samples in order to estimate the number of SNPs available for genome-wide association studies in the Japanese population and to examine the performance of the current SNP Array 6.0 platform and the genotype calling algorithm "Birdseed".

Results: About 20% of the 909,622 SNP markers on the array were revealed to be monomorphic in the Japanese population. Consequently, 661,599 SNPs were available for genome-wide association studies in the Japanese population, after excluding the poorly behaving SNPs. The Birdseed algorithm accurately determined the genotype calls of each sample with a high overall call rate of over 99.5% and a high concordance rate of over 99.8% using more than 48 samples after removing low-quality samples by adjusting QC criteria.

Conclusion: Our results confirmed that the SNP Array 6.0 platform reached the level reported by the manufacturer, and thus genome-wide association studies using the SNP Array 6.0 platform have considerable potential to identify candidate susceptibility or resistance genetic factors for multifactorial diseases in the Japanese population, as well as in other populations.

Background

Together with technology developments on large-scale single nucleotide polymorphism (SNP) genotyping [1,2], there have been a number of genome-wide association studies (GWAS) to identify candidate susceptibility or resistance genetic factors for multifactorial diseases [3-7]. It is estimated that eleven million SNPs with a greater than 1% minor allele frequency (MAF) are located in the

human genome [8]. Over six million SNPs have been uploaded on public SNP databases through the Human Genome Project and international SNP discovery projects. Among these SNPs, over 900 K SNPs across the human genome are selected with an average MAF of 19.6%, 18.2% and 20.6% in the HapMap Caucasians, Asians and Africans, respectively, and can be simultaneously genotyped using Affymetrix Genome-Wide Human SNP Array 6.0 platform [9]. Several studies have evaluated the coverage of commercial platforms using HapMap population data and genotype data of non-reference Caucasian populations [10-12]. Results from these studies indicated that in a non-reference Caucasian population, as well as the HapMap populations, commercial SNP typing platforms offered similar levels of genome coverage. However, the number of genotyped Japanese individuals in the HapMap project was only 45 samples, which may lead to inaccurate estimation of the number of SNPs with low MAF in the Japanese population.

The SNP Array 6.0 platform offers the genotype calling algorithm "Birdseed" to determine the genotypes of 909,622 SNPs [9]. The Birdseed algorithm performs a multiple-chip analysis to estimate signal intensity for each allele of each SNP, fitting probe-specific effects to increase precision, and then makes genotype calls by fitting a Gaussian mixture model in the two-dimensional A-signal vs. B-signal space, using SNP-specific models to improve accuracy. There was a report that 45% of SNPs observed to be significantly associated with the disease did not agree with Hardy-Weinberg equilibrium (HWE) using the previous version of Mapping 500 K Array set [13]. Some of the miss-called SNPs would be induced by genotype calling algorithms and are likely to be ranked as significantly associated with the disease (false-positive). Therefore, there are strong demands for accurate genotype calls using the Birdseed algorithm.

The SNP Array 6.0 platform has three check points prior to hybridization on GeneChip arrays in order to exclude experimental errors; PCR amplicon size check by electropherograms, DNase I digested fragment size check by electropherograms and quantity check of the purified PCR products. The platform also includes Quality Control (QC) probes for 3,022 SNPs to assess the overall quality for a sample based on the Dynamic Model (DM) algorithm. There are assay criteria to exclude experimental errors and low-quality samples; however, we empirically know that some samples, which pass these criteria, have low-quality genotyping results.

In this study, we genotyped 400 non-HapMap Japanese samples using the SNP Array 6.0 platform in order to evaluate the number of SNPs available for GWAS in the Japanese population, to examine an appropriate approach for acquiring accurate genotype calls using the Birdseed genotype calling algorithm, and to evaluate the assay criteria for preventing low-quality genotyping data.

Results

Genotyping 400 Japanese samples using SNP Array 6.0 platform

We collected 2 sets of 200 Japanese samples for genotyping using the SNP Array 6.0 platform. The average concentration of genomic DNA for the 1st set of 200 samples was 54.8 ng/μl and that for the 2nd set of 200 samples was 52.7 ng/μl. One of the critical points for the SNP Array 6.0 platform to acquire high quality genotyping data is to prepare a uniform quantity of 250 ng genomic DNA for Nsp I and Sty I digestion steps. When an almost 10-fold excess amount of genomic DNA was used, the average overall call rate drastically decreased to about 80% for both Nsp I and Sty I digestion steps with the Mapping 500 K Array (data not shown).

The average concentration of purified PCR products for the two sets of 200 samples was 524.4 ng/μl (range 412.8 to 718.0 ng/μl) and 497.3 ng/μl (range 256.6 to 939.8 ng/μl), respectively (Figure 1a and Figure 2a). In total, 11 samples (2 samples for the 1st set and 9 samples for the 2nd set) showed low QC call rates below the default 86% QC criteria (Figure 1b and Figure 2b). The genotype calls of 909, 622 SNPs for each individual were determined using the Birdseed genotype calling algorithm, embedded in the Affymetrix Genotyping Console 2.0 software (Affymetrix). The 198 samples of the 1st set that were over 86% QC criteria were used to assign genotypes and had an average overall call rate of 99.58%, ranging from 96.42 to 99.90% (Figure 1c). For the 2nd set, 191 samples were over 86% QC criteria and the average overall call rate was 97.54%, ranging from 89.52 to 99.27% (Figure 2c). When genotype calls were determined for every 48 samples analyzed simultaneously in the same batch, the average overall call rate was improved to 99.71% (range, 98.37 to 99.94%) for the 1st set, and 98.66% (range, 94.86 to 99.76%) for the 2nd set (Figure 1d and Figure 2d).

Assay criteria for experimental errors occurring on running batches

The SNP Array 6.0 platform has three check points prior to hybridization on GeneChip arrays in order to remove samples with experimental errors. However, some samples that pass these check points still have relatively low-quality genotyping results with lower overall call rates than 97%; 1 sample for the 1st set of 200 samples and 59 samples for the 2nd set of 200 samples. When genotype calls were determined for every 48 samples simultaneously analyzed in the same batch, the average overall call rate of 48 samples for batch #1 from the 2nd set was 97.21%, which was almost 2% lower than other batches