Enhanced pregenomic RNA levels and lowered precore mRNA transcription efficiency in a genotype A hepatitis B virus genome with C1766T and T1768A mutations obtained from a fulminant hepatitis patient

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The viral factors associated with the development of fulminant hepatitis B are not fully understood. We recently found four unique mutations [G to A at nucleotide 1742 (G1742A), C1766T, T1768A and T1809C] in the basal core promoter (BCP) region of a genotype A hepatitis B virus (HBV) strain (FH) obtained from a 53-year-old man with fatal fulminant hepatitis. To elucidate the association of the mutations of the FH genome with the disease, we constructed a 1.3-fold FH genome and its five variants by replacing one or two mutated nucleotides with wild-type nucleotide(s) via site-directed mutagenesis, and transfected human hepatoma cells (HepG2/C3A) with the constructs. There were no discernible differences between FH and two variants (FH_A1742G and FH_C1809T) with regard to viral replication and protein expression. However, in comparison to three other variants (FH_T1766C, FH_A1768T and FH_T1766C/A1768T) with wild-type nucleotide(s) at 1766 and/or 1768, the FH genome exhibited a 2.5–5-fold enhancement of viral replication by heightened pregenomic RNA synthesis and a 1.5–2.5-fold reduction in the hepatitis B e antigen (HBeAg) synthesis by the downregulation of the precore mRNA level. An immunofluorescence analysis revealed the increased and predominant cytoplasmic localization of the core protein in the FH genome. The present study demonstrates that the C1766T/T1768A mutations in the BCP region of genotype A HBV enhance viral replication, downregulate HBeAg expression and are responsible for the predominant localization of the core protein in the cytoplasm, which are likely associated with the development of fulminant hepatitis.

INTRODUCTION

Hepatitis B virus (HBV) is a circular, partially dsDNA virus of approximately 3200 nt that belongs to the prototype group of the family Hepadnaviridae (Mason et al., 2012). The HBV genome is classified into eight well-established genotypes (A–H) (Arauz-Ruiz et al., 2002; Norder et al., 1994; Okamoto et al., 1988; Stuyver et al., 2000). With the exception of genotypes E, G and H, the genotypes are divided into several subgenotypes, such as A1–A6 in genotype A, B1–B9 in genotype B and C1–C16 in genotype C (Mulyanto et al., 2012).

Approximately 2 billion people worldwide have been exposed to HBV, which affects an estimated 350 million persistently infected individuals worldwide (Lavanchy, 2004). HBV induces various acute liver diseases ranging from subclinical to fulminant hepatitis, and chronic liver diseases such as chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. Approximately 1 % of patients who are acutely infected with...
HBV develop fulminant hepatitis B, in which the rapid destruction of the liver parenchyma cells proves lethal for many patients (Lee, 1993). Although the pathogenesis of fulminant hepatitis B remains to be fully elucidated, the enhanced replication of the virus (as a viral factor) and the host’s immune response are considered to play key roles (Rivero et al., 2002).

Some mutations in the HBV genome have been shown to be associated with the development of fulminant hepatitis B as viral factors: (i) G1896A, a precore mutation that makes a stop codon (TGG to TAG) to abolish the translation of hepatitis B e antigen (HBeAg) gene (Carman et al., 1989; Okamoto et al., 1990), is reported to be associated with fulminant hepatitis (Kosaka et al., 1991; Liang et al., 1991; Omata et al., 1991). (ii) Mutation(s) in the basal core promoter (BCP) region (nt 1613–1849) markedly enhance viral propagation (Kramvis & Kew, 1999; Quarleri, 2014). The major double core promoter mutations (A to T at nt 1762 and G to A at nt 1764; A1762T/G1764A), which are frequently found in HBeAg-negative chronic hepatitis patients (Okamoto et al., 1994) and fulminant hepatitis patients (Sato et al., 1995), were reported to be related to an increase in the viral replication level as a result of the upregulation of pregenomic RNA (pgRNA) production, based on in vitro experiments (Buckwold et al., 1996; Moriyma et al., 1996), which is accompanied by a reduction in the level of HBeAg. (iii) Mutation(s) that lead to the abnormal accumulation of core protein in the cytoplasm can activate strong and multi-specific T-cell responses (Missale et al., 1993; Sugiyama et al., 2007).

We recently experienced the case of a 53-year-old Japanese man with fatal fulminant hepatitis B (Hoshino et al., 2016) that was caused by a genotype A HBV strain without any mutations of A1762T/G1764A or G1896A, which is reported to be associated with the development of fulminant hepatitis in rare cases (Ozasa et al., 2006; Sugauchi et al., 2006; Tamada et al., 2012). Notably, several unique mutations were found in the HBV genome of the fulminant strain, including those in the BCP region. The present study was performed to elucidate the significance of these mutations in the pathogenesis of fulminant hepatitis using a plasmid-based reverse genetics system for HBV.

RESULTS

Identification of the unique mutations in the fulminant strain of HBV, FH

The full-genome sequence of the HB14-0620 strain (FH) obtained from the index patient was determined and deposited to GenBank/EMBL/DDBJ databases under accession number LC051141. Comparison of the FH strain with the 31 strains of the same subgenotype (A1) deposited in GenBank/EMBL/DDBJ revealed five unique nucleotide mutations (Figs 1a and S1, available in the online Supplementary Material). A mutation (A to G) found at nt 79 in the FH genome (Fig. 1a) changes Asn to Asp at amino acid residue 332 of the P gene product, but not Gly at amino acid residue 30 of the overlapping preS2 protein, a mutation that was found in a chronic hepatitis patient in Maranhão state, Northeast Brazil (AFR43422) (Barros et al., 2014) and a symptom-free blood donor in the USA (AFH87373) (Delwart et al., 2012). Thus, it was considered that this nucleotide mutation did not have a significant effect on the pathogenesis of fulminant hepatitis.

The other four nucleotide mutations, G to A (none of the 31 strains in Fig. 1a), C to T (two), T to A (three) and T to C (none), were found at nt 1742, 1766, 1768 and 1809 in the BCP region of HBV, respectively. With the exception of the FH genome that was found in the present study, no strains had all four of these mutations (Fig. 1a).

Construction of the plasmids containing the 1.3-fold genome of the FH strain and its variants substituted with wild-type nucleotide(s) and the transfection of hepatoma cells with these constructs

To investigate the significance of the mutations of A at nt 1742, T at nt 1766, A at nt 1768 and C at nt 1809 in the FH strain, the pMD20 plasmids containing the 1.3-fold genome of the FH strain or its variants with substitution(s) to wild-type nucleotide(s), FH_A1742G, FH_T1766C, FH_A1768T and FH_C1809T (Fig. 1b), were constructed, as described in detail in Methods (see Fig. 7).

Comparison of the amount and molecular forms of HBV DNA among the constructs

The amount of the HBV virions released in the culture supernatant at 4 and 6 days post-transfection was measured by a real-time PCR (Fig. 2a). Three constructs with FH and its two variants (FH_A1742G and FH_C1809T) had nearly identical levels of HBV DNA in the culture supernatant, while levels of HBV DNA in culture supernatant for the other three constructs (FH_T1766C, FH_A1768T and FH_C1809T) were reduced by 70–98, 48–55 and 68–74 %, respectively, at both day 4 and day 6 post-transfection in comparison to FH. The amount of HBV DNA in the cell lysates of these three variants was 0.3–0.5-fold lower than that of FH (data not shown), which was consistent with the HBV DNA levels that were detected in the culture medium. Taken together, it was considered that the combined mutation of T at nt 1766 and A at nt 1768 enhanced the synthesis of HBV virions to 2.5–5-fold that of wild-type with nucleotides of C at nt 1766 and T at nt 1768. The amount of HBV DNA in the culture supernatant continued to increase until 10 days post-transfection and was maintained at a steady state until 14 days post-transfection (data not shown).

To compare the amount and molecular forms of HBV DNA among the constructs, a Southern blot analysis was performed using DNA extracted from the culture supernatant and intracellular core particle-associated HBV DNA that was prepared at 6 days post-transfection (Fig. 3). The
**Fig. 1.** Unique nucleotides in the FH (HB14-0620) sequence and the construction of a FH (HB14-0620) plasmid and its mutants. (a) The alignment of the nucleotide sequences at nt 71–87 and nt 1735–1816 of the FH strain (top) obtained in the present study and 31 reported HBV strains of the same subgenotype (A1). The nucleotide positions are numbered in accordance with the FH strain. Dots represent nucleotides that are identical to those of the FH strain (top). (b) A schematic diagram showing the genomic organization with four coding regions (P, precore/C, X and preS1/preS2/S) and five distinct species of HBV mRNA transcripts as well as a plasmid (pMD20) with the 1.3-fold FH genome and its variants substituted with wild-type nucleotide(s).
signal intensities, which reflect the amount of HBV released in the culture supernatant and the cell lysate, were quantified by a RT-PCR in each of the corresponding variants. The major form of HBV DNA released in the culture supernatant was matured relaxed circular (RC), and the ratio of RC to all of the forms [RC+L (double-stranded linear DNA)+SS (ssDNA)] was 61±3% in all of the constructs, including FH (Fig. 3a). In contrast, the major form of HBV DNA in the cell lysate was found to be L, which was a replicative intermediate. The ratio of L to all of the forms was 47±2% in all of the constructs (Fig. 3b). These results indicated that the different amounts of HBV DNA caused by the mutations T at nt 1766 and A at nt 1768 directly reflected the quantity of the replicated HBV DNA, which was not related to the differences in the progress of maturation of the genome in any of the constructs.

**Comparison of the hepatitis B surface antigen, HBeAg and HBV core antigen levels produced by the constructs**

At 6 days post-transfection, FH and its two variants (FH_A1742G and FH_C1809T) secreted nearly equal levels of hepatitis B surface antigen (HBsAg). In contrast, the other constructs (FH_T1766C, FH_A1768T and FH_T1766C/A1768T) secreted higher levels of HBsAg (2.3-, 2.2- and 3.2-fold, respectively) in comparison to FH (Fig. 2b). In addition, FH_T1766C, FH_A1768T and FH_T1766C/A1768T secreted higher levels of HBeAg (5.1-, 3.9- and 5.1-fold, respectively) at 6 days post-transfection (Fig. 2c). These results indicate that the mutations of T at nt 1766 and A at nt 1768 found in the FH genome are related to the decreased secretion of HBsAg and HBeAg, while the G1742A and T1809C mutations have no discernible effects on protein expression.

A Western blot analysis for HBV core antigen (HBcAg) and HBeAg was performed using cell lysates and culture supernatants that were collected at 6 days post-transfection. Bands corresponding to HBcAg (20 kDa) and HBeAg (17 kDa) were observed in the cell lysate (Fig. 4a) and in the culture supernatant (Fig. 4b), respectively. The intensity for HBcAg in three variants (FH_T1766C, FH_A1768T and FH_T1766C/A1768T) was reduced in comparison to FH, FH_A1742G and FH_C1809T. In contrast, the 17 kDa band of HBeAg in the culture supernatant was clearly visible in the variants of FH_T1766C, FH_A1768T and FH_T1766C/A1768T.
A1768T (Fig. 4b). This corroborated the results obtained by the ELISA (Fig. 2c). Ito et al. (2009) have reported that genotype A differs from other HBV genotypes in producing multiple size forms of HBeAg. Thus, the weak bands above the major band are most likely alternative cleavage forms of HBeAg (Fig. 4b).

**Distribution of HBcAg and HBeAg in HepG2/C3A cells**

An immunofluorescence assay was performed using confocal microscopy to compare the distribution of HBcAg and HBeAg in cells among the FH and its variants (Fig. 5). The core particles, detectable by mAb 3120 recognizing the surface epitope of the core particles (Takahashi et al., 1983), were predominantly and uniformly located with a strong signal in the cytoplasm of cells transfected with the FH construct. The variants bearing A1742G or C1809T showed a distribution and intensity that were similar to FH. In contrast, the signals presented by the three other variants (FH_T1766C, FH_A1768T and FH_T1766C/A1768T) were predominantly located in the cytoplasm, but in a dotted pattern (Fig. 5a).

HBeAg, which is detectable by mAb 905 (Imai et al., 1982), was located evenly in a mist form with a weak signal in both the cytoplasm and nucleus of the cells transfected with the constructs containing the FH, FH_A1742G or FH_C1809T genome (Fig. 5b). On the other hand, the HBeAg signals were strong in the FH_T1766C, FH_A1768T and FH_T1766C/A1768T constructs, and were predominantly and uniformly located in the cytoplasm. A smaller amount of secreted HBeAg (Fig. 2c) and a weak intracellular HBeAg signal (Fig. 5b) indicated the impaired production of HBeAg in the FH genome with C1766T and T1768A mutations.

**Comparison of the transcription efficiency among the constructs**

The four species of mRNAs with lengths of 3.5, 2.5, 2.1 and 0.7 kb are transcribed from the HBV genome (Ganem & Varmus, 1987). To investigate the effects of the mutations found in the FH genome on the transcription efficiency of these mRNAs, total RNA extracted from the transfected cells was subjected to a Northern blot analysis. With regard to the 2.1 kb mRNA encoding HBsAg, the signal was weaker in the FH and FH_C1809T constructs than in FH_T1766C/A1768T, which indicates that the secretion of HBsAg was decreased in the culture supernatant of the FH- and FH_C1809T-transfected cells (Fig. 2b). However, contrary to our expectations, the amount of 3.5 kb mRNA did not differ among the six constructs (Fig. 6a). The 3.5 kb mRNA is composed of pgRNA and precore mRNA (Ou et al., 1990; Seeger et al., 1986; Will et al., 1987). The pgRNA is the template for genome
replication by reverse transcription as well as encoding the core and polymerase protein. The slightly longer precore mRNA encodes the protein precursor of HBeAg. The relative amount of pgRNA and precore mRNA was therefore determined by two sets of reverse transcription (RT)-PCRs using the same amount of total RNA (Fig. 6b). The results showed that the amount of pgRNA plus precore mRNA was similar among all of the variants, which was consistent with the results obtained by the Northern blot analysis (Fig. 6a); however, a 1.5–2.5-fold enhancement of the precore mRNA was found in the FH_T1766C, FH_A1768T and FH_T1766C/A1768T variants. These results indicated that the combined nucleotide substitutions, C to T at nt 1766 and T to A at nt 1768, reduced the transcription level of precore mRNA and upregulated viral genome replication via the increased transcription of pgRNA — an amount that was equivalent to the amount of precore mRNA reduction. On the other hand, FH_A1742G or FH_C1809T had little or no noticeable effect on the amount of pgRNA and precore mRNA.

**DISCUSSION**

The present study revealed that the double mutations C1766T/T1768A in the BCP region of genotype A HBV enhance viral replication, downregulate HBeAg expression and are responsible for the increased and predominant localization of the core protein in the cytoplasm. These activities were likely to be associated with the development of fulminant hepatitis in a patient infected with genotype A HBV.

It has been reported that HBV genotypes affect the outcome of liver disease (Kao et al., 2000). In Japan, among 301 reported cases of acute hepatitis B, 10 and 33 cases were caused by HBV of subgenotype A1 and A2, respectively (Ozasa et al., 2006). Only one of the A1-infected and none of the A2-infected patients developed fulminant hepatitis. It is hypothesized that the limited number of cases of fulminant hepatitis due to genotype A HBV infections can be attributed to the rare occurrence of the precore mutation (G1896A) corresponding to the higher stability of the epsilon stem–loop structure (Li et al., 1993). In fact, the FH genome in the present study had G at nt 1896. Although double BCP mutations of A1762T/G1764A are known to be related to the enhancement of HBV replication (Buckwold et al., 1996; Moriyama et al., 1996) and are frequently found in patients with fulminant hepatitis B (Sato et al., 1995), the nucleotides at nt 1762 and nt 1764 in the core promoter of the FH strain that were obtained from the index patient in the present study were both wild-type (A and G, respectively). In addition, T1753V (A, C or G), T1754S (C or G), T1961V, C1962D (A, G or T) and A2339G are found in patients with fulminant hepatitis B who are infected with HBV genotype B (mainly subgenotype B1) or, less frequently, genotype C (subgenotype C2) (Imamura et al., 2003; Inoue et al., 2012; Kusakabe et al., 2009). However, no such mutations were identified in the FH genome. In contrast, four unique nucleotide mutations G to A, C to T, T to A and T to C were found at nt 1762, 1766, 1768 and 1809, respectively, in the BCP region of the FH genome (Fig. 1b). Of note, unique double mutations (C1766T/T1768A; alternatively termed C1768T/T1770A due to a 2 nt insertion), accompanied by a precore G1896A mutation, have previously been identified in a strain of genotype D HBV and have been suggested to be associated with
fulminant hepatitis (Baumert et al., 1996); this encouraged us to investigate the association of the four BCP mutations that were found in the FH strain (including C1766T/T1768A), with fulminant hepatitis.

Contrary to our speculation, in the present study using 1.3-fold HBV genomes of the FH strain and its mutants substituted with wild-type nucleotide(s), two unique BCP mutations (G1742A and T1809C) that were identified in the FH genome were found to have no appreciable effect on viral replication or protein expression (Figs 2, 3, 4, 5 and 6). However, the combined mutations of C1766T/T1768A in the FH genome were found, by a RT_PCR and ELISA (Fig. 2), to be associated with the enhancement of viral replication and the reduction of HBeAg expression (Figs 2, 3 and 4). The RT_PCR and ELISA results were confirmed by Southern blotting (Fig. 3) and Western blotting (Fig. 4b), respectively. Supporting the finding of a reduced HBeAg production, an immunofluorescence analysis revealed that HBeAg was located evenly in the mist form with a weak signal in both the cytoplasm and nucleus of the FH-transfected cells, while the higher levels of HBeAg were predominantly and uniformly located in the cytoplasm of the cells transfected with FH_T1766C, FH_A1768T or FH_T1766C/A1768T (Fig. 5b).

In contrast, higher levels of HBcAg were detectable in the lysates of the FH-transfected cells (Fig. 4a). The enhanced expression and the predominant and uniform distribution of core proteins were visible in the FH-transfected cells. These findings were explained by the increased transcription of pgRNA, which encodes core protein and DNA polymerase/reverse transcriptase (Ou et al., 1990; Seeger et al., 1986; Will et al., 1987), and the decreased transcription of precore mRNA, which encodes HBeAg protein (Fig. 6), although Baumert et al. (1996) reported that the C1768T/T1770A (corresponding to our C1766T/T1768A) mutations had no significant effect on the precore RNA transcription as well as the HBeAg synthesis. Of note, there was a slight discrepancy between the increase (approximately twofold) in pgRNA (Fig. 6) and the enhancement (approximately fivefold) of viral replication (Fig. 2). An increase in encapsidation may be a viral factor that enhances viral replication (Baumert et al., 1998; Buckwold et al., 1997; Hasegawa et al., 1994; Scaglioni et al., 1997). Mutations of C1766T/T1768A can substitute Phe
to Tyr at amino acid residue 132 in the product of the overlapping HBx gene, which might be independently associated with an increase in encapsidation (Baumert et al., 1996).

The increase in core protein synthesis and the predominant localization of core particles in the cytoplasm were clearly observed in the FH strain (Figs 4a and 5a). The increase in core protein might be largely a result of a post-transcriptional or translational effect of the T1766/A1768 mutations, and could be associated with enhanced replication (Baumert et al., 1998). Other viral factors that have been suggested to be associated with the development of fulminant hepatitis include the retention of the core particles and the core particle-associated HBV DNA in the cells (Inoue et al., 2009), a frameshift insertion/deletion in the precore region (Inoue et al., 2011) and an amino acid substitution in the core protein (Inoue et al., 2012; Sugiyama et al., 2007). As for the FH strain, it was suggested that the predominant localization of the core particles in the cytoplasm that was found upon the transfection of the FH construct (Fig. 5), which is consistent with previous reports on the A1742G/T1766C/A1768T construct (Kawai et al., 2003; Liu et al., 2009), might strongly activate multi-specific T-cell responses (Missale et al., 1993; Sugiyama et al., 2007).

The decrease in HBsAg production was found in the FH strain (Fig. 2). The decrease in 2.5 kb mRNA levels was very small, but the 2.1 kb mRNA levels of the FH strain were 15–22 % (data not shown) lower than those in FH_T1766C, FH_A1768T and FH_T1766C/A1768T (Fig. 6), which may have resulted in smaller levels of HBsAg production. In contrast, the decrease in HBsAg production was not found in a genotype D strain with C1766T/T1768A mutations (Baumert et al., 1998). This difference might be attributed to the genotype or hepatoma cell line that was used as the host cell.

In the present study, we constructed the FH variant, along with five revertants (FH_A1742G, FH_T1766C, FH_A1768T, FH_T1766C/A1768T and FH_C1809T). However, since there were no discernible effects in the back mutations of A1742G and C1809T (into the consensus of subgenotype A1), we did not construct a recombinant plasmid with all four positions reverted to the wild-type A1 sequence. As for the experimental design, it might have been better to have such a revertant to check for any possible additive or synergistic effects.
**Fig. 7.** Schematic diagram showing the creation of a recombinant plasmid containing the 1.3-fold FH genome. An inverted long-distance PCR with primers HB279 and HB280 (see Table 1 for nucleotide sequences) was followed by restriction digestion using SapI and self-ligation using T4 DNA ligase to make full-length circular FH HBV DNA. Using ligated full-length FH HBV DNA as a template, two sets of PCR amplifications, with primers P5/P6 and P2/P4, were performed to obtain fragments of 1606 and 3036 bp, respectively. Each fragment was then subcloned into a plasmid pMD20 T-vector to create recombinant plasmids pMD_HB14-0620(P5/6) and pMD_HB14-0620(P2/4). Both of the recombinant plasmids were digested with KpnI and BspEI, and a shorter fragment of pMD_HB14-0620(P5/6) (1263 bp) was subcloned into the KpnI and BspEI sites of pMD_HB14-0620(P2/4) to create the 1.3-fold FH genome construct. An asterisk represents a mutated nucleotide that was used to make variants with substitution to a wild-type nucleotide (FH_A1742G, FH_T1766C, FH_A1768T, FH_T1766C/A1768T and FH_C1809T: see Fig. 1).
Other investigators (Parekh et al., 2003; Tsai et al., 2009) have analysed the phenotype of the A1762T/G1764A/C1766T triple core promoter mutation in the background of genotype A2. The findings of greatly increased genome replication, reduced HBeAg production and altered transcription of 3.5 kb precore vs pgRNAs were very similar to those described in the present study for subgenotype A1. These findings support the conclusions in the present study and help explain the underlying mechanism for increased replication (Tsai et al., 2009). Most subgenotype A1 isolates harbour the G1809T/C1812T double mutation relative to the consensus sequence from other HBV genotypes, which may modestly reduce HBeAg production (Ahn et al., 2003). Therefore, although the C1809T back mutation did not have any major biological effects in the present study, the genotype A1 background with G1809C/C1812T mutations may contribute to low HBeAg expression, a contributing factor for fulminant hepatitis.

In conclusion, the present study demonstrated that the novel BCP mutations, C1766T/T1768A, of a subgenotype A1 HBV strain (HB14-0620) recovered from a patient with fulminant hepatitis B were responsible for enhanced pgRNA levels, while also decreasing the precore mRNA transcription efficiency, which resulted in a heightened viral replication, a reduction of HBeAg expression level and the abnormal accumulation of core particles in the cytoplasm. These phenotypic changes caused by the C1766T/T1768A mutations might have been associated with the development of fulminant hepatitis B in the patient infected with genotype A HBV.

### METHODS

**Serum sample.** A serum sample was collected at admission from a 53-year-old Japanese male with fulminant hepatitis B who was infected with genotype A (subgenotype A1) HBV. The sample was stored at −80°C for subsequent testing. Informed consent for inclusion in the present investigation was obtained from this patient. The detailed clinical course of the patient has been reported elsewhere (Hoshino et al., 2016).

**Determination of HBV full-length sequences.** The complete nucleotide sequence of HBV was determined as described previously (Mulyanto et al., 2010). The three overlapping regions of HBV DNA (primer sequences at both ends excluded) spanned nt 2333–3221 and 1–667 (1556 bp), nt 480–1795 (1316 bp) and nt 1699–2380 (682 bp), respectively. The amplified products on both strands were sequenced directly using a BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems).

**Creation of FH and mutant constructs.** A plasmid containing the 1.3-fold HBV genome (nt 1073–3221/1–2667) was constructed according to the method reported by Günther et al. (1995) with the following modifications. Total nucleic acids were extracted from 50 μl of serum from a patient with FH (HB14-0620) using a commercially available kit (SMITEST EX-R&D, Medical & Biological Laboratories) and subjected to a PCR with a high-fidelity polymerase, KOD FX Neo (TOYOBO). The full-length HBV genome was amplified by a PCR using primers HB279 and HB280 (Table 1), which were designed based on the conserved sequences of the HBV genomes and which possess the restriction endonuclease Sapi site. The amplicons digested with Sapi (New England Biolabs) were self-ligated with T4 DNA ligase (TaKaRa Bio), and subjected to two sets of PCRs with primers (P2/P4 and P5/P6); the amplified fragments were 3036 bp (nt 2267–3221/1–2067) and 1606 bp (nt 1073–2666) in length, respectively. After the addition of an adenine to

### Table 1. Primers used in the present study

Capital letters denote HBV- or GAPDH-specific sequences, while the lower case letters indicate sequences containing restriction sites (underlined) as described in Note. Nucleotides that were substituted with wild-type are indicated in bold.

<table>
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<th>Name</th>
<th>Sequence (5’ to 3’)</th>
<th>Note</th>
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<td>HB279</td>
<td>aagcttgagcttcTTTTTACCTCTGACC&lt;br&gt;TAATCATCTCTCTGCTATGGTCCAC&lt;br&gt;TGGTGCAAGCACCATATTATTGCC</td>
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<td>Nol nt 2049–2067 (antisense)</td>
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<td>GTAGCGGTAAGAATCTAGCAGG</td>
<td>nt 2646–2666 (antisense)</td>
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<td>P6</td>
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<td>HB14-0620-Pcp</td>
<td>GTGTGCGGCAACACCATC&lt;br&gt;nt 1796–1813 (sense)</td>
<td>nt 1796–1813 (sense)</td>
</tr>
<tr>
<td>GAPDH-F</td>
<td>CCAATTGGAAGAGGGCTGGG&lt;br&gt;nt 485–502 (sense)/GAPDH_NM_001289746</td>
<td>nt 485–502 (sense)/GAPDH_NM_001289746</td>
</tr>
<tr>
<td>GAPDH-R</td>
<td>CAAGTTGTACATGGATGCC&lt;br&gt;nt 660–679 (antisense)/GAPDH_NM_001289746</td>
<td>nt 660–679 (antisense)/GAPDH_NM_001289746</td>
</tr>
</tbody>
</table>
the 3'-ends with Taq DNA polymerase (TaqKaRa Bio), the two DNA fragments were subcloned into pMD20 T-vector (TaqKaRa Bio) to construct pMD_HB14-0620(P2/4) and pMD_HB14-0620(P5/6), respectively. The fragment excised from pMD_HB14-0620(P5/6) with the restriction endonucleases, KpnI and BspEI (New England Biolabs), was inserted into the KpnI–BspEI sites of pMD_HB14-0620(P2/4) to create a construct with a 1.3-fold FH genome. The method used for the creation of the FH construct is detailed in Fig. 7. Because the FH isolate had four unique mutations of A at nt 1742, T at nt 1766, A at nt 1768 and C at nt 1809 in the BCP region, the mutated nucleotides were converted to wild-type nucleotides by an inverse long-distance PCR according to a previously described method (Sasaki et al., 2001). The two obtained subclones, pMD_HB14-0620(P2/4) and pMD_HB14-0620(P5/6), a high-fidelity DNA polymerase (KOD polymerase Plus, TOYOBO) and the appropriate primers with wild-type nucleotide(s) (Table 1) were used to create mutant constructs with a wild-type nucleotide of G at nt 1742 (FH_A1742T), C at nt 1766 (FH_T1766C), T at nt 1768 (FH_A1768T), C at nt 1766 and T at nt 1768 (FH_T1766C/A1768T) or T at nt 1809 (FH_C1809T) (Fig. 1b). All of the constructs were sequenced to confirm the presence of introduced substitution(s).

Cell culturing and the transfection of FH and mutant DNAs. Human hepatoma cells (HePG2/C3A, ATCC no. CRL-10741; American Tissue Culture Collection) were cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% (v/v) FBS (EQUITECH-BIO) at 37°C in a humidified 5% CO2 atmosphere. Two days after seeding the cells in six-well plates at 5×105 cells well−1, each FH construct and its corresponding mutant constructs (2.5 µg well−1) were transfected separately into the cells using TransIT-LT1 Transfection Reagent (Mirus). After being washed four times with 0.7 ml of culture medium 2 days later, the cells were cultured in 2 ml of culture medium. A half volume of the supernatant was replaced with fresh medium at 4 days post-transfection and the whole culture supernatant and cells were harvested at 6 days post-transfection. The collected culture supernatants and cells were used for the subsequent analyses. All of the transfection experiments were performed in triplicate.

Detection and quantification of intracellular replicative intermediates of HBV. The core particle-associated HBV DNA in cells was isolated as described previously (Abdelhamed et al., 2002; Inoue et al., 2009). Cells collected at 6 days post-transfection were washed with ice-cold PBS and lysed in 300 µl of lysis buffer (50 mM Tris/HCl, 1 mM EDTA, 1% Nonidet P-40) per well. The cell lysate was cleared by centrifugation at 10,000 rpm for 5 min at 4°C. Five units of DNase I (TaqKaRa Bio) were added to 160 µl of the lysate and incubated at 37°C for 1 h to remove core particle-unassociated DNA. Total DNA was extracted with Ex RR&D and 3 µl of the 50-µl DNA solution was subjected to a Southern blot analysis using a probe of full-length HBV DNA at 37°C and cells were used for the subsequent analyses. All of the transfection experiments were performed in triplicate.

mAbs. The mAb 3120 was directed to the determinant β of HBcAg, which is expressed on the surface of HBV core particles (Takahashi et al., 1983). The mAbs 904 and 905 were directed to determinants a and b, respectively, of HBcAg (lami et al., 1982). The mAb C33 was raised against a 21-mer synthetic peptide representing aa 126–146 of HBcAg (Yoshikawa et al., 1993). Three mAbs (824, 5124 and 7906) were raised against the common determinants of HBsAg (Luengrojanakul et al., 1990; Ohnuma et al., 1990; Usuda et al., 1986).

Quantification of extracellular HBV DNA, HBsAg and HBeAg. The virions of HBV in 100 µl of the culture supernatant were immunocaptured with 10 µl of Protein G Mag Sepharose gel (GE Healthcare), to which 0.1 mg of mAb 824 was bound according to the manufacturer’s protocol. Nucleic acids of the captured virions were extracted with SMI-TEST EX-R&D and dissolved in 20 µl of UltraPure distilled water (Invitrogen). The HBV DNA in the 2 µl DNA solution was quantified by an in-house RT-PCR using a LightCycler System (Roche Diagnostics), as described previously (Akahane et al., 2002). Ten microliters of the DNA solution was also subjected to a Southern blot analysis, as described above. The levels of HBsAg and HBeAg in the culture supernatant were quantified by an ELISA, using in-house ELISA reagents for HBsAg and HBeAg, respectively. Briefly, HBsAg was tested using three mAbs against the common determinants of HBsAg with mAbs 7906 and 5124 as immobilized antibodies and mAb 824 as a labelled antibody. HBeAg was determined by sandwiching it between mAb 904 and mAb 905. The OD was measured at 450 nm (OD450).

Northern blot analysis of HBV mRNAs and the semi-quantification of 3.5 kb mRNA by an RT-PCR. Total RNA was extracted from cultured cells at 6 days post-transfection using TRIzol reagent (ambion) and dissolved in 50 µl of UltraPure distilled water. One microgram of total RNA was subjected to a Northern blot analysis using a 6.7 kb HBV RNA probe (AB205152: nt 1401–2069) labelled with a T7-Flash Transcription Kit (AmpliScribe Epicentre) to detect HBV mRNAs. The relative amount of pgRNA and precore mRNA was determined by an RT-PCR according to the method of Laras et al. (2002), with slight modifications. Briefly, to remove contaminating DNA, 2.5 µg of the total RNA preparation was treated with 2 units of TURBO DNase (ambion). Purified RNA (100 ng) was used for cDNA synthesis with an antisense primer HB14-0620_BC1 (Table 1) and Superscript III (Invitrogen) in 10 µl of reaction mixture containing an RNase inhibitor, RNAsin Plus (Promega). Two microlitres of the cDNA product was subjected to two sets of PCRs with sense (HB14-0620_PGP or HB14-0620_PCP) and antisense (HB14-0620_BC1) primers. The reactions were carried out using KOD FX Neo for 35 cycles (98°C, 10 s; 50°C, 15 s; 68°C, 30 s). Two microlitres of the PCR product was analysed by agarose gel electrophoresis. The total RNA without cDNA synthesis was subjected to RT-PCR for pgRNA plus precore mRNA to rule out DNA contamination. An RT-PCR for mRNA of a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Entrez Gene: 2640) for 10 min at 94°C, 15 s; 68°C, 30 s) was performed with two primers of GAPDH-F and GAPDH-R (Table 1). An RT-PCR for precore mRNA, pgRNA plus precore mRNA and GAPDH mRNA produced 179, 149 and 195 bp fragments, respectively.

Western blot analysis. Proteins in the cell lysate and culture supernatant were separated by 12 or 15% SDS-PAGE and blotted onto PVDF membranes (0.45 µm, Millipore). The membrane was incubated with mAb C33 (5 µg ml−1) or mouse anti-β-actin IgG (diluted 1:1000, Sigma-Aldrich) in PBS(−) containing 2.5% skim milk (Becton, Dickinson and Company) and 0.1% Tween 20 at 37°C for 1 h. After washing with PBS(−) containing 0.1% Tween 20, the membrane was incubated with ECL HRP-linked anti-mouse IgG from sheep (diluted 1:20,000, GE Healthcare). Protein bands were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). The signal was analysed with ImageQuant LAS 500 image analyser.

Immunofluorescence assay. An immunofluorescence assay was performed according to the methods of Yamada et al. (2009), with slight modifications. Briefly, cultured cells that were harvested at 3 days post-transfection were seeded onto collagen-coated eight-well chamber slides (AGC SCITECH) at ×104 cells well−1. One day later, the cells were washed with ice-cold PBS(−), fixed in 4% paraformaldehyde in PBS(−) (Wako Pure Chemical Institutes) for 15 min, washed twice with PBS(−), permeabilized with 0.2% Triton X-100 (Wako Pure Chemical Institutes) in PBS(−) for 10 min at room temperature, and finally washed three times with PBS(−). Non-specific binding was blocked with 1% BSA in PBS(−) at 37°C for 30 min and incubated with either diluted mAb 3120 (2 µg ml−1) or mAb 905 (5 µg ml−1) as the primary antibody for 1 h at 37°C and stained with Alex Fluor 488-
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REFERENCES


HBV C1766T/T1768A mutations and enhanced pregenomic RNA


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