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Enhanced intracellular retention of a hepatitis B virus strain associated with fulminant hepatitis

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ABSTRACT

A plasmid carrying 1.3-fold HBV genome was constructed from a HBV strain that caused five consecutive cases of fulminant hepatitis (pBFH2), and HepG2 cells were transfected with pBFH2 or its variants. The pBFH2 construct with A1762T/G1764A, G1862T, and G1896A showed the largest amount of core particle-associated intracellular HBV DNA, but no significant increase of extracellular HBV DNA in comparison with the wild construct, suggesting that these mutations might work together for retention of the replicative intermediates in the cells. The retention might relate to the localization of hepatitis B core antigen (HBcAg) in the nucleus of HepG2, which was observed by confocal fluorescence microscopy. HBcAg immunohistochemical examination of liver tissue samples obtained from the consecutive fulminant hepatitis patients showed stronger staining in the nucleus than acute hepatitis patients. In conclusion, the fulminant HBV strain caused retention of the core particles and the core particle-associated HBV DNA in the cells.

Introduction

Hepatitis B virus (HBV) causes a spectrum of liver diseases such as acute self-limiting or fulminant hepatitis, chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. Fulminant hepatitis, which is lethal during a short period in many cases, occurs in approximately 1% of patients with acute HBV infection (Lee, 1993). The pathogenesis that leads to fulminant hepatitis B is considered to result from enhanced replication of the virus (Baumert et al., 1996; Hasegawa et al., 1994) and the exuberant immune response of the host (Rivero et al., 2002), but it is not fully understood.

HBV contains a small (3.2 kb), circular, partially double-stranded DNA genome. This genome includes four, partly overlapping open reading frames: the precore/core gene encoding for the hepatitis B e antigen (HBeAg) and the core antigen (HBcAg), the polymerase gene encoding for the polymerase protein, the preS/S gene encoding for the hepatitis B surface antigen (HBsAg), and the X gene encoding for the X protein. In the course of replication, four kinds of viral RNAs, 3.5, 2.4, 2.1, and 0.8 kb in size, are transcribed (Ganem and Varmus, 1987). There are two types of 3.5 kb RNAs whose 5′ termini and functions are different: the pregenomic RNA (pgRNA) and the precore mRNA. The pgRNA, which is encapsidated as the template for reverse transcription to generate viral DNA, serves as the mRNA for HBcAg and the polymerase protein (Summers and Mason, 1982). The precore mRNA is translated into the precore/core fusion protein that is post-translationally modified to HBeAg (Roossinck et al., 1986; Standring et al., 1988). The transcription of the pgRNA and the precore mRNA are regulated by the core promoter corresponding to nucleotide (nt) 1613–1849 (Kramvis and Kew, 1999). The double core promoter mutations of A1762T/G1764A, which were frequently observed in HBeAg-negative chronic hepatitis patients (Okamoto et al., 1994) and fulminant hepatitis patients (Sato et al., 1995), were reported to reduce the production of HBeAg and enhance the replication of HBV in an in vitro study (Buckwold et al., 1996; Moriyama et al., 1996). It was also documented that the precore mutation of G1896A, which makes a stop codon and abrogates HBeAg (Carman et al., 1989), was associated with fulminant hepatitis (Liang et al., 1991; Omata et al., 1991), and the enhanced replication of HBV with G1896A in vitro has been described (Ozasa et al., 2006; Scaglioni et al., 1997).

Recently, we reported five consecutive cases of fulminant hepatitis B that were caused by the same strain of HBV (Nagasaki et al., 2008). The full-length sequences of HBV obtained from them

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were 99.8–100% identical to each other. This strain belonged to genotype B2 (Ba), which was reported to cause fulminant hepatitis less frequently than genotypes A, C, D, and other subgenotypes of genotype B (Ozasa et al., 2006). The fulminant strain was found to have several mutations in the core promoter and precore regions. The aim of this study is to investigate the significance of these mutations in the pathogenesis of fulminant hepatitis, using an in vitro culture system.

Results

Mutations in the fulminant strain of HBV

In the fulminant HBV strain FH-2, many mutations throughout the genome were observed. The full-length sequence (accession number: AB302943) (Nagasaki et al., 2008) was compared with a genotype B2 consensus sequence that was deduced from 52 full-length sequences deposited on GenBank/EMBL/DDBJ. A total of 45 nucleotide mutations, including the double core promoter mutations of A1762T/G1764A and the precore stop mutation of G1896A, were found. In the precore region, a distinctive mutation of G1862T located within the bulge of the ε signal as an RNA structure (Fig. 1C) was also found. Other nucleotide mutations were not found in the core promoter and precore regions except for G1632C. Because nt 1632 varies in the known genotype B2 strains, the nucleotide was considered not to have an important role in the pathogenesis of fulminant hepatitis.

As for amino acid (aa), 17 mutations in the polymerase gene, 7 mutations in the preS/S gene, 6 mutations in the precore/core gene, and 6 mutations in the X gene were observed (Table 1). Focusing on the core promoter and precore regions, an amino acid mutation of residue 17 of Val to Phe (V17F) in the precore region was found besides the precore stop mutation and the double core promoter mutations (corresponding to aa 130 and 131 in the X gene). V17F in the precore region corresponds to the G1862T mutation. We suspected that the nonsynonymous mutations of G1862T might have some effects on the development of fulminant hepatitis, besides A1762T/G1764A and G1896A.

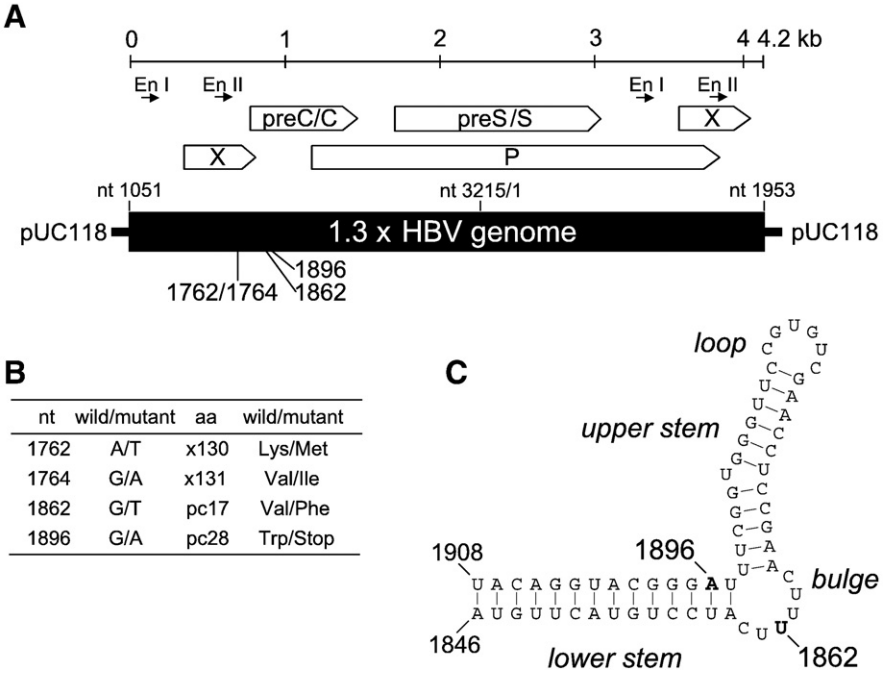
Validation of the replication capacity and the transfection efficiency

A plasmid containing 1.3-fold genome of the FH-2 strain, named pBFH2, was transfected to HepG2 or Huh7 cells, and the replication capacity of the strain was compared in these cell lines. Because the amount of HBsAg and HBV DNA in the culture medium was significantly larger in HepG2 than Huh7 (Fig. 2A), HepG2 was used in the following experiments.

To investigate the significance of the mutations of A1762T/G1764A, G1862T, and G1896A in the FH-2 strain, pBFH2 and its variants were transfected to HepG2 cells and compared. First, the efficiency of transfection was validated using the SEAP reporter system. It was shown that the differences in the activity of SEAP between pBFH2 and its variants coincided with that of expressed HBsAg (Fig. 2B). Therefore, the assay of HBsAg was thought to be suitable for the validation of the transfection efficiency in this system.

Intracellular replicative intermediates of HBV

Southern blotting analysis was performed using HepG2 collected 3 days after transfection. Intracellular core particle-associated HBV DNA was shown as replicative intermediates such as relaxed circular DNA, double-stranded linear DNA, and single-stranded DNA (Fig. 3A). The amount of intracellular HBV DNA was measured by densitometry (Fig. 3B). When compared with the all-wild construct (A1762/G1762, G1862, and G1896), the intracellular HBV DNA was decreased significantly in the construct with the single mutation of G1862T. This finding was in agreement with a previous report (Guarnieri et al., 2006). However, the suppression of HBV replication was overcome by the coexisting mutation of A1762T/G1764A or G1896A. Moreover, only the all-mutant original construct, pBFH2, showed significantly larger amounts of intracellular HBV DNA than the all-wild construct. The double mutations of



nt	wild/mutant	aa	wild/mutant
1762	A/T	x130	Lys/Met
1764	G/A	x131	Val/Ile
1862	G/T	pc17	Val/Phe
1896	G/A	pc28	Trp/Stop

Fig. 1. Construction of a plasmid (pBFH2) and mutations in the strain. (A) Schema of the plasmid containing 1.3-fold HBV genome and the positions of nucleotide mutations found in the core promoter and the precore region. (B) Nucleotide mutations and corresponding amino acid mutations in the core promoter and the precore region in pBFH2. (C) The structure of the ε signal as an RNA secondary structure. It consists of two base-paired segments (lower and upper stems), a bulge, and a loop. nt 1862 is within the bulge and nt 1896 is within the lower stem. En, enhancer; preC/C, precore/core; P, polymerase.

Table 1
Differences of amino acids between the B2 consensus sequence and the FH-2 strain.

Gene	aa position	B2 consensus[a]	FH-2
Polymerase	50	Ile	Thr
	93	Lys	Glu
	104	Asn	Thr
	118	Asn	Lys
	150	Thr	Ile
	212	Pro	Ser
	261	Gly	Asp
	266	His	Asn
	301	Ala	Thr
	464	Asn	Asp
	470	Asn	His
	474	Thr	Asn
	480	Asn	Asp
	566	Leu	Ile
	678	Ser	Arg
	679	Lys	Asn
	809	Thr	Ser (Phe)[b]
preS/S	81	Ala	Thr
	120	Met	Ile
	132	Gln	His
	214	Asn	Ser
	250	Cys	Tyr
	294	Pro	Thr
	358	Val	Ala
Precore/core	17	Val	Phe
	28	Trp	Stop
	106	Glu	Gln
	108	Pro	Gln
	113	Leu	Ala
	210	Ser	Pro
X	37	Leu	Val
	44	Val	Ala
	48	Asp	Ile
	87	Gly	Arg
	130	Lys	Met
	131	Val	Ile

[a] Deduced from 52 full-length sequences of genotype B2 HBV registered on GenBank/EMBL/DDBJ.
[b] Found in the other isolates obtained from consecutive cases of fulminant hepatitis.

A1762T/G1764A, or the triple mutations of A1762T/G1764A and G1896A, had a tendency to increase the replicative intermediates, but not significantly. The single G1896A mutation did not seem to increase them.

Released HBV virions and HBeAg

The amount of the yielded HBV virions in the culture supernatant of pBFH2-transfected HepG2 was assayed by real-time PCR and compared with pBFH2-variant constructs (Fig. 4). The single mutation of G1862T reduced it significantly whereas the double mutation of A1762T/G1764A increased it. Notably, the amount was not increased significantly for pBFH2, in contrast to the result of intracellular HBV DNA. These data suggested that pBFH2 might cause accumulation of the replicative intermediates in the cells due to the mutations in the core promoter and precore region.

The secreted HBeAg was reduced in the pBFH2-variant constructs with A1762T/G1764A and/or G1896A expectedly (Fig. 4), but the effect of A1762T/G1764A was limited in this study. Consistent with a previous report (Guarnieri et al., 2006), G1862T did not seem to affect the expression of HBeAg.

Distribution of HBcAg in HepG2 cells

To investigate the mechanism of the retention of viral replicative intermediates in the cells, the relationship between the distribution of HBcAg in the cells and the mutations of HBV was analyzed using confocal microscopy (Fig. 5). The all-wild construct showed weak HBcAg staining mainly in the nucleus. The construct with only A1762T/G1764A showed the predominant distribution of HBcAg in the cytoplasm, which was concordant with a previous report (Kawai et al., 2003; Liu et al., 2009). The predominant distribution of HBcAg in the nucleus was observed in the construct with the single mutation of G1896A. Although the construct with only G1862T showed HBcAg staining mainly in the cytoplasm, strong staining of HBcAg was observed in the nucleus of the transfected cells of the all-mutant construct, pBFH2. The relationship between the predominance of the HBcAg distribution and the amount of intracellular/extracellular HBV DNA is shown in Table 2. The constructs demonstrating predominant HBcAg distribution in the cytoplasm, such as the construct having only A1762T/G1764A, had a tendency to yield a large amount of extracellular HBV DNA in comparison with intracellular HBV DNA. In contrast, pBFH2, which produced the largest amount of intracellular HBV DNA, showed HBcAg distribution in the nucleus. Whereas the HBcAg distribution in the cytoplasm might lead to the efficient release of the HBV particles, its distribution in the nucleus might be related to the retention of replicative intermediates in the cells. The same results were obtained also using a mouse monoclonal anti-HBcAg antibody (Hyb-3120; Institute of Immunology, Tokyo, Japan), which recognizes a capsid conformation-specific epitope and not HBeAg (Conway et al., 2003) (data not shown). Taken together, the presence of all of the mutations of A1762T/G1764A, G1896A, and G1862T was considered to work together to accumulate HBV DNA in the cells via retention of HBV core particles in the nucleus.

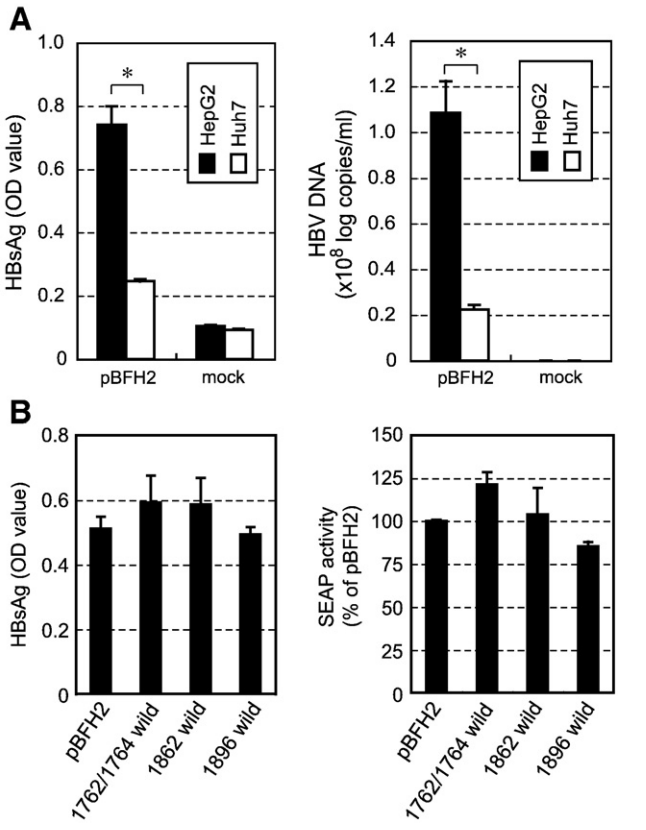


Fig. 2. (A) Comparison of replicative capacity of the FH-2 strain in HepG2 and Huh7. HBsAg and HBV DNA in the culture medium were assayed by ELISA and real-time PCR, respectively. *, $P<0.05$. (B) Validation of transfection efficiency using the SEAP reporter system. The SEAP activity in the culture supernatant was compared with HBsAg, and it was evaluated to determine whether the HBsAg assay was appropriate for the validation of transfection efficiency. Values represent means of triplicate experiments ± standard deviation.

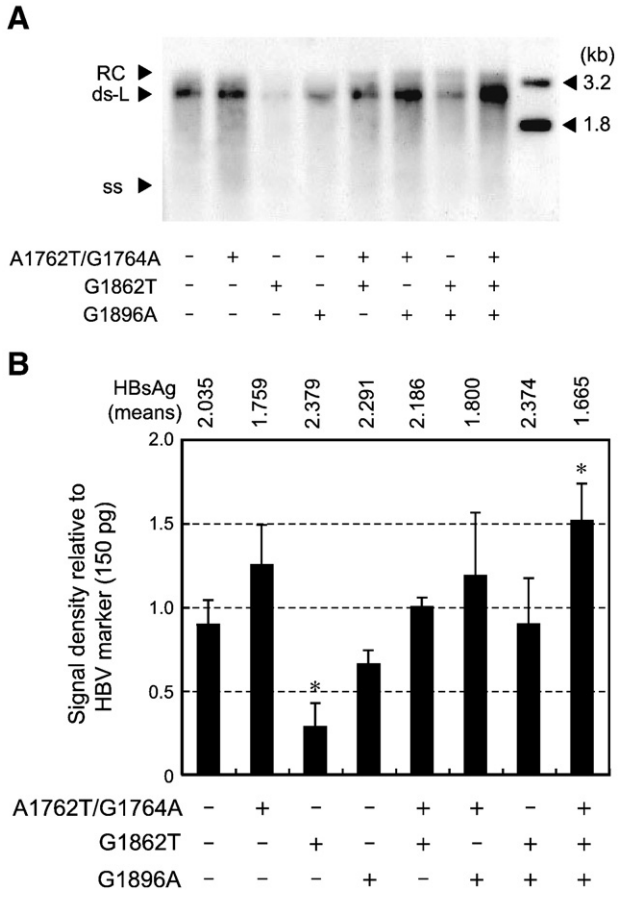


Fig. 3. Detection of replicative intermediates of HBV by Southern blotting analysis. (A) A result of a representative experiment of Southern blotting analysis. + indicates the mutant nucleotides in each nucleotide and − indicates the wild-type nucleotides. RC, relaxed circular HBV DNA; ds-L, double-stranded linear HBV DNA; ss, single-stranded HBV DNA. (B) Amount of replicative intermediates measured by densitometry, in comparison with the density of 150 pg HBV marker. The bars indicate the mean value of triplicate experiments ± standard deviation. Means of HBsAg assayed by ELISA were shown as markers for transfection efficiency. *, $P<0.05$ in comparison with the all-wild construct.

Distribution of HBcAg in tissue samples

To evaluate the results of in vitro study, immunohistochemical examination of liver specimens was performed using the same primary antibody to HBcAg (Fig. 6). In the samples obtained from three of five consecutive fulminant hepatitis B patients, both the nucleus and the cytoplasm of hepatocytes were stained (Figs. 6A–C). In contrast, nuclear staining was rarely observed in acute hepatitis B patients (Figs. 6D–F). In a chronic hepatitis B patient for positive control, nuclear staining was observed (Fig. 6G). Although the cytoplasmic HBcAg was observed more strongly in the tissue samples of fulminant hepatitis than the pBFH2-transfected cells in vitro, it was shown that HBcAg was retained in the nucleus of hepatocytes of these fulminant hepatitis patients. It was considered that the findings from the tissue samples supported the results from pBFH2-transfected cells, in which the core particle-associated replicative intermediates of HBV were retained.

Discussion

A large body of evidence has demonstrated that HBV-related liver disease is immune-mediated (Chisari, 1997; Kondo et al., 1997; Perrillo, 2001). Whereas strong and multispecific T cell responses are observed during acute HBV infection, patients with chronic hepatitis B tend to have weak and narrowly focused immune responses (Chisari, 1997). However, immunocompromised hosts such as patients undergoing hemodialysis can develop fulminant hepatitis B (Igaki et al., 2003), indicating that both viral factors as well as the host immune response are involved in the pathogenesis of fulminant hepatitis. In our study, all of the five consecutive patients with fulminant hepatitis B were over 60 years old and might have had relatively weaker immune responses to HBV. Therefore, the HBV strain obtained from them is considered to be appropriate for the study of the viral factors that lead to fulminant hepatitis.

The fulminant strain of genotype B2 in this study had several mutations including A1762T/G1764A and G1896A, and additionally, G1862T was found in the precore region. It was reported that G1862T was found frequently in the HBV of fulminant hepatitis patients without A1762T/G1764A and G1896A in China, where genotype B2 HBV is prevalent (Hou et al., 2002). In that report, G1862T caused a reduction of HBeAg, which might be associated with the development of fulminant hepatitis. However, another report using a genotype D strain described that the mutation did not change the expression of HBeAg but reduced the expression of the core protein and impaired HBV replication (Guarnieri et al., 2006). These reports are controversial and further analysis of this mutation is needed.

This study demonstrated that the precore G1862T mutation caused the accumulation of the core particle-associated HBV DNA in the cells, in conjunction with the core promoter A1762T/G1764A and precore G1896A mutations. nt 1862 could have two possible functions in the replication of HBV based on the position in the precore

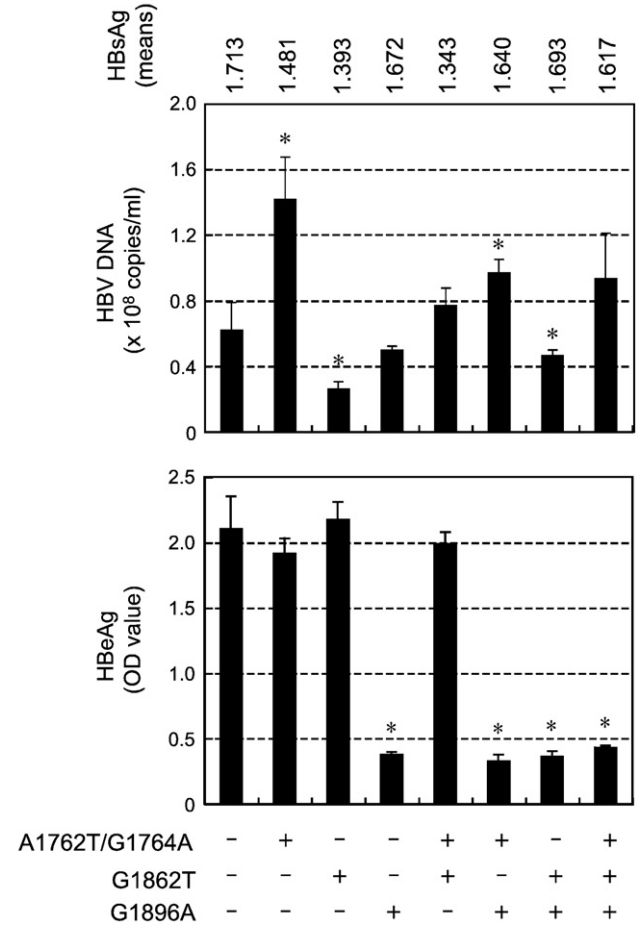


Fig. 4. Released HBV particle and HBeAg in the culture supernatant. The amount of HBV particle was measured by real-time PCR and HBeAg was assayed by ELISA. In the top, the means of HBsAg in the culture supernatant are shown. Values represent means of triplicate experiments ± standard deviation. *, $P<0.05$ in comparison with the all-wild construct.

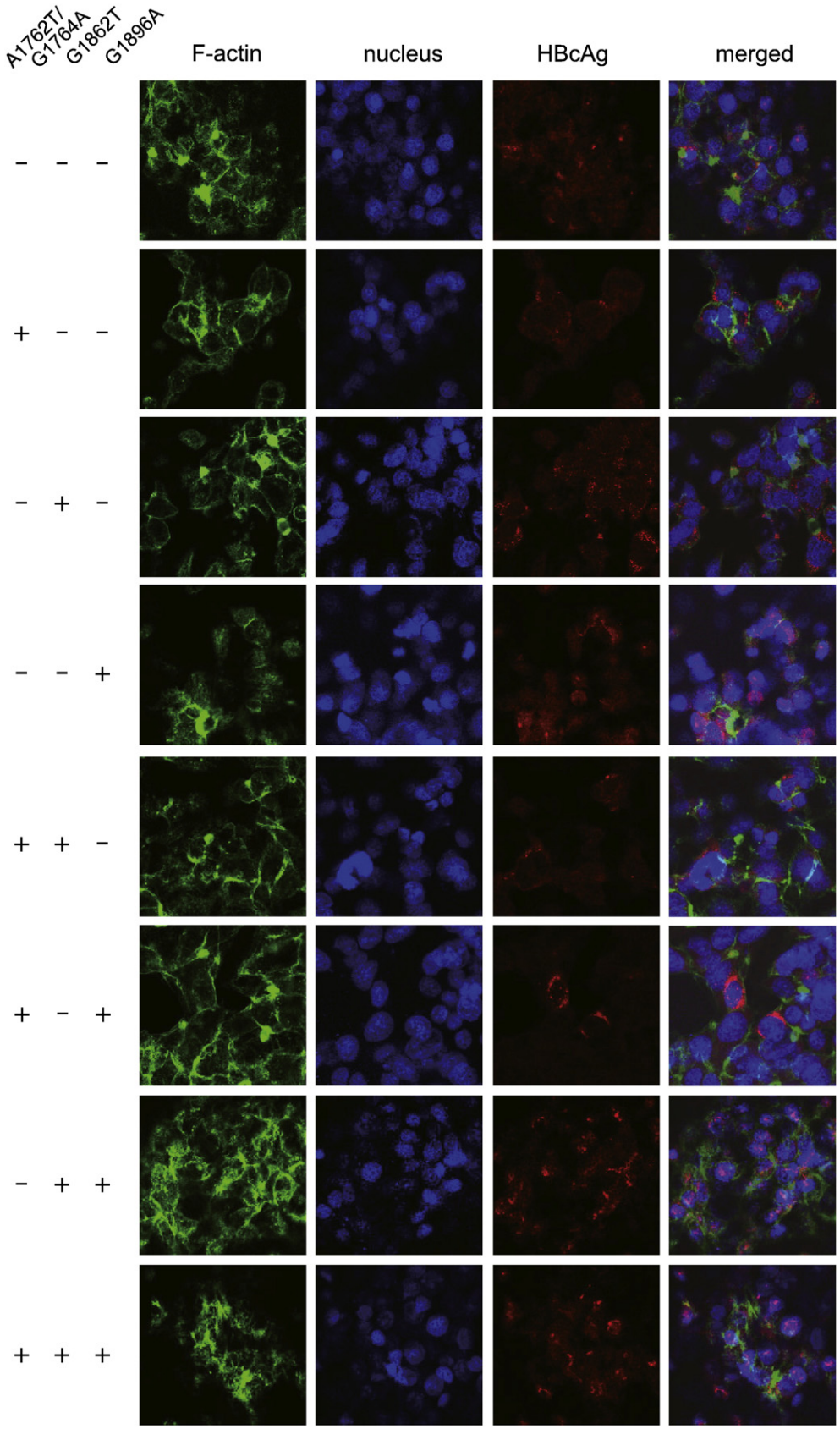


Fig. 5. Comparison of the distribution of HBcAg in the transfected HepG2 cells observed by confocal microscopy according to the HBV mutations. HBcAg was stained using a rabbit polyclonal anti-HBcAg antibody (Dako), and F-actin and nucleus were stained simultaneously.

Table 2
The summarized results of the intracellular/extracellular HBV DNA and distribution of HBcAg in the nucleus according to HBV mutations.

HBV mutations			Intracellular	Extracellular	HBcAg distribution[b]
A1762T/G1764A	G1862T	G1896A	HBV DNA[a]	HBV DNA[a]	
−	−	−	+	+	N>C
+	−	−	+	++	C>N
−	+	−	±	±	C>N
−	−	+	+	+	N>C
+	+	−	+	+	C>N
+	−	+	+	++	C>N
−	+	+	+	±	N>C
+	+	+	++	+	N>C

[a] ++ indicates that the amount of HBV DNA was significantly larger than the all-wild (A1762/G1764, G1862, and G1896) construct, and ± indicates that the amount was significantly lower than the all-wild construct. + indicates no significant change relative to the all-wild construct.
[b] N>C indicates that the cells having HBcAg in the nucleus were observed more predominantly than those having HBcAg in the cytoplasm, and C>N indicates the opposite frequency.

sequence: (i) its mutation converts aa 17 of valine to phenylalanine in the precore peptide and can affect processing of the precore/core protein into HBeAg (Chen et al., 2008; Hou et al., 2002) and (ii) it is within the bulge of the ε signal, which works as the template for the primers to synthesize the negative-strand DNA (Nassal and Rieger, 1996), and is recognized by HBV polymerase in the encapsidation of the pgRNA into the core particle (Rieger and Nassal, 1995). As for the former, aa 17 in the precore peptide is located at the −3 position of the signal peptidase cleavage site, and phenylalanine in this position is considered a "forbidden" amino acid, which may abrogate the cleavage of the precore/core protein by the signal peptidase. Although the effect of G1862T on the production of HBeAg is controversial (Chen et al., 2008; Guarnieri et al., 2006; Hou et al., 2002), it could be supposed that the single mutation of G1862T has a suppressive effect on HBV replication, at least in in vitro studies using genotype D strains (Chen et al., 2008; Guarnieri et al., 2006). A protein of 22 kDa (p22) including precore peptide, which is a product from the precore/core protein other than HBeAg, was reported to inhibit encapsidation (Kimura et al., 2005; Scaglioni et al., 1997). Hence, it was suspected that the possibly increased p22, which might not be cleaved due to G1862T, might lower the capacity of HBV encapsidation and replication. The finding in this study that the replication capacity was overcome by the precore stop mutation G1896A, which abrogates the precore/core protein, supports this speculation. In view of the function of the bulge of the ε signal, G1862T could make the extended templates for the initiation of the negative-strand DNA replication. Whereas the primer of TGAA is made using the 3′-part of the bulge as the template in wild-type HBV, the extended primer of TGAAA or TGAAAA which can anneal to the direct repeat 1 (DR1) and the continued 5′ nucleotides (5′-CT*TTTTCACCTCTGC*CT-3′, italic-typed nucleotides are the DR1 sequence and underlined nucleotides are complement to the extended primer) can be made in the presence of G1862T. Although it is unknown whether the extended primer can actually initiate the extension of the negative-strand DNA, the primer may have some positive effects on reverse transcription. Some of the possible effects of G1862T on the viral replication can be affected by other mutations and the phenotype may be changed.

The core promoter mutation of A1762T/G1764A, which is known to enhance the transcription of pgRNA and reduce the precore mRNA (Moriyama et al., 1996), was described as being associated with the cytoplasmic distribution of HBcAg (Kawai et al., 2003; Liu et al., 2009). In this study, the localization of HBcAg was changed to the nucleus when both G1862T and G1896A were present. Whereas HBcAg is a 21 kD protein (p21) and can be transported across the nuclear membrane by diffusion, the core particle, which consists of approximately 180 subunits of a core protein, cannot diffuse across it (Forbes, 1992; Kawai et al., 2003). It has been speculated that high density HBcAg resulting from the increased pgRNA in the presence of A1762T/G1764A might favor the formation of the core particle in the cytoplasm, and the release of the particle as an infectious virus particle after envelopment. A part of the incompletely processed precore/core protein, p22, can also assemble into the core particle, but the particle cannot encapsidate the pgRNA (Scaglioni et al., 1997). The empty core particle could be detected also in this study using the polyclonal anti-HBcAg antibody. As G1862T reduces HBcAg expression (Guarnieri et al., 2006) and G1896A abrogates the precore/core protein, the presence of these mutations may lower the density of p21 and p22 leading to inefficient assembly of the core particle in the cytoplasm. After that, unassembled p21 is transported across the nuclear membrane by the function of the putative nucleolar localization signal of HBcAg (Ning and Shih, 2004) and be assembled in the nucleus, followed by formation of the core particle. As the particles cannot be transported across the nuclear membrane, they may accumulate in the nucleus.

The core promoter mutations of A1762T/G1764A change overlapping X protein, and there is a possibility that the change of X protein may have effect on HBV replication or localization. A recent

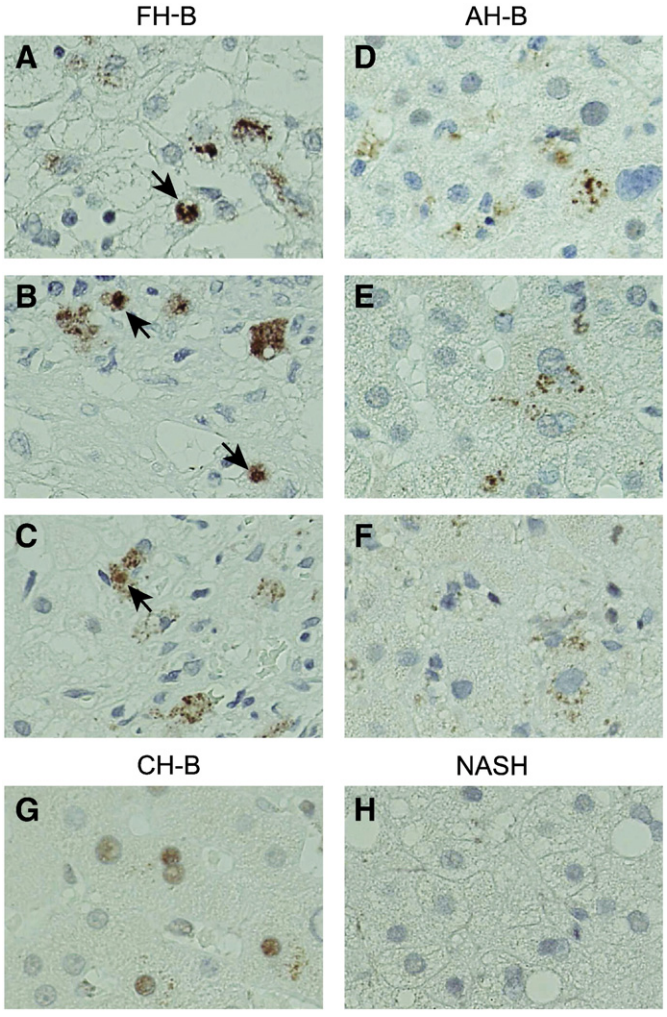


Fig. 6. HBcAg immunohistochemical examination of the liver tissue samples obtained from fulminant hepatitis B (FH-B) patients and acute hepatitis B (AH-B) patients using a rabbit polyclonal anti-HBcAg antibody (Dako). (A–C) Tissue samples of three of five consecutive fulminant hepatitis B patients from whom the FH-2 strain was isolated. The arrows indicate the hepatocytes with nuclear HBcAg staining. (D–F) Tissue samples obtained from three acute hepatitis B patients. (G) A sample obtained from a chronic hepatitis B (CH-B) patient as positive control. (H) A sample of a nonalcoholic steatohepatitis (NASH) patient as negative control.

report showed that the core promoter mutations of A1762T/G1764A do not affect expression of the X gene or impair its stimulatory effect on viral genome replication (Hussain et al., 2009). The effect of the mutant X protein on the viral localization remains unclear and has to be elucidated in the future study.

We also performed HBcAg immunohistochemical examination of the liver tissue samples, and it was confirmed that HBcAg was retained in the hepatocytes of fulminant hepatitis patients, from whom the fulminant HBV strain was isolated. In these samples, HBcAg was observed in the cytoplasm besides the nucleus. The different distribution pattern of HBcAg between in vitro and in vivo might be due to many differences of conditions such as the characteristics of cells and the absence/presence of immune system. Generally, it is thought that HBV-infected hepatocytes are targeted by immune system including T cells (Chisari, 1997), and that the immune response is strongly induced in fulminant hepatitis patients. The retained HBcAg in the cells could induce such immune response. Alternatively, it is speculated that the retained viral proteins might have direct cytopathic effects. Ning and Shih (2004) reported that cells showing the nucleolar localization of HBcAg were often apoptotic, suggesting that the presence of HBcAg in the nucleus may perturb cytokinesis. It was also suggested that the large surface protein or X protein of HBV induced apoptosis (Chirillo et al., 1997; Foo et al., 2002). A strain of HBV that was associated with a fatal outbreak of fulminant hepatitis showed enhanced replication and induced apoptosis in primary Tupaia hepatocytes (Baumert et al., 2005). Interestingly, Sugiyama et al. (2006) showed that the endoplasmic reticulum stress, which was evaluated by the Grp78 promoter activity in genotype A to D HBVs obtained from HBeAg positive patients, was the highest in genotype B2. If the endoplasmic reticulum stress is enhanced further by the retained intracellular viral proteins including HBcAg, apoptosis or inflammation might be promoted, resulting in fulminant hepatitis. Further studies are needed to clarify whether such retention is caused by other fulminant HBV strains.

In conclusion, the fulminant HBV strain that was isolated from consecutive fulminant hepatitis patients retained the core particles and the core particle-associated HBV DNA in the cells. The mutations of A1762T/G1764A, G1862T, and G1896A might work together for the retention. These findings may have important implications for understanding the mechanism leading to fulminant hepatitis.

Materials and methods

Construction of plasmids

Using serum of one of the consecutive patients with fulminant hepatitis B (FH-2) in Japan (Nagasaki et al., 2008), total DNA was extracted with QIAamp DNA Blood Mini Kit (QIAGEN GmbH, Hilden, Germany) and subjected to nested polymerase chain reaction (PCR) for two overlapping fragments; the amplified fragments were nt 1051–3215/1–327 (2492 nt; fragment A) and nt 180–1953 (1774 nt; fragment B). PCR was performed with high fidelity polymerase, PrimeSTAR HS DNA polymerase (TaKaRa Bio, Inc., Shiga, Japan). The amplification products were cloned into pUC118 vectors, and digested with XbaI. The fragments A and B were ligated, and finally, a plasmid containing 1.3-fold HBV genome (nt 1051–3215/1–1953) was constructed and named pBFH2 (Fig. 1A).

QuikChange II-E Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to introduce nucleotide substitutions into pBFH2. Each mutation found in the core promoter and precore regions, A1762T/G1764A, G1862T, and G1896A (Fig. 1B), was converted into wild-type nucleotides, and to construct plasmids with combined nucleotide substitutions, these converted plasmids were used next as templates. As a result, seven variant constructs were generated from pBFH2, and all constructs were sequenced to confirm the nucleotide substitutions. There were two copies of the core promoter and precore regions in the plasmids, and the mutations in both copies were converted by the site-directed mutagenesis.

Cell culture and transfection

Human hepatoma HepG2 or Huh7 cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% bovine serum at 37 °C and 5% CO_2. For the assay of HBV replication, six-well plates were seeded with 5×10^5 HepG2 or Huh7 cells each. On the next day, 1.5 μg of plasmid DNA was transfected to these cells using TransIT LT-1 Transfection Reagent (Mirus, Madison, WI), and the culture supernatant and cells were collected 3 days later. The transfection efficiency was evaluated by Great EscAPe SEAP Reporter System 3 (Clontech, Mountain View, CA), in which 10 ng/ml of a reporter plasmid expressing secreted alkaline phosphatase (SEAP) was cotransfected. Experiments were performed at least in triplicate.

Detection of intracellular replicative intermediates of HBV

The core particle-associated HBV DNA in the cells was isolated as described previously (Abdelhamed et al., 2002) with slight modifications. Three days after transfection the cells were washed with phosphate-buffered saline (PBS) and lysed in 400 μl of lysis buffer (50 mM Tris–HCl, 1 mM EDTA, 1% Nonidet P-40) per well. The lysed cells were centrifuged at 14,000 rpm for 5 min and the supernatant was collected. To remove unprotected DNA, 10 units of DNase I was added to 160 μl of the supernatant, followed by incubation at 37 °C for 1 h. The reaction was stopped by EDTA, and total DNA was extracted with a QIAamp DNA Blood Mini Kit. After ethanol-precipitation, it was analyzed by Southern blot analysis using a full-length HBV DNA probe labeled with PCR DIG Probe Synthesis Kit (Roche Diagnostics). The signal of HBV DNA was analyzed with the LAS-1000 image analyzer (Fuji Photo Film, Tokyo, Japan) and quantified by densitometry with ImageJ 1.39u (The National Institutes of Health, Bethesda, MD).

Quantification of extracellular HBV DNA, HBsAg, and HBeAg

To digest the input plasmid DNA in the culture supernatant, 5 μl of the supernatant was treated with 5 units of DNase I (TaKaRa Bio, Inc.) at 37 °C for 1 h, and the reaction was stopped with EDTA. Then, total DNA was extracted with a QIAamp DNA Blood Mini Kit, and 10 μl of 200 μl DNA solution was subjected to real-time PCR using a LightCycler system (Roche Diagnostics, Mannheim, Germany) as described previously (Jardi et al., 2001). HBsAg and HBeAg in 50 μl of the culture supernatant were assayed by enzyme-linked immunosorbent assay (ELISA), using an HBsAg ELISA kit (Hope Laboratories, Belmont, CA) and ELISA kit for HBeAg (BioChain Institute, Inc., Hayward CA), respectively.

Confocal fluorescence microscopy

At 48 h post-transfection, the culture slides were washed in PBS and the cells were fixed in ethanol for 10 min at room temperature (RT). After fixation, the cells were washed and incubated in blocking solution, 10% (v/v) goat serum prepared in PBS, for 30 min at RT. The cells were incubated with a diluted (1:500) rabbit polyclonal anti-HBcAg antibody (Dako, Glostrup, Denmark) as the primary antibody for 1 h at RT, washed in PBS, and incubated with Alexa Fluor 546 goat anti-rabbit IgG (Molecular Probes, Eugene, OR) as the second antibody for 1 h at RT. At the same time, the F-actin and nucleus were stained with Alexa Fluor 488 phalloidin (Molecular Probes) and TO-PRO-3 iodide (Molecular Probes), respectively. Images were captured by confocal microscopy (Nikon, Tokyo, Japan) with EZ-C1 software.

Immunohistochemistry

Tissue samples were obtained from three of five consecutive cases of fulminant hepatitis B previously reported by us (Nagasaki et al., 2008). As controls, samples from three acute hepatitis B patients and a chronic hepatitis B patient were evaluated. For negative control, a sample from a nonalcoholic steatohepatitis patient was also used. Each tissue was preserved for routine pathological evaluation using paraffin-embedded samples. For HBcAg immunohistochemical examination, after treatment with antigen retrieval solution (Dako) and quenching endogenous peroxidase activity by methanol–peroxide solution, paraffin-embedded liver sections (2 μm) were incubated with a diluted (1:700) rabbit polyclonal anti-HBcAg antibody (Dako) at 4 °C overnight. After rinsing with PBS, Histofine Simple Stain MAX PO (M) (Nichirei, Tokyo, Japan) was added for 1 h at RT. Nuclear counterstaining was performed using hematoxylin for light microscopy after detecting reactions with VECTOR NovaRED (Vector Laboratories, Inc., Burlingame, CA). These liver specimens were observed with a digitalized light microscope BZ-8000 (Keyence, Osaka, Japan).

Statistical analysis

Statistical analyses were performed using Mann–Whitney U test for comparison of continuous variables between two groups. Differences were considered to be statistically significant when $P<0.05$.

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