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ORIGINAL ARTICLE

Specific mutations of basal core promoter are associated with chronic liver disease in hepatitis B virus subgenotype D1 prevalent in Turkey

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ABSTRACT

The role of hepatitis B virus (HBV) genetics in the clinical manifestations of infection is being increasingly recognized. Genotype D is one of eight currently recognized major HBV genotypes. The virus is ubiquitous worldwide, but shows different features in different regions. One hundred and ninety-eight patients with chronic HBV infection were enrolled in this study, 38 of whom had been diagnosed with cirrhosis of the liver and/or hepatocellular carcinoma. HBV DNA was isolated from the patients' blood samples and the entire genome and/or the basal core promoter/core promoter region sequenced. Phylogenetic analysis of the complete genomes revealed that subgenotype D1 is the most prevalent subgenotype in Turkey, but there was no definite phylogenetic grouping according to geography for isolates from different regions within Turkey, or for isolates in Turkey relative to other parts of the world. Turkish isolates tended to be genetically similar to European and central Asian isolates. Overall, HBV-infection in Turkey appears to be characterized by early HBeAg seroconversion, a high incidence of the A1896 core promoter mutation and a small viral load. Genotype D characteristic mutations A1757 and T1764/G1766 were found in the BCP region. T1773 was associated with T1764/G1766 and a larger viral load. In conclusion, infection with HBV genotype D in Turkey has a similar clinical outcome to that of Europe and central Asia. Genotypic mutations in genotype D may be linked with disease prognosis in Turkey, but further studies with higher sample numbers and balanced clinical groups are needed to confirm this.

Key words basal core promoter, genotype D, hepatitis B virus, Turkey.

Hepatitis B virus infection is a global public health problem, affecting more than 350 million people worldwide. The clinical manifestations of this infection vary greatly and include acute self-limiting disease, an inactive carrier state and CH with progression to LC and HCC (1, 2). An accumulating body of evidence indicates that the viral genotype (3, 4) and specific mutations in the viral genome (5, 6) are important viral factors contributing to the development of HCC. The main eight genotypes of HBV (A–H) have been identified based

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List of Abbreviations: γ-GTP, γ-glutamyl transpeptidase; AFP, alpha-fetoprotein; Alb, albumin; ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate aminotransferase; BCP/CP, basal core promoter/core promoter; CH, chronic hepatitis; DB, direct bilirubin; EIA, enzyme-linked immunoassay; Glob, globulin; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinomas; HCV, hepatitis C virus; Kozak, Kozak sequence used in translation initiation site of eukaryotic mRNA; LC, liver cirrhosis; PLT, platelet; PT-INR, Prothrombin time-international normalized ratio; TB, total bililubin; TP, total protein.

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on comparison of complete genomes, most genotypes having a distinct geographic distribution (7). There are some indications of correlations between HBV genotypes and clinical manifestations of this infection; one study showing that HBV genotype D is more strongly associated with severe liver disease and HCC than is genotype A (8). However, other studies found no association between genotype and clinical manifestations of this infection (9, 10). Specific mutations in the HBV genome reportedly affect both translation of the HBeAg and replication of HBV, thereby influencing the clinical manifestations of HBV infection and contributing to development of HCC (11, 12).

The aim of the current study was to investigate the distribution of HBV genotypes and subgenotypes in chronic hepatitis B patients in different regions of Turkey and to compare these distributions with those of HBV genotypes from other parts of the world. Our aim was to make it possible to draw inferences about disease transmission within Turkey, and between Turkey and other countries. This topic is particularly interesting, given Turkey's location at the crossroads of Europe and Asia. We also investigated the prevalence of BCP/CP mutations in patients with and without LC and/or HCC.

MATERIALS AND METHODS

Patients

In all, 198 patients with CHB were enrolled in the study. All were attendees at four clinical centers in geographically distinct parts of Turkey, namely Samsun (north), Ankara (center), Gaziantep (south) and Istanbul (west). The patients' ages ranged from 16 to 73 years. In 38 of the patients, LC or HCC had been diagnosed before enrollment (Table 1).

Diagnoses based on HBsAg seropositivity for longer than 6 months, clinical findings and liver biopsies were used to classify the patients into two clinical groups: (i) CH patients with persistently high serum ALT concentrations but no evidence of LC or HCC; and (ii) LC and/or HCC patients (hereafter referred to as LC/HCC patients) with clinical evidence of cirrhosis (e.g., coarse liver architecture, nodular liver surface and blunt liver edge) based on evidence of hypersplenism (e.g., splenomegaly demonstrated by ultrasonography or computed tomography and platelet counts of $< 100{,}000$ platelets mm^3) and complementary clinical information (e.g., ascites, jaundice, encephalopathy or esophageal varices), and/or HCC diagnosed on the basis of results of imaging studies together with high serum AFP concentrations (≥ 400 ng/mL). Sera were collected from each individual and stored immediately at $-70°C$ until use. The serological and biochemical tests were performed at Ondokuz Mayis University (Kurupelit, Turkey). Molecular analyses were performed at the Department of Virology, Liver Unit, Nagoya City, University Graduate School of Medical Science, Nagoya, Japan. The study was approved by the Ethics Committee of the School of Medicine, Ondokuz Mayis University. Informed consent was obtained from all subjects and the study was conducted in accordance with the declaration of Helsinki (as revised in Tokyo 2004).

Table 1. Summary of samples collected

City (location)	N (%)
Samsun (north)	63 (31.8)
Ankara (center)	76 (38.4)
Gaziantep (south)	20 (10.1)
Istanbul (west)	39 (19.7)
Total	198 (100)

Serological analysis

Hepatitis B surface antigen, anti-HBs, HBeAg, anti-HBe, anti-HBc IgG, anti-Delta, and anti-HCV in patient serum samples were detected by ARCHITECT (Abbott Diagnostics, Lake Forest, IL, USA). Biochemical markers, including concentrations of anti-HCV, HBeAg, TP, Alb, Glob, PT-INR, AST, ALT, γ-GTP, ALP, TB, DB, and HBV DNA and PLT counts in all samples were measured at the local hospitals.

Genotyping of hepatitis B virus

Hepatitis B surface antigen-positive samples were subjected to HBV genotyping using commercially available EIA kits (Institute of Immunology, Tokyo, Japan). This method allows discrimination among the seven major HBV genotypes (A–G) by monoclonal antibodies targeted to the pre-S2 epitopes (2). HBV genotype H was not determined in this study because the EIA kit is unable to identify it.

Sequencing and phylogenetic analysis

Nucleic acids were extracted from 100 μL of serum using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Complete genomes were amplified using primer sets as described previously (13). Partial HBV genomes were also amplified in enhancer II/core promoter and precore regions as described previously (13).

PCR products were directly sequenced with the ABI PRISM BigDye v3.1 kit (Applied Biosystems, Foster City, CA, USA) on an ABI 3100 DNA automated sequencer. All sequences were analyzed in both forward and reverse directions. Complete and partial genomes were assembled

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using GENETYX Version 11.0 (GENETYX Corporation, Tokyo, Japan) Additional sequences were retrieved from the DNA Data Bank of Japan, EMBL Nucleotide Sequence Submissions and GenBank nucleic acid sequence databases for phylogenetic analysis. Phylogenetic relationships between sequences were determined using the neighbor-joining method using MEGA 4 software (14).

Quantification of serum hepatitis B virus DNA

Hepatitis B virus DNA was quantified using real-time detection PCR as previously described (15), with modifications as previously described (16). The detection limit of this assay was 100 copies/mL.

Statistical analysis

Statistical differences were evaluated by Fisher's exact test and the X^2 test, with Yates' correction for continuity where appropriate. Differences were considered significant for P-values < 0.05. All statistical analyses were performed using version 8.0 of the Stata Software package (StrataCorp LP, College Station, TX, USA).

RESULTS

Clinical characteristics of chronic hepatitis B patients in Turkey

Sera were collected from a wide area of Turkey. Figure 1 and Table 1 illustrate the locations of the four cities and the number of collected samples, respectively. The clinical characteristics of the 198 HBsAg-positive patients are summarized in Table 2. The LC/HCC patients were significantly older than the CH patients ($P < 0.0001$). Most (89.5%, 34 of 38 patients) were male. The concentrations of ALT, AST, and HBV DNA were not significantly different in LC/HCC patients compared to CH patients.

Hepatitis B virus genotypes

Hepatitis B virus genotypes were successfully determined in 185/198 HBsAg-positive patients by the EIA genotyping method. Genotyping was not possible for the remaining 13 patients because no HBV PCR products were detected in their samples.

Phylogenetic analysis of hepatitis B virus isolates based on complete genome sequencing

Whole HBV genomes were obtained from 36 of the HBsAg samples from four different geographical regions of Turkey, including 9 strains from Ankara, 15 from Samsun, 4 from Istanbul and 8 from Gaziantep (Fig. 2). All but one of these strains clustered with database reference strains representing genotype D, subgenotype D1. One strain isolated from a patient in Samsun clustered with subgenotype D3 references.

Fig. 1. Geographical locations from which samples were collected. All samples were collected at four clinical centers in geographically distinct parts of Turkey: Samsun (north), Ankara (center), Gaziantep (south), and Istanbul (west).

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Table 2. Clinical characteristics of chronic viral hepatitis patients from Turkey with and without liver cirrhosis and/or hepatocellular carcinoma

Characteristic	Total (n = 198)	CH (n = 160)	LC/HCC (n = 8)	P-values
Age (years)†	41.4 ± 15	37.1 ± 13	58.9 ± 9	< 0.001
Male‡	132 (66.7)	98 (61.2)	34 (89.5)	< 0.001
Anti-HCV‡	1 (0.5)	1 (6%)	0	NS
Genotyped (HBV/D)‡	185 (93.4)	151 (94.4)	34 (89.5)	NS
HBeAg‡	68 (34.3)	59 (36.9)	9 (23.7)	NS
TP (g/dL)†	7.4 ± 0.8	7.6 ± 0.6	6.5 ± 1	< 0.001
Alb (g/dL)†	3.9 ± 0.7	4.1 ± 0.5	3.0 ± 0.7	0.001
Glob (g/dL)†	3.5 ± 0.6	3.5 ± 0.6	3.4 ± 0.6	NS
PLT count ($\times 10^3$ μL)†	150 ± 107	164 ± 112	94 ± 65	< 0.001
PT-INR†	1.1 ± 0.3	1.0 ± 0.2	1.5 ± 0.2	0.01
AST (IU/mL)†	93 ± 194	90 ± 208	107 ± 119	NS
ALT (IU/mL)†	123 ± 226	133 ± 247	80 ± 75	NS
	55 ± 47	52 ± 48	69 ± 37	NS
ALP (IU/L)†	133.1 ± 83.4	134 ± 84	130 ± 82	NS
TB (mg/dL)†	1.9 ± 5.5	1.1 ± 2.4	5.4 ± 10.9	< 0.001
DB (mg/dL)†	1.2 ± 4.7	0.5 ± 1.5	4.1 ± 9.9	< 0.001
HBV DNA ($\log_{10}$ copies/mL)†	5.4 ± 24	5.9 ± 25	0.2 ± 0.8	NS

NS, not significant.
†mean ± SD ‡number (%) of patients (percentage).

Basal core promoter and core region sequence analysis

To investigate genetic differences between LC/HCC and CH patients, the BCP/CP regions of HBV were successfully sequenced in samples from 22 LC/HCC patients and 52 age-, sex- and HBeAg-status-matched non-LC/HCC patients. Matching control subjects for these characteristics is important because HBV mutation rates are dependent on them. A summary of mutations observed in the BCP/CP region is presented in Table 3. There was a tendency toward a difference in the prevalence of the T1764G1766 double mutation (P = 0.065) and a statistically significant difference in prevalence of the A1896 mutation (P = 0.03) between LC/HCC and non-LC/HCC patients, a higher prevalence being found in LC/HCC patients. There was also a significant difference in the prevalence of the C1773 mutation, which was more frequently present in CH patients than in controls (P = 0.05).

Further, viral and host characteristics of the HBeAg-positive and HBeAg-negative patients were compared by using the samples from which the BCP/CP sequence were obtained (Table 4). Significant differences between the groups were observed in terms of the prevalence of V1753, A1757, and A1896 mutations (P = 0.011, 0.024, and 0.0001, respectively).

The T1773 mutation is associated with HBeAg-negative patients and is less often found in patients with advancing liver disease and infection with HBV genotypes B and C (17). However, in the present study, an excess of the T1773 mutation was not observed in patients with mild liver damage (Table 3) or HBeAg-negative patients (Table 4). On the other hand, specific mutation patterns were observed in HBeAg-negative patients infected with HBV/D. All the HBeAg-negative samples shown in Table 4 were allocated to two groups based on their 1773 mutation patterns (T1773 or C1773) and analyzed to determine any correlations with other mutations in the core promoter region. As shown in Table 5, the T1773 mutation coupled with the double mutation, T1764/G1766. In addition, the prevalence of A1757/T1764/G1766 mutations in the T1773 mutation group was statistically significant. The T1773 group had a larger viral load than did the C1773 group without the T1764/G1766 double mutation.

DISCUSSION

Viral hepatitis is one of the most prevalent and serious infectious diseases in the world and presents a serious public health problem. HBV infection follows different routes of inter- and intra-community transmission, various geographical, social and cultural factors playing important roles. The epidemiology of HBV genotypes provides useful information about population-specific behaviors, which may have direct or indirect roles in HBV transmission (18).

In this study, we investigated the genetic characteristics of HBV in a cohort of patients with CH with and without LC/HCC in Turkey. Phylogenetic analysis of complete genomes was carried out on HBV isolates from patients in different regions of Turkey to determine the distribution and transmission of different HBV genotypes within different areas of the country, and between Turkey and other parts of the world. In the present study in Turkey, all HBV genotypes (subgenotypes) were D1 type except for one isolate. There were no specific phylogenetic groupings of HBV isolates according to geography within Turkey. Previous studies have reported a high prevalence of genotype D1 (approximately 89%) in Mongolia (19, 20). They showed that HCV and Delta virus co-infections with HBV infection confer a high risk of HCC. These studies differ from the present study in that they investigated cases of co-infection with HBV and HCV or with HBV and Delta virus to assess association with HCC whereas we assessed cases of monoinfection. However, in Turkey genotype D1 infection without HCV co-infection is characterized by early HBeAg seroconversion, a small viral load upon seroconversion

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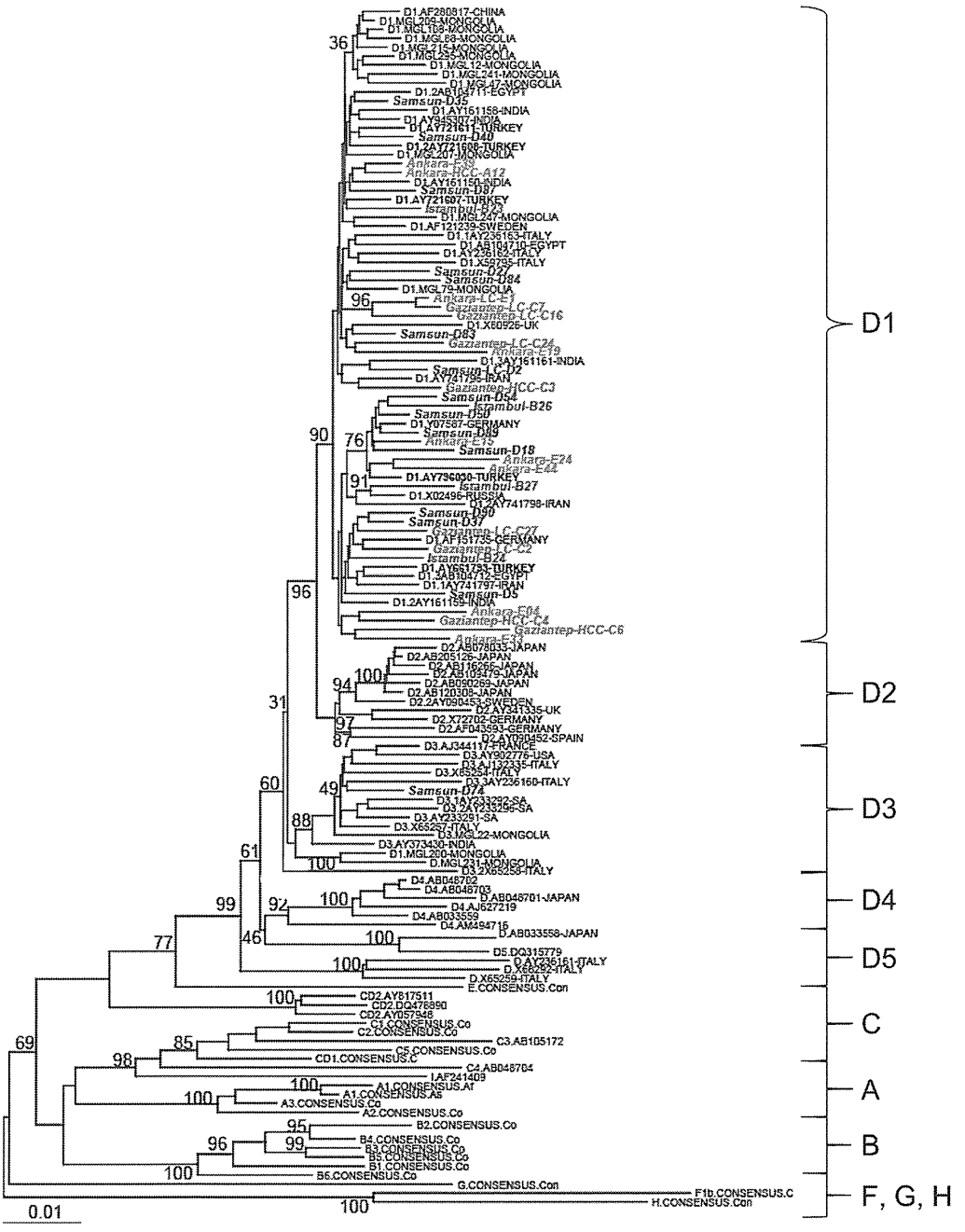

Fig. 2. Neighbor-joining phylogram based on complete hepatitis B virus genomes from Turkey and other countries. Sequences from the current study are color-coded according to the region of Turkey from which they were isolated (blue, Samsun; green, Ankara; orange, Gaziantep; red, Istanbul), and other Turkish sequences are labeled in bold. The study isolates were subjected to bootstrap re-sampling with all available complete genome sequences obtained from the EMBL, DDBJ, and GenBank nucleic acid sequence databases. Sequences used for the phylogenetic tree are indicated under the corresponding accession numbers from sequence databases and country of origin.

and a relatively low incidence of LC/HCC in those infected, which might indicate that HCV and Delta virus co-infection change the pathogenesis of HBV genotype D1.

Two previous studies on Iranian and Mongolian genotype D isolates reported a genotype-specific pattern of the functionally important BCP/CP region, with A1757 and T1764/G1766 (21, 22). Our *in vitro*

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Table 3. A comparison of BCP/PC mutations between age-, sex-, and HBeAg-status-matched chronic viral hepatitis patients from Turkey with and without LC and/or HCC

	CH (n = 52)	LC/HCC (n = 22)	P-values
Age (years, mean ± SD)	46.6 ± 11	57.1 ± 10.1	NS
Male	38 (73.1)	20 (90.9)	NS
HBeAg	21 (40.4)	7 (31.8)	NS
T1653	3 (5.8)	3 (13.6)	NS
C1727	12 (23.1)	3 (13.6)	NS
C1752	12 (23.1)	3 (13.6)	NS
V1753	14 (26.9)	5 (22.7)	NS
G1757	13 (25)	3 (13.6)	NS
T1762/A1764	19 (36.5)	7 (31.8)	NS
T1764/G1766	7 (13.5)	7 (31.8)	NS (0.065)
C1773	27 (51.9)	6 (27.3)	NS (0.053)
Kozak	8 (15.4)	2 (9.1)	NS
H1862	5 (9.6)	5 (22.7)	NS
A1896	22 (42.3)	16 (72.7)	0.017

NS, not significant.
Numbers in brackets represent percentages (%).
V base contains A, C or G bases. H base contains A, C or T bases. Kozak, Kozak sequence used in translation initiation site of eukaryotic mRNA.

Table 4. A comparison of BCP/PC mutations between age-matched patients with chronic viral hepatitis from Turkey with positive or negative HBeAg status

	HBeAg-positive (n = 47)	HBeAg-negative (n = 50)	P-values
Age (years, mean ± SD)	37.6 ± 14.4	37.6 ± 14.4	Matched
LC/HCC	7 (14.9)	14 (28)	NS
Male	30 (63.8)	37 (74)	NS
T1653	2 (4.3)	4 (8)	NS
C1727	7 (14.9)	10 (20)	NS
C1752	9 (19.1)	9 (18)	NS
V1753	5 (10.6)	16 (32)	0.011
A1757	43 (91.5)	37 (74)	0.024
T1762/A1764	10 (21.3)	17 (34)	NS
T1764/G1766	9 (19.1)	11 (22)	NS
C1773	16 (34)	24 (48)	NS
Kozak	2 (4.3)	8 (16)	NS (0.057)
T1862	7 (14.9)	7 (14)	NS
A1896	6 (12.8)	38 (76)	<0.001

NS, not significant. Numbers in bracket represent percentages (%).
V base contains A, C or G bases. Kozak, Kozak sequence used in translation initiation site of eukaryotic mRNA.

Table 5. Association between T1773 and T1764/G1766 double mutation

	T1773 (n = 26)	C1773 (n = 24)	P-values
LC/HCC	9 (34.6)	5 (20.8)	NS
Male	22 (84.6)	15 (62.5)	NS
Age (years, mean ± SD)	36.9 ± 12.5	38.3 ± 14.3	NS
T1653	0	4 (16.7)	0.030
V1753	7 (25.9)	9 (37.5)	NS
A1757	20 (76.9)	17 (70.8)	NS
T1762/A1764	8 (29.6)	9 (37.5)	NS
T1764/G1766	11 (40.7)	0	< 0.001
Kozak	2 (7.4)	6 (25)	NS (0.095)
T1862	6 (22.2)	1 (4.2)	NS (0.054)
A1896	19 (70.4)	19 (79.2)	NS
A1757 + T1762/A1764	3 (11.1)	4 (16.7)	NS
A1757 + T1764/G1766	11 (40.7)	0	0.001
Al 757 + wild 1762/1764/1766	6 (23.1)	13 (54.2)	0.024
G1757 + T1762/A1764	5 (18.5)	5 (20.8)	NS
Gl 757 + wild 762/1764/1766	1 (3.7)	2 (8.3)	NS
HBV DNA $\log_{10}$ copies/mL	5.4 ± 1.8	4.6 ± 1.3	0.009

NS, not significant. Numbers in brackets represent percentages (%).
V base contains A, C or G bases. Kozak, Kozak sequence used in translation initiation site of eukaryotic mRNA.

experiments indicated that the A1757 and T1764/G1766 mutations are associated with the levels of viral. In the present study, we observed an association between T1773 and T1764/G1766 and a higher viral load in Turkish patients, but identified no clear correlations between mutations in the BCP, PC, and/or core region and disease prognosis. This may have been a result of the uneven group sizes of the samples or a specific pattern of viral mutation that is dependent on geographical area. Further *in vitro* and clinical studies are needed to clarify the role of the 1773 mutation.

In this study, we observed an accumulation of T1773 mutations in CH patients and no statistically significant difference between HBeAg positive and HBeAg negative patients, in contrast to a previous paper on Taiwanese subjects (17). These discrepancies might be related to the different study populations because HBV mutation patterns are dependent on genotype and race. Turkey is a high prevalence area for HBV/D according to nation-wide collection of samples, whereas Taiwan area is known to have a high prevalence of HBV/B and C. As previous studies have reported (21, 22), HBV/D1 has a unique mutation pattern in the BCP/CP region. The T1762/A1764 double mutation frequently occurs in HBV/B and C, whereas the T1764/G1766 double mutation tends to occur in HBV/D1. The amount of HBV-DNA in the A1757/T1764/G1766/T1773 mutation group was higher than that in the non-A1757/T1764/G1766/T1773 group; these findings are in concordance with those of Sendi *et al.* (22). Therefore, the specific mutation pattern of HBV/D1 might provide advantages in viral replication. Detection of coordinated mutations such as A1757/T1764/G1766/T1773 suggests the possibility that a

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mechanism such as secondary structure or a distinct transcriptional factor binding in the BCP/CP region of HBV/D1 is having an effect. Computer simulation shows binding of hepatocyte nuclear factor 3 on A1757/T1764/G1766/T1773.

Hepatitis B virus has a compact and constrained genome (23), and correlations between particular mutations in *cis*-acting elements of the virus and different phenotypic features of the virus have been shown clinically (17, 24–26), *in vitro*, and *in vivo* (27–29). In addition to viral factors, environmental factors such as exposure to aflatoxin (30) and the prevalence of co-infections (19, 31), may play important roles in causing regional differences in the clinical manifestation of HBV infections. Recent progress associated with the human genome project indicates the importance of host genetic factors in the outcome of HBV infections (32). There is still much to discover about HBV genotype D infection. We recommend that future work focus on characterizing the disease at a sub-genomic level in different parts of Asia in which genotype D is endemic, and broadening studies to include host factors.

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DISCLOSURE

All authors have no conflicts of interest.

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Mechanism of the dependence of hepatitis B virus genotype G on co-infection with other genotypes for viral replication

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SUMMARY. Hepatitis B virus (HBV) is classified into several genotypes. Genotype G (HBV/G) is characterised by worldwide dispersion, low intragenotypic diversity and a peculiar sequence of the precore and core region (stop codon and 36-nucleotide insertion). As a rule, HBV/G is detected in co-infection with another genotype, most frequently HBV/A2. In a previous *in vivo* study, viral replication of HBV/G was significantly enhanced by co-infection with HBV/A2. However, the mechanism by which co-infection with HBV/A2 enhances HBV/G replication is not fully understood. In this study, we employed 1.24-fold HBV/A2 clones that selectively expressed each viral protein and revealed that the core protein expressing construct significantly enhanced the replication of HBV/G in Huh7 cells. The introduction of the HBV/A2 core promoter or core protein or both genomic regions into the HBV/G genome showed that both the core promoter and core protein are required for efficient HBV/G replication. The effect of genotype on the interaction between foreign core protein and HBV/G showed that HBV/A2 was the strongest enhancer of HBV/G replication. Furthermore, Western blot analysis of Dane particles isolated from cultures of Huh7 cells co-transfected by HBV/G and a cytomegalovirus (CMV) promoter–driven HBV/A2 core protein expression construct indicated that HBV/G employed HBV/A2 core protein during particle assembly. In conclusion, HBV/G could take advantage of core proteins from other genotypes during co-infection to replicate efficiently and to effectively package HBV DNA into virions.

Keywords: co-transfection, core protein, genotype A, genotype G, hepatitis B virus, replication.

INTRODUCTION

Hepatitis B virus (HBV) infection affects more than 350 million people and is one of the major causes of acute and chronic liver disease. Acute HBV infection in adults is usually self-limiting, while chronic HBV infection can cause chronic hepatitis, liver cirrhosis or hepatocellular carcinoma [1]. As the clinical course in infected individuals depends on a complex interplay among various factors including viral, host and environmental factors, molecular characteristics of HBV including the genotype could become increasingly important in our understanding of HBV clinical implications [2].

Abbreviations: CMV, cytomegalovirus; CP, core promoter; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; SEAP, secreted alkaline phosphatase.

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Eight major HBV genotypes (A–H) have been identified by a sequence divergence >8% in the entire HBV genome [3,4] and have a relatively distinct geographical distribution, which may be associated with anthropological history [5]. Hepatitis B virus genotype G (HBV/G) was first described in 2000 by studies carried out in France [6]. It is usually detected during co-infection with other genotypes, most frequently with HBV/A2 [7,8]. Co-infection with HBV/C and H has also been reported [9–11]. One of the features distinguishing HBV/G from other genotypes is the 36-nucleotide (nt) insertion in its core gene [6,12]. Recent studies indicated that the 36-nt insertion increased core protein translation without enhancing mRNA abundance [13], and insertion of the 36-nt in the core region of genotypes A and D impaired genome replication, despite upregulation of core protein expression, indicating that the 36-nt insertion could alter core protein expression without altering the mRNA expression [14]. The other feature of the HBV/G genome that is unique is the possession of two stop codons in the precore region that prevents the expression of hepatitis B e antigen (HBeAg) [6,12]. Nevertheless, some HBV/G carriers are

HBeAg positive, which is explained by co-infection with an HBeAg-expressing HBV/A strain [7].

As previously reported, HBV/G monoinfection in uPA/SCID mice that had been transplanted with human hepatocytes (hereafter referred to as chimeric mice) resulted in very low level viral replication, but HBV replication increased markedly when the animals were co-infected with HBV/A2, C or H [11,15]. Furthermore, the co-infection induced more pronounced fibrosis, which concurs with findings from studies of immunosuppressed patients [16]. However, as it is still unclear how the interaction between HBV/G and other genotypes enhances the replication of HBV/G and affects the virological and clinical manifestation within an individual, we conducted *in vitro* studies using 1.24-fold HBV clones to elucidate the mechanism of HBV/G replication during co-infection.

MATERIALS AND METHODS

Plasmid constructs of HBV DNA and sequencing

Hepatitis B virus DNA was extracted from 100 μL of serum using the QIAamp DNA blood kit (Qiagen GmbH, Hilden, Germany). Four primer sets were designed to amplify two fragments (A and B) covering the entire HBV/G genome. PCR with nested primers was performed using TaKaRa LA Taq polymerase (Takara Biochemicals, Kyoto, Japan) for 35 cycles (30 s at 95° C, 30 s at 60° C and 2 min at 72° C). The primer pairs and protocols for plasmid construction are outlined in the Supporting Information. As reported previously [17], these fragments were added to the pUC19 vector, which had been deprived of promoters (Invitrogen Corp., Carlsbad, CA, USA), by digestion with *Hin*dIII and *Eco*RI, resulting in the 1.24-fold HBV genome – required to transcribe the oversized pregenome and precore messenger RNA. Cloned HBV DNA sequences were confirmed with Prism BigDye (Applied Biosystems, Foster City, CA, USA) using the ABI 3100 automated sequencer.

HBV DNA mutagenesis and construct design

HBV/A2 and HBV/G clones containing the 1.24-fold HBV genome were constructed using isolates obtained from a co-infected Caucasian patient from the San Francisco cohort described in our previous study (patient #1) [7]. The study design conformed to the 1975 Declaration of Helsinki and was approved by our institutional ethics committee. Written informed consent was obtained from the patient. The HBV/A2 clones isolated from the patient's blood specimen did not possess any precore or core promoter mutations that are known to affect HBeAg expression. To study the interaction between the different genotype isolates, the following viral protein expression constructs were prepared (outlined in Fig. 1) in HBV/A2 recombinant plasmids: HBV/A2-S, HBV/A2-core, HBV/A2-pol and HBV/A2-X and were each able to selectively translate one of the four viral proteins (the large surface, precore/core, polymerase and X proteins, respectively), whereas translation of the other three was prevented by the introduction of point mutations that produced corresponding stop codons (Fig. 1a). The following stop codons were used: (i) for surface protein: change from TTA to TAG in the 15th codon of the S gene (T198A) [18], (ii) for core protein: change from AAG to TAG in the 96th codon of the core gene (A2186T), (iii) for polymerase: change from CA-ACAA to TAATAA in the 283rd and 284th codons of the pol gene (C2558T/C2592T) and (iv) for X protein: change from CAA to TAA in the 7th codon of the HBx gene (C1395T) [19]. All of the above HBV/A2 recombinant plasmids possessed a TCTG motif after nucleotide position 1876, which abolished genome replication by altering the ε loop (CTGT to TCTG, nt 1877–1880) [20]. The 'HBV/A2-N' clone contained all six mutations and was used as an experimental negative control. All of the mutations in this study (substitutions, insertions and deletions) were created by overlapping PCR extension followed by the exchange of endonuclease enzyme-restricted fragments, as described previously [13,21].

Three cytomegalovirus (CMV) promoter-driven expression clones were constructed containing the whole core genes (not including the precore section) of HBV/G (nt 1901–2488), HBV/A2 (nt 1901–2458) and HBV/C (nt 1901–2452): CMV-HBV/G/core, CMV-HBV/A2/core and CMV-HBV/C/core, respectively (Fig. 1b).

Three replicating recombinant constructs were created by recombination of different genomic sections of HBV/G and HBV/A2 (Fig. 1c). The 'HBV-G/A2-CP' clone was a HBV/G-based construct in which the leading fragment containing the core promoter (CP) region (nt 1413–1806) was replaced with that of HBV/A2. The 'HBV-G/A2-CP +core' clone was also an HBV/G-based construct, in which the leading fragment containing the core promoter (CP), precore and core region (nt 1413–2821) of HBV/G was replaced with those of HBV/A2. The 'HBV-G/A2-core' clone was an HBV/G-based construct in which the fragment of the precore and core region (nt 1806–2821) was replaced with those of HBV/A2.

Cell culture and transfection

After 16 h of culture, Huh7 cells were transfected with 5 μg of DNA construct per 10-cm diameter dish using the Fugene 6 transfection reagent (Promega, Madison, WI, USA) according to the manufacturer's protocol and harvested 3 days later. Transfection efficiency was measured by co-transfection with 0.5 μg of a reporter plasmid expressing secreted alkaline phosphatase (SEAP) and normalised with subsequent SEAP measurement from culture supernatant using a SEAP reporter assay kit (TOYOBO, Osaka, Japan) [17]. Three experiments were conducted for each clone.

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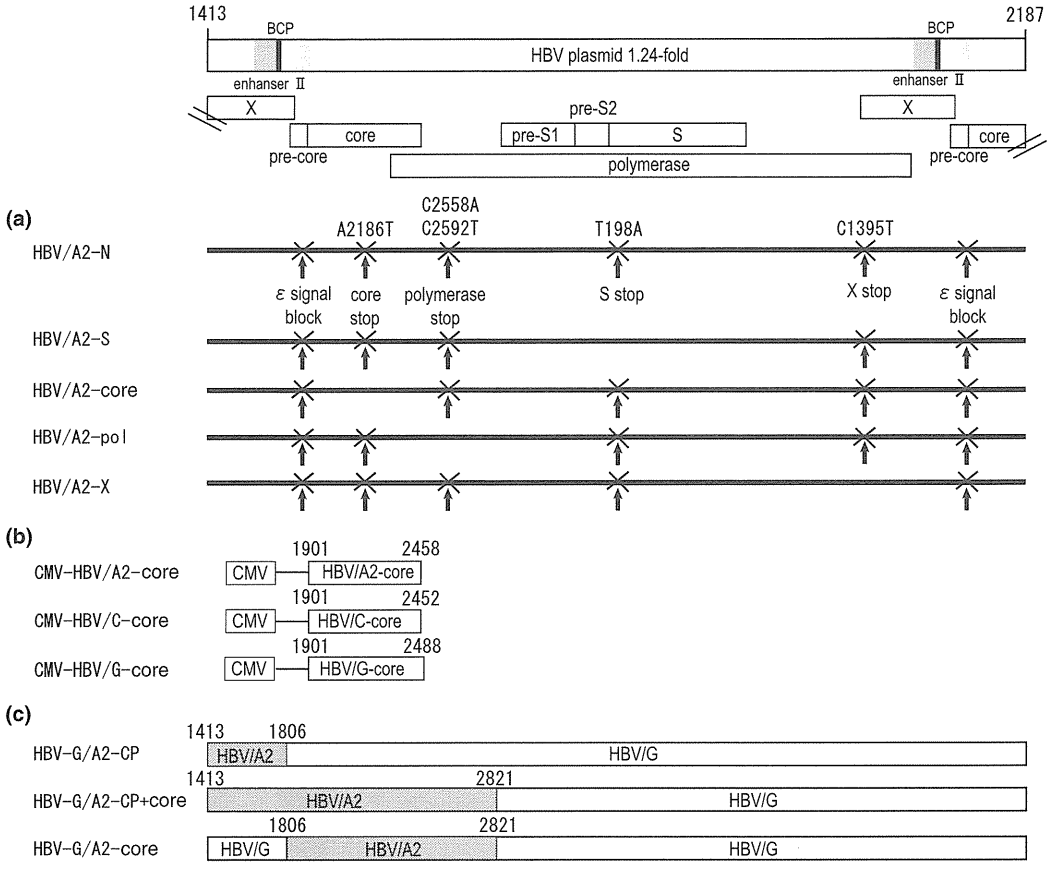

Fig. 1 HBV constructs (1.24-fold) and CMV-driven HBV core protein expression constructs used for the present study. CP, core promoter; BCP, basal core promoter; CMV, cytomegalovirus promoter. Stop codons for the corresponding HBV protein are indicated by crosses and arrows. All HBV/A2 recombinant plasmids consisted of the packaging-negative mutation (ε signal block). In three recombinant constructs between HBV/A2 and HBV/G, the corresponding recombinant genomic parts are shown by the grey bar. CMV-core constructs produce core protein without generating HBeAg in the absence of the preceding ε signal.

Determination of HBV markers

The expression levels of hepatitis B surface antigen (HBsAg) and HBeAg were determined by chemiluminescent enzyme immunoassay using commercial assay kits (Fujirebio Inc., Tokyo, Japan). The detection limit of the HBsAg assay is 0.05 IU/mL. HBV core-related antigen (HBcrAg) was measured in serum using a previously described chemiluminescent enzyme immunoassay [22]. The detection limit of the HBcrAg assay is 1.0 kU/mL.

Southern blot hybridisation

Southern blot hybridisation was performed with full-length probes for each genotype/subgenotype according to previously described methods [23]. In brief, cells were harvested and lysed in 1.5 mL of lysis buffer containing 50 mM Tris–HCl (pH 7.4), 1 mM EDTA and 1% NP-40. Half of the cell lysate was treated with 100 μg/mL of RNase A and 200 μg/mL of DNase I for 2 h at 37° C, in the presence of 6 mM Mg acetate. Then, HBV DNA was released by proteinase K digestion, extracted with phenol and precipitated with ethanol after the addition of 20 μg of glycogen. DNA was separated on a 1.2% agarose gel, transferred to a positively charged nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany) and hybridised with an alkaline phosphatase-labelled full-length HBV/G or HBV/A2 fragment generated with a Gene Images AlkPhos direct labelling module (GE Healthcare, Hertfordshire, UK). The detection was performed with CDP-Star, ready-to use (Roche Diagnostics GmbH). The signals were analysed by using a LAS-3000 image analyzer (Fuji Photo Film, Tokyo, Japan).

Western blot analysis

Serum or culture medium samples were subjected to SDS-PAGE under 15–25% polyacrylamide gel electrophoresis conditions. The proteins in the gel were electroblotted onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Billerica, MA, USA) at 15 V for 45 min. The

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membrane was then blocked and probed using alkaline phosphatase-conjugated HB50 (for HBcAg) or HB91 (for HBcrAg) monoclonal antibody [22] at room temperature for 1 h, before being washed and incubated with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate solution (KPL, Gaithersburg, MD, USA) for 15 min (for HBcrAg) or 90 min (for HBcAg).

Sucrose density gradient ultracentrifugation

Aliquots (1.7 mL) of 10%, 20%, 30%, 40%, 50% or 60% (w/w) sucrose in 10 mM Tris–HCl, 150 mM NaCl and 1 mM EDTA (pH 7.5) were carefully layered in a 12–mL ultracentrifuge tube and left at room temperature for 6 h. The culture supernatant of Huh7 cells that had been co-transfected with the 1.24-fold HBV genome construct (HBV/G or HBV/A) and/or the CMV-HBV/A2-core plasmid was layered onto this sucrose gradient, and ultracentrifugation was performed at 200 000 × *g* for 15 h at 4° C in a Beckman Sw40Ti rotor (Beckman Coulter, Brea, CA, USA). Fractions were collected from the top to the bottom of the gradient. The density of each fraction was calculated from its weight and volume. Each fraction was diluted 10-fold and tested for HBcrAg, HBsAg, HBeAg and HBV DNA.

Immunoprecipitation

Immunoprecipitation was carried out using magnetic beads coated with monoclonal anti-HBs from the 'Magnosphere™ MS300/Caboxyl' kit (JSR Corp., Tokyo, Japan) [24]. A 100-μL aliquot of sample was mixed with 100 μL of a magnetic bead suspension. The mixture was then incubated for 1 h at room temperature under gentle agitation and then magnetically separated. The core protein in the precipitate was analysed by Western blotting.

RESULTS

The replication of HBV/G is enhanced by HBV/A2 in co-transfection experiments

In this study, HBV/G and HBV/A2 genome clones (1.24-fold) were constructed from the serum of a HBV carrier that had been co-infected with HBV/G and HBV/A2. The HBV/G-d36 clone is a HBV/G genome-based construct in which the genotype-specific 36-nt insertion was deleted. We performed co-transfection with HBV/A2 and HBV/G clones and assessed virological features. Because of an over 12% sequence divergence between genotype A and G at the nucleotide level [12], the blot was hybridised successively with genotype-specific probes to DNA of each genotype. However, due to the unbiased binding of each probe at lower efficiency in Southern blot analysis [although the replication of HBV/A2 was higher than that of HBV/G, relative value of HBV/A2 with probe G became lower (0.63), as well as the detection of HBV/G with probe A was very weak (0.24)], each probe of genotype G or A was used for hybridisation with the HBV/A2 and HBV/G clones (Fig. 2a). The density of single-strand HBV DNA detected by the genotype-specific probes in Southern blot analysis revealed that co-transfection with HBV/A2 resulted in increased replication of the wild-type HBV/G

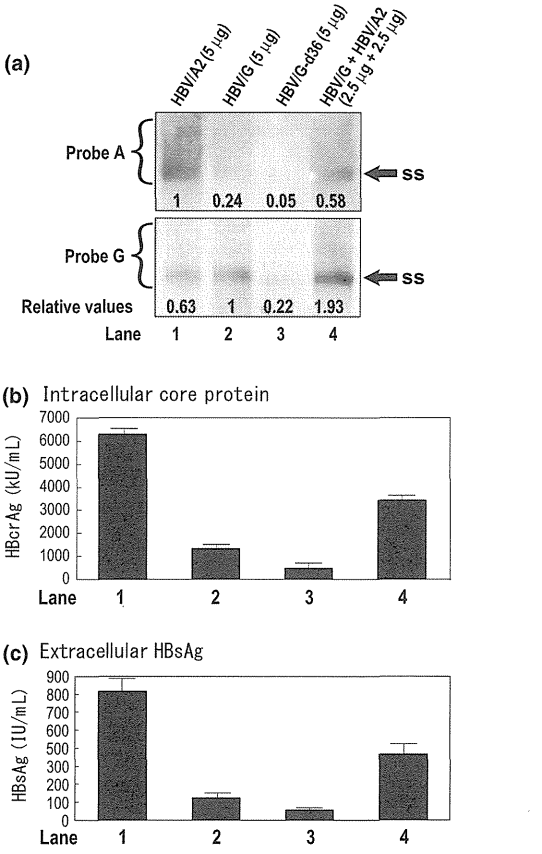

Fig. 2 (a) Southern blot analysis for replicative activity among HBV/G monotransfection, HBV/G-d36 monotransfection, HBV/A2 monotransfection and co-transfection with HBV/A2 and HBV/G (3 days after transfection). HBV/G-d36 clone was a deletion mutant lacking the 36-nt unique insertion in the core gene of the wild-type HBV/G clone. Hybridisation of the blot with genotype-specific probes of genotype A2 (upper) and G (lower). The density values shown at the bottom were measured to the probe-specific DNA sample. Single-stranded (SS) DNA is indicated by arrows. (b) Intracellular expression of core protein was estimated by detecting HBV-core-related antigen (HBcrAg) [22] as measured by a commercial chemiluminescent enzyme immunoassay (mean and standard deviation, $n = 3$). (c) HBsAg levels in the supernatant as detected by a commercial chemiluminescent enzyme immunoassay (mean and standard deviation, $n = 3$). All experiments were tested at least three times.

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clone, compared with monotransfection of the wild-type HBV/G (Fig. 2a). The intracellular expression of core protein in the cell lysates and the expression of HBsAg in the culture supernatant were also enhanced by the co-transfection with both HBV/A2 and HBV/G clones (Fig. 2b and 2c). Removing the 36-nt insertion from the wild-type HBV/G genome resulted in a significant reduction in viral replication and core protein expression compared with the wild-type HBV/G clone. These results are in agreement with the observations of a previous study [13].

The core protein of HBV/A2 is essential for efficient replication of HBV/G

To determine how HBV/A2 rescues HBV/G replication during co-transfection, we constructed four HBV/A2 recombinant plasmids that selectively expressed one of the four viral proteins, whereas translation of the other three was prevented by the introduction of stop codons (Fig. 1a). All of these plasmids were prevented from coding for the viral pregenomic RNA containing the 'packaging-negative' mutation in the ε signal loop to abrogate encapsidation (see Materials and methods). Huh7 cells were co-transfected with the wild-type HBV/G clone and one of the four plasmids expressing a single viral protein. According to Southern blot analysis, the expression of intracellular HBV DNA was greatly increased when HBV/G was co-transfected with HBV/A2-core compared with the other three expression plasmids or the experimental control (pUC19 or HBV/A2-N) (Fig. 3a). The intracellular expression of core protein in the cell lysates was also the highest when HBV/G was co-transfected with HBV/A2-core (Fig. 3b). The expression of HBsAg in the culture supernatant was only increased when HBV/G was co-transfected with the HBV/A2-S plasmid (Fig. 3c). These results indicated that the core protein translated from the HBV/A2 recombinant plasmid can enhance HBV/G replication.

The core protein of HBV/A2 is more effective than those of HBV/C and HBV/G at promoting HBV/G replication

To compare the effects of genotype on the ability of the core protein to increase HBV/G replication in co-transfection experiments, we generated three genotype-specific core protein expression constructs (HBV/G, HBV/A2 and HBV/C) driven by the CMV promoter, which produced core protein in the absence of a preceding ε signal (Fig. 1c). Huh7 cells were co-transfected with HBV/G and one of the three core protein expression vectors. Southern blot analysis showed that the level of intracellular HBV DNA was highest during co-transfection with CMV-HBV/A2/core, followed by CMV-HBV/G/core, and was lowest for CMV-HBV/C/core (Fig. 4a), although the expression of core protein in the cell lysates was the highest during co-transfection with CMV-HBV/C/core, followed by CMV-HBV/G/core, and CMV-HBV/A2/core (Fig. 4b). As anticipated, there was no difference in the expression levels of HBsAg in any co-transfection experiment (Fig. 4c).

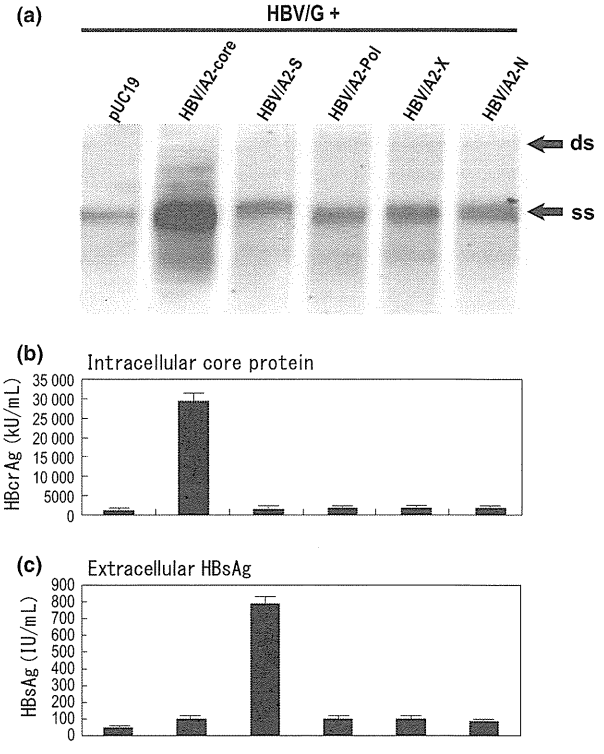

Fig. 3 (a) Southern blot analysis for replication competence of HBV/G clones co-transfected with each of the four HBV/A2 recombinant plasmids: HBV/A2-S, HBV/A2-core, HBV/A2-pol and HBV/A2-X selectively expressing only one of the four viral proteins (large surface, precore/core, polymerase or X protein, respectively). The 'HBV/A2-N' contained all the six mutations to be used as an experimental control. All of the above HBV/A2 recombinant plasmids had the 'packaging-negative' mutation in the ε signal to abrogate encapsidation. (b) Intracellular expression of core protein was measured as described in Fig. 2b. (c) The expression of HBsAg in the culture supernatant was detected as described in Fig. 2c.

A comparison of viral replication among HBV/G and recombinant HBV/G clones

To examine the effects of genetic recombination and the roles of the core promoter, precore and core genomic regions in the interaction of HBV/G and HBV/A2 during co-transfection, we employed three HBV/G and HBV/A2 chimaeric replicating constructs (see Materials and methods), which are shown in Fig. 1c. After the transfection experiment, Southern blot analysis of cell lysates indicated an abundant level of DNA expression in HBV/G/A2-CP/core–transfected cells compared with those in the cells transfected with HBV/G-wild type, HBV/G/A2-CP and HBV/G/A2-core (Fig. 5a). As shown in Fig. 5b, the highest

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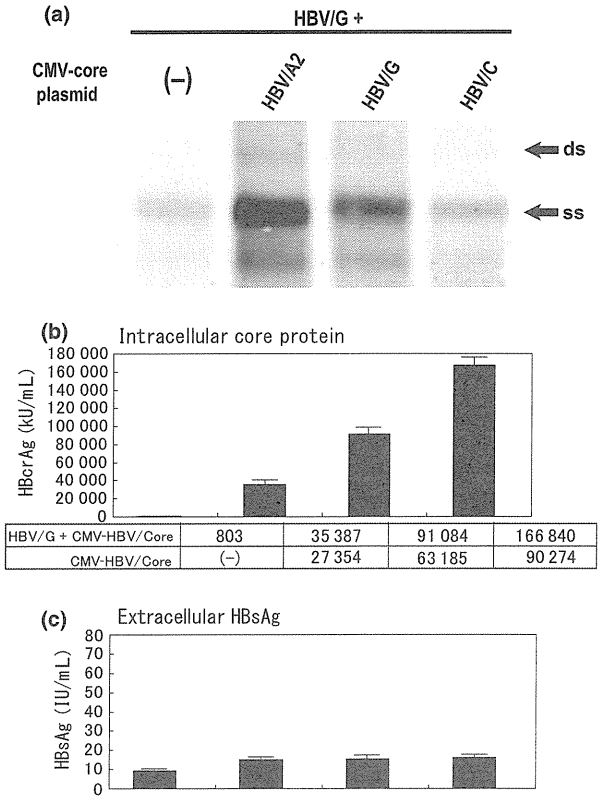

Fig. 4 (a) Southern blot analysis for the expression of intracellular HBV/G DNA during co-transfection with the three core protein expression constructs for each genotype (HBV/G, HBV/A2 and HBV/C) driven by the CMV promoter, which produced core protein in the absence of a preceding ε signal. (b) Intracellular expression of core protein. (c) The expression of HBsAg in the culture supernatant.

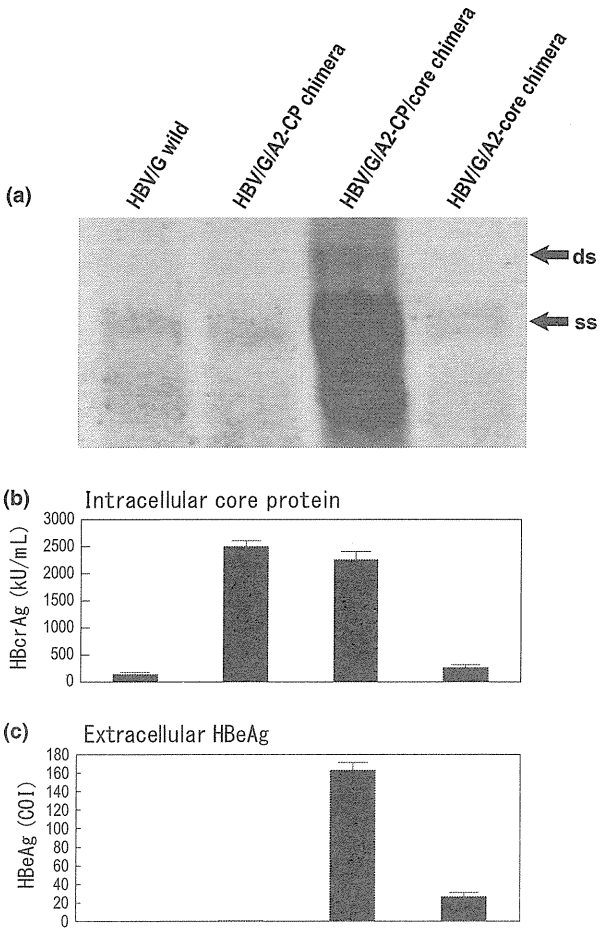

Fig. 5 (a) Southern blot analysis for HBV replication among HBV/G and three chimeric replicating constructs created by recombination of different genomic sections of HBV/G and HBV/A2 (see Materials and methods). The 'HBV/G/A2-CP' clone was a HBV/G-based construct in which the fragment containing the core promoter (CP) region but not the precore or core was replaced by the corresponding sequence from HBV/A2. The 'HBV/G/A2-Core' clone was an HBV/G-based construct in which the section of the precore and core region was replaced with that of HBV/A2. For the 'HBV/G/A2-CP/core' clone, the CP, precore and core region of HBV/G were replaced with that of HBV/A2. (b) Intracellular expression of core protein. (c) Extracellular expression of HBeAg levels detected by a commercial chemiluminescent enzyme immunoassay (mean and standard deviation, $n = 3$).

levels of core protein (HBcrAg) expression were observed for the HBV/G/A2-CP and HBV/G/A2-CP/core-transfected cultures, which was in sharp contrast with the low levels observed in the HBV/G/A2-core and the wild-type HBV/G cultures. The discrepancy between viral replication and core production of the HBV/G/A2-CP clone might indicate insufficient virion assembly. Figure 5c shows the HBeAg levels measured in culture supernatants. The expression of HBeAg was the highest in the HBV/G/A2-CP/core culture distantly followed by that in the HBV/G/A2-core culture. The HBV/G/A2-CP and wild-type HBV/G clones expressed HBeAg protein at levels close to or below the level of detection. Nevertheless, a high HBcrAg titre was detected in the cell lysate of the HBV/G/A2-CP clone, although its DNA level was as low as that of the wild-type HBV/G clone (Fig. 5a). These results indicated that low replication of HBV/G might be explained by low synthesis of HBV/G core protein due to weak core promoter activity or dysfunction, as well as insufficient virion assembly due to the larger core protein of HBV/G (12-aa unique insertion).

Dane particles produced by HBV/G during co-transfection with HBV/A2 were packed in HBV/A2 protein

To investigate the effects of HBV/A2 core protein during HBV/G viral assembly, we tried to define whether the Dane particles in B Huh7 cells that had been co-transfected with wild-type HBV/G and the CMV-HBV/A2-core plasmid

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(source of the Fig. 4 lane 2) contained HBV/A2 or HBV/G core protein. To extract the Dane particles, we employed ultracentrifugation of the culture media through a 10–60% (w/w) sucrose density gradient followed by immunoprecipitation using anti-HBs-coated magnetic beads. We thereby extracted Dane particles, which were then analysed using Western blotting. The obtained fractions were tested for HBcrAg, HBsAg and HBV DNA (Fig. 6a). HBcrAg appeared in the high-density fractions, and its levels peaked in the same fraction (fraction 22) as HBV DNA. As reported previously, the fraction in which the levels of HBV DNA and HBcrAg peaked contained Dane particles [22]. To eliminate contamination of the Dane particles with 'naked' core particles or core protein, they were specifically retrieved from sucrose high-density fraction 22 by means of immunoprecipitation using anti-HBs-coated magnetic beads. The media supernatant obtained from the culture of cells that had been subjected to CMV-HBV/A2/core clone monotransfection was also subjected to sucrose gradient ultracentrifugation using the same protocol. Sucrose high-density fraction 22, in which the HBcrAg concentration peaked, presumably contained 'naked' core particles or core protein (Fig. 6b). This fraction was collected and processed in the same manner via anti-HBs-coated magnetic bead separation and was used as negative control for this procedure (Fig. 6c, lane 4). To discriminate between HBV/G and HBV/A2 core proteins on Western blot analysis probed with anti-HBc antibody, we employed cell lysates produced from cells that had been transfected with the wild-type HBV/G clone and those produced with the HBV/A2 clone as controls. As can be seen on the Western blotting image (Fig. 6c), HBV/G-transfected cells (lane 1) produced larger proteins than the HBV/A2-transfected cells (lane 2), which can be explained by the 12-aa insertion in the core protein of HBV/G coded by its 36-nt unique insertion. Interestingly, the most saturated band associated with the Dane particles produced by HBV/G that had been co-transfected with CMV-HBV/A2/core (lane 3) was the same size as that for HBV/A2, suggesting that HBV/G competitively produces Dane particles consisting of HBV/A2 core protein during virion assembly.

DISCUSSION

HBV/G was first isolated in 2000 in France and the USA and was later found in Thailand, Japan and Mexico, indicating its global dissemination and association with specific risk groups, such as injection drug users (IDU) and men who had sex with men (MSM) [25]. Studies have also demonstrated that throughout the world, HBV/G strains possess unprecedented genetic homology and are mainly detected during co-infection with another genotype that is endemic in the area. Further studies have suggested that genotype G represents a 'replication-defective' variant of HBV that requires co-infection with another genotype to establish a persistent infection. We and others have reported *in vitro* and *in vivo* experimental evidence of this HBV/G dependence [13–15]. The unique 36-nt insertion within core coding region increases core protein level and genome replication in genotype G but impairs replication, not core protein expression, in other genotypes [14]. These results strongly suggest the 36-nt/12-aa insertion has pleiotropic effects on core protein expression, genome replication and virion secretion [14]. To obtain clues about the mechanism by which genotype G works in combination with genotype A to effect its replication, we performed co-transfection experiments using Huh7 cells.

Using HBV/A2 viral proteins expressing plasmids, we determined that a HBV/A2 plasmid that selectively expressed core protein was capable of increasing the replication of the wild-type HBV/G (Fig. 3a). The replication of HBV/G during co-transfection was not affected by other viral elements of HBV/A2 because of the presence of the 'packaging-negative mutation' in the epsilon-coding region and stop codons preventing the translation of the other three viral proteins (the polymerase, surface and X proteins). The specific role of the core protein was further confirmed in experiments with CMV promoter–driven core expressing constructs, in which the core protein alone enhanced HBV/G replication in the absence of HBV pregenomic RNA. Interestingly, co-transfection of HBV/G with the CMV-HBV/A2/core expression construct produced the highest levels of intracellular DNA, even though this combination produced the lowest intracellular core protein level, compared with the CMV-core constructs of the other two genotypes (HBV/G and HBV/C) (Figs 4a,b). The replication of HBV/G was the highest during co-transfection with the CMV-HBV/A2/core expression construct, which agreed with the results of experiments using other genotype (HBV/D, HBV/B1) CMV-core constructs (data not shown). Thus, the core protein of HBV/A2 was confirmed to play an important role in upregulating HBV/G replication and performed this task more efficiently than the other genotypes. These experimental results might explain why HBV/A is the genotype that is most frequently found in co-infections with HBV/G [12,26].

Moreover, HBV/G core protein overexpression achieved by the co-transfection of HBV/G with CMV-HBV/G/core did not enhance replication, suggesting that HBV/G core protein is functionally defective; that is, results in insufficient viral packaging. To investigate the functional defect in the HBV/G core protein, we exchanged the core gene of the wild-type HBV/G for the corresponding gene of HBV/A2 (HBV/G/A2-core); the introduction of the HBV/A2 core promoter together with core coding region into the HBV/G genome (HBV/G/A2-CP/core) significantly enhanced replication. However, the replication of the recombinant construct (HBV/A2 core coding region; HBV/G/A2-core) did not differ from that of the wild-type HBV/G, suggesting that the replacement of HBV/G/A2-core alone was not

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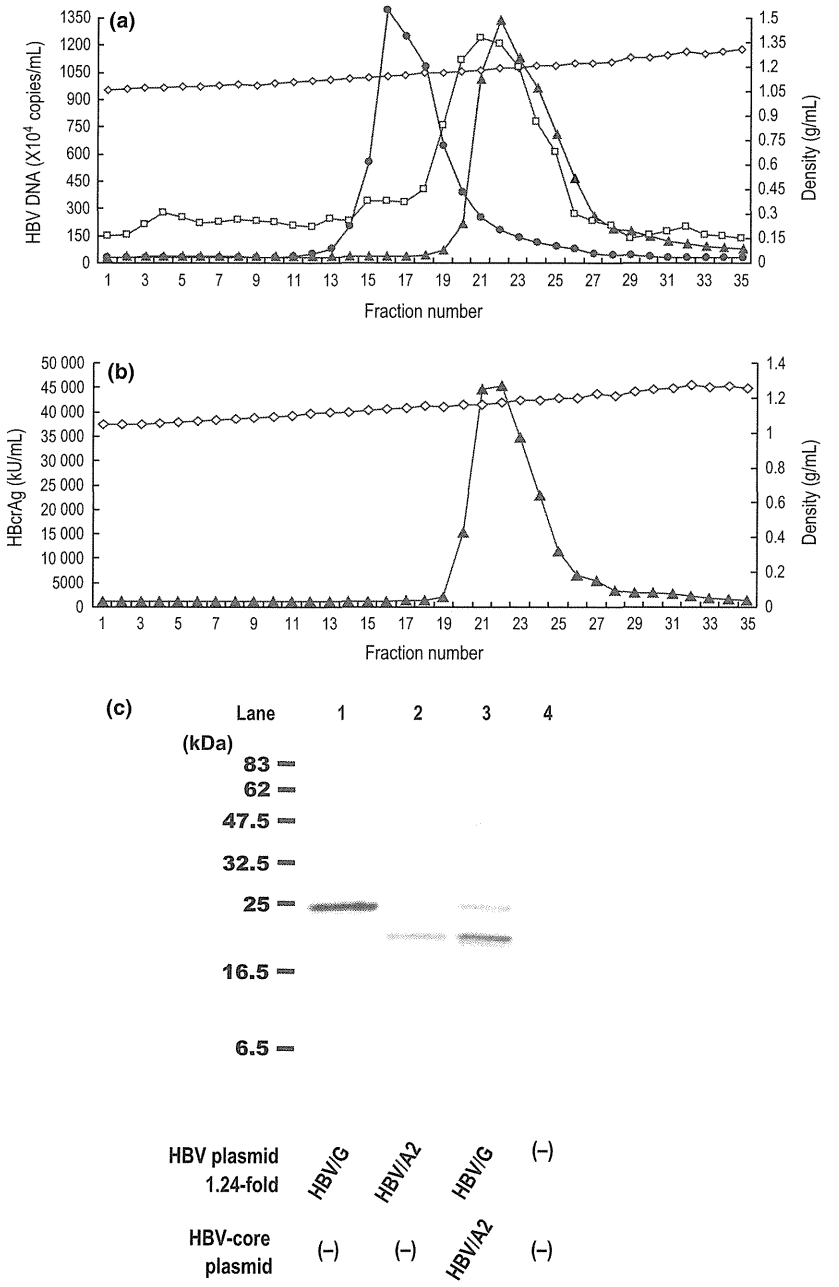

Fig. 6 (a) Sucrose gradient analysis of the culture media of Huh7 cells that had been co-transfected with wild-type HBV/G and the CMV-HBV/A2-core plasmid. It was subjected to ultracentrifugation through a 10–60% (w/w) sucrose density gradient. Density of each fraction is shown as a line with diamond symbols. Fractions were diluted 10-fold and tested for HBsAg (•) (IU/mL), HBcrAg (▲) (KU/mL) and HBV DNA (□) (10^4 copies/mL). (b) Sucrose gradient analysis of culture supernatant obtained from the cells that were subjected to CMV-HBV/A2/core monotransfection using the same protocol. (c) Western blot analysis for HBV core protein was probed by anti-HBc antibody. HBV/G/core and HBV/A2/core were obtained from cell lysates that were transfected with the wild-type HBV/G clone and the wild-type HBV/A2 clone, respectively. The 'HBV/G + CMV-HBV/A2/core' was obtained from sucrose high-density fraction 22 (Fig. 6a) that had been co-transfected with wild-type HBV/G and the CMV-HBV/A2-core plasmid by means of immunoprecipitation using anti-HBs-coated magnetic beads.

enough for viral replication because the core promoter of HBV/G was not capable of generating sufficient amounts of core protein to enhance HBV replication. As well, an HBV/G/A2-CP construct containing the HBV/A core promoter region in the context of the wild-type HBV/G genome did not enhance replication, even though its core protein

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production was significantly increased (Figs 5a,b). Although it was previously reported that the 36-nt insertion of the HBV/G core gene was required for both efficient core protein expression and HBV/G replication [13], in this study, the discrepancy between viral replication and core production of the HBV/G/A2-CP clone might indicate insufficient virion assembly due to the larger core protein of HBV/G (12-aa unique insertion). *Trans*-complementation experiments carried out by Gutelius *et al.* [14] demonstrated an association between enhanced core protein level and reduced replication capacity only when the core and polymerase proteins are expressed from the same RNA template. Thus, it was indicated that HBV/G itself could not replicate sufficiently due to a defect in its core protein and/or the core promoter of HBV/G.

Finally, we investigated whether HBV/G utilises the core protein of HBV/A2 for virion packaging. Dane particles obtained from the culture supernatants of cells that had been co-transfected with HBV/G and CMV-HBV/A2/core were assessed by Western blotting, and it was found that the Dane particles of HBV/G contained HBV/A core proteins. Thus, it was implied that HBV/G replication is enhanced by the core protein of HBV/A because it is more suitable for virion packaging than its own core protein, suggesting that the core protein of HBV/A is a key element enhancing the replication of HBV/G during co-infection. Interestingly, our experiments demonstrated that there were large differences in core protein expression among the CMV-core constructs of different genotypes, despite the fact that all of the CMV-core constructs had the same CMV promoter (Fig. 4b). In a previous report, it was speculated that the core protein binds to its own mRNA to influence protein translation [13]. For example, dihydrofolate reductase protein has been found to downregulate its own translation by binding to cognate mRNA [27,28]. Therefore, we predict that the core protein of HBV/A2 enhances HBV/G replication by affecting viral promoters or transcription in addition to its role in virion packaging.

In conclusion, enhanced replication of HBV/G requires the HBV/A2 core protein during co-infection with HBV/A2. Our findings provide a possible explanation that the core protein of HBV/A2 is more suitable for virion packaging rather than that of HBV/G, and the replication of HBV/G occurs at a very low level, which may be due to defects in its core protein functions and/or core promoter activity. Further experiments are warranted to clarify the detailed roles of the enhanced HBV/G replication by co-infection with the other genotype and the clinical manifestation of HBV/G infection.

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CONFLICT OF INTEREST STATEMENT

None declared.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Data S1. Plasmid construct of HBV/G.

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