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SUPPORTING INFORMATION

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

Table S1 Clonal analysis of HBV RT region of samples from the patient with lamivudine and adefovir resistance.

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MAJOR ARTICLE

Enhanced Replication of Hepatitis B Virus With Frameshift in the Precore Region Found in Fulminant Hepatitis Patients

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Background. The genotype B of hepatitis B virus (HBV) was reported to associate with fulminant hepatitis (FH). We aimed to clarify the characteristics of HBV obtained from FH patients in an area of Japan where genotype B HBV is prevalent.

Methods. Using serum samples of 16 HBV-associated FH patients, partial HBV sequences were determined. The effects of HBV mutation/insertion/deletion were evaluated using an in vitro HBV replication system.

Results. Of the 16 HBV isolates, 31% belonged to subgenotype B1/Bj, 38% were subgenotype B2/Ba, and 31% were subgenotype C2/Ce. Notably, the single nucleotide insertion/deletion that resulted in a frameshift of the precore protein was found exclusively in 60% of B1/Bj strains. An in vitro study showed that all of the frameshift mutants had significantly higher amounts of HBV DNA than did the wild type. One of the isolates had a novel insertion of A between nucleotides 1900 and 1901, which resulted in a 3-nucleotide change within the Kozak sequence of the core protein and enhanced the core protein expression in vitro.

Conclusions. The frameshift insertion/deletion in the precore region enhanced HBV replication and might be associated with the development of FH by the subgenotype B1/Bj HBV.

Hepatitis B virus (HBV) is one of the most common viruses affecting the human health. It causes a spectrum of chronic liver diseases including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. Acute HBV infection induces acute self-limited hepatitis or fulminant hepatitis (FH), and the pathogenesis leading to the development of fulminant hepatitis B (FHB) is still being investigated. Although enhanced replication of the virus [1, 2] and an exuberant immune response by the host [3] are considered to be the main pathogeneses, various issues are not fully understood.

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HBV contains a 3.2-kb, circular, partially double-stranded DNA genome; according to the heterogeneity of the nucleotide sequence, at least 8 (A–H) genotypes [4, 5] and, tentatively, 2 new genotypes (I and J) [6, 7] are classified. HBV genotypes are considered to affect the liver disease outcome [8], and the association of genotype B or subgenotype B1/Bj with FH was reported from Japan [9–11]. It has also been reported that several HBV mutations, such as T1753V (not T), T1754V, A1762T/G1764A, G1862T, G1896A, G1899A, and A2339G, were associated with FH [9–12]. In particular, the mutation of G1896A in the precore region, which makes a stop codon and abrogates hepatitis B e antigen (HBeAg), has been well documented [13–15]. HBV with G1896A was reported to have high replication capacity in vitro [10, 16]. However, in general clinical settings, chronic hepatitis patients with HBV with G1896A, which is the main cause of seroconversion of HBeAg to antibody against HBeAg (HBeAb), have lower viral load [17]. The reason for this discrepancy has not yet been elucidated clearly.

A difference in worldwide geographic distribution of the HBV genotypes has been noted. Also, in Japan, where HBV of genotype C prevails, there is a difference

in the distribution: it is known that the percentage of genotype B is higher in the northeast area [18]. However, little is known about the virological features of HBV obtained from FH patients in this area. Therefore, we aimed to investigate the characteristics of HBV, especially those of genotype B, that cause FH in our hospital in northeast Japan.

METHODS

Serum Samples

From January 1996 to November 2010, 60 patients were admitted to our hospital for acute HBV infection. Of them, 15 (25%) were diagnosed with FH. As there was an HBV carrier who developed FH, a total of 16 serum samples from FH patients were used in this study. The diagnosis of FH was made based on the following findings: coma grade II or higher and a prothrombin time <40% developing within 8 weeks after onset.

Determination of HBV Partial Sequences

The partial sequences of HBV were determined as described previously [19] with modifications. To amplify the 396-nucleotide sequence in the S gene (nucleotides 272–667; the nucleotide numbers are in accordance with a genotype C HBV isolate of 3215 nucleotides [AB033550]), total DNA extracted from 50 μL of serum was subjected to nested polymerase chain reaction (PCR) with the primers described previously. To amplify the 255-nucleotide sequence in the core promoter/precore region (nucleotides 1673–1927), the first round of PCR was carried out with primers B015 (5'-CAC GTY GCA TGG ARA CCA CCG TGA-3' [Y = C or T; R = A or G]) and B008 (5'-GTC AGA AGG CAA AAA AGA GAG TAA CTC-3'), and the second round was carried out with primers B016 (5'-GTC TTR CAT AAG AGG ACT CTT GGA CT-3') and B007 (5'-AAA GAG AGT AAC TCC ACA GAA GCT CC-3'). The amplification products were sequenced on both strands directly on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems), located in the Biomedical Research Core of Tohoku University Graduate School of Medicine. Sequence analysis and evaluation of the epsilon (ϵ) signal stability, which was calculated as minimum free energy, were performed using Genetyx-Mac (Version 12.2.6; Genetyx Corp). The sequence data from the current report have been assigned to the GenBank/EMBL/DDBJ with the accession numbers AB602749–AB602759 (partial S region sequence) and AB602760–AB602770 (partial core promoter/precore sequence).

Construction of Plasmids

A plasmid containing the 1.3-fold HBV genome (nucleotide 1051-3215/1-1953) was constructed as described previously [20] using serum of a self-limited acute hepatitis patient (AH-2; accession number of the full-genome sequence, AB602818) with HBV of the subgenotype B1/Bj in our hospital. Because the isolate had a mutation of G1899A in the precore region, the mutation was converted to the wild-type nucleotide using Quick Change II-E Site-Directed Mutagenesis Kit (Stratagene) as described previously [20], and the clone was used as a subgenotype B1/Bj wild-type clone.

The wild clone was used as template to construct a clone with a mutation of G1896A, an insertion of A between nucleotides 1837 and 1838 (1838insA), a deletion of a single nucleotide at 1846 (1846del), or an insertion of A between nucleotides 1900 and 1901 (1901insA). A clone with 1901insA was used as the next template to introduce the additional mutation of T1855C. All constructs were sequenced to confirm the introduced mutation/insertion/deletion.

Cell Culture and Transfection

Human hepatoma HepG2 cells were cultured as described previously [20]. On the next day, after seeding cells in 24-well plates at 1.25×10^5 cells/well, 0.5 μg/well of plasmid DNA was transfected using FuGENE HD Transfection Reagent (Roche Diagnostics), and the culture supernatant and cells were collected 3 days later. For Southern blot analysis, cells were seeded in 6-well plates at 5.0×10^5 cells/well, and 1.5 μg/well of plasmid DNA was transfected. In this system, the transfection efficiency could be evaluated with the level of hepatitis B surface antigen (HBsAg) in the culture supernatant [20]. The experiments were performed at least in triplicate.

Assay of HBV Markers

Five microliters of the supernatant was treated with 5 units of DNase I (TaKaRa Bio) at 37°C for 2 hours to digest the input plasmid DNA in the culture supernatant, and the reaction was stopped with ethylenediaminetetraacetic acid. Then, total DNA was extracted with a QIAamp DNA Blood Mini Kit (QIAGEN GmbH), and the amount of HBV DNA was quantified with real-time PCR using a StepOnePlus Real Time PCR System (Applied Biosystems) [21]. HBsAg and HBeAg in 50 μL of the culture supernatant were assayed by enzyme-linked immunosorbent assay [20]. To detect the intracellular replicative intermediates of HBV, the core particle–associated HBV DNA in the cells was isolated as described previously [20]. After DNase I treatment for the removal of unprotected DNA, extracted total DNA was analyzed by Southern blotting using a full-length HBV DNA probe labeled with PCR DIG Probe Synthesis Kit (Roche Diagnostics).

In Vitro Cell-Free Protein Expression

To investigate whether the change of the Kozak sequence around the initiation codon of the core protein affects the protein expression, TNT T7 Quick for PCR DNA (Promega) was used. The template of transcription/translation was a purified PCR product that was amplified from the subgenotype B1/Bj wild clone. To make the wild-type template, PCR was performed with a forward primer CoreKW (5'-GGA TCC TAA TAC GAC TCA CTA TAG

GGA ACA TGG GGC ATG GAC ATT GAC CCT T-3'), including the T7 promoter sequence, spacer, and the Kozak sequence (underlined) including the initiation codon of the core protein, followed by the partial core sequence and a reverse primer CoreR (5'-CTA TCT AGA CTA ACA TTG GGA TTC CCG A-3') including the termination codon of the core protein. To make templates with G1896A, G1899A, and 1901insA, forward primers CoreK-5A (the underlined sequence of CoreKW was changed to TAGGGCATGG), CoreK-2A (the underlined sequence was changed to TGGGACATGG), and CoreK-1A-2C-6G (the underlined sequence was changed to GGGGCAATGG) were used, respectively. The expressed protein was analyzed with Western blotting using a rabbit polyclonal anti–hepatitis B core antigen (HBcAg) antibody (Dako) as the primary antibody.

Statistical Analysis

Statistical analyses were performed using Mann–Whitney *U* test for comparison of continuous variables between 2 groups. Differences were considered to be statistically significant when $P < .05$.

RESULTS

Characteristics of the Fulminant Hepatitis Patients

The clinical characteristics of the 16 FH patients are shown in Table 1. The mean age was 53.0 years (range, 29–71), and 13 (81%) were male. The mean peak total bilirubin was 14.7, the mean peak alanine aminotransferase was 4932, and the mean lowest prothrombin time was 18.6%. Nine (56%) patients died of fulminant hepatitis. Lamivudine was administered to 4 patients (numbers 12–15), and entecavir was administered to 1 patient (number 16). After 2003, living related liver transplantation has been performed for 4 FH patients, and all of the patients were rescued. Two of them (numbers 12 and 13) showed rapid progression and were considered so-called hyperacute cases, but were rescued with liver transplantation without complications [22]. The HBV isolates from these patients were named BFJT followed by the onset year, excluding 5 cases referred to as FH-1 to FH-5 in a previous report by us [23].

Determination of HBV Genotype

Based on the partial sequences in the S region of HBV isolates from FH patients, a phylogenetic tree was constructed (Figure 1). Of the 16 HBV isolates, 5 (31%) belonged to subgenotype B1/Bj, 6 (38%) belonged to subgenotype B2/Ba, and 5 (31%) were subgenotype C2/Ce. The 5 isolates of subgenotype B2/Ba were grouped into a cluster: these patients were considered to have the same source of infection [23]. In this study, 69% of the FH patients were infected with genotype B HBV, which was much higher than previously reported in Japan (22%–33%) [10, 11]. It was also higher than the reported percentage (21%) of genotype B in acute hepatitis B patients in northeast Japan [24].

Mutation, Insertion, and Deletion in the Core Promoter and Precore Region

The analysis of the partial sequences in the core promoter/precore region showed that there were several mutations in the HBV isolates. The mutations that were reported previously to have an association with FH are shown in Table 2. Because the 5 isolates of subgenotype B2/Ba were almost identical, they were counted as a single strain. The mutations at nucleotides 1753,

Table 1. Characteristics of the Fulminant Hepatitis B Patients

Patient no.	Age/sex	Date of onset	Peak T. Bil (mg/dL)	Peak ALT (IU/L)	Lowest PT (%)	Possible infection source	Liver transplantation	Outcome	HBV isolate name
1	65/M	December 1996	19.2	1764	18.0	Unknown	No	Died	BFJT1996-1
2	29/M	October 1997	8.8	6900	5.0	Unknown	No	Died	BFJT1997-1
3	65/M	February 1999	16.9	2162	22.4	Unknown	No	Died	BFJT1999-1
4	28/F	August 1999	8.9	7120	17.1	Sexual contact	No	Rescued	BFJT1999-2
5	61/M	May 2000	32.8	3750	26.0	Unknown	No	Died	BFJT2000-1
6	69/F	May 2000	10.6	4190	6.0	Iatrogenic	No	Died	FH-1
7	71/M	July 2000	29.0	3530	38.0	Iatrogenic	No	Died	FH-2
8	66/M	October 2000	13.9	6950	10.1	Iatrogenic	No	Died	FH-3
9	50/M	December 2000	13.3	13 420	10.0	Blood transfusion	No	Died	BFJT2000-2
10	71/F	December 2000	17.0	3380	27.0	Iatrogenic	No	Rescued	FH-4
11	60/M	February 2001	6.4	10 290	12.7	Iatrogenic	No	Died	FH-5
12	45/M	July 2003	11.8	6450	9.3	Sexual contact	Yes	Rescued	BFJT2003-1
13	34/M	August 2003	9.5	7150	9.0	Sexual contact	Yes	Rescued	BFJT2003-2
14	32/M	July 2005	10.8	278	22.1	Sexual contact	Yes	Rescued	BFJT2005-1
15	38/M	August 2006	5.1	728	39.8	Unknown	No	Rescued	BFJT2006-1
16	64/M	April 2009	20.5	857	24.7	Carrier	Yes	Rescued	BFJT2009-1

NOTE. ALT, alanime aminotransferase; HBV, hepatitis B virus; PT, prothrombin time; T. Bil, tital bilirubin.

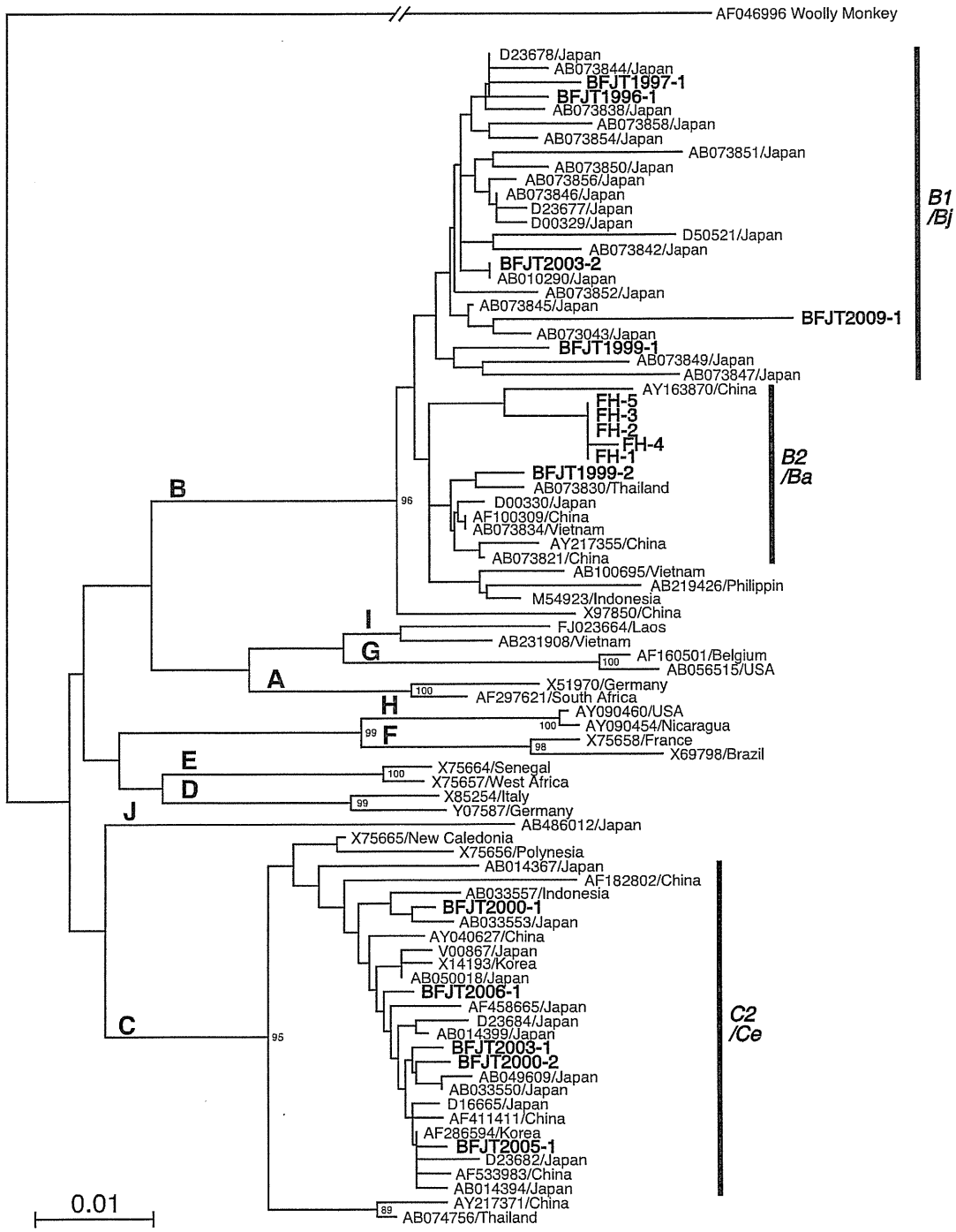


Figure 1. Phylogenetic tree constructed by the neighbor-joining method based on the partial 396-nucleotide sequences in the S region of 84 hepatitis B virus isolates. In addition to the 16 isolates found in this study, which are indicated in bold type for visual clarity, 68 reported isolates of genotypes A–J were included for comparison. Bootstrap values are indicated for the major nodes as a percentage obtained from 1000 resamplings of the data.

1754, 1762/1764, 1862, 1896, and 1899 were found in 17%, 33%, 42%, 8%, 67%, and 25% of the 12 isolates, respectively. Among the subgenotypes, there were differences in the distribution of the mutations: T1754G was found only in subgenotype B1/Bj (4 of 5, 80%), the mutations at nucleotide 1762/1764 were found in subgenotype B2/Ba and C2/Ce (5 of 7, 71%), and G1899A was found only in subgenotype B1/Bj (3 of 5, 60%). Interestingly, an insertion/deletion of a single nucleotide in the precore region (1838insA, 1846del, and 1901insA) was also found only in subgenotype B1/Bj (3 of 5, 60%). The surrounding nucleotide

Table 2. Mutations, Insertions, and Deletions of Hepatitis B Virus Found in the Fulminant Hepatitis Patients

		Nucleotide no.[a]						
Isolate name	Subgenotype	1753	1754	1762/1764	1862	1896	1899	Frameshift[b]
BFJT1996-1	B1/Bj	T	T	A/G	G	G	**A**	**1846del**
BFJT1997-1	B1/Bj	T	**G**	A/G	G	G	G	**1901insA**
BFJT1999-1	B1/Bj	T	**G**	A/G	G	**A**	**A**	...
BFJT2003-2	B1/Bj	T	**G**	A/G	G	**A**	G	**1838insA**
BFJT2009-1	B1/Bj	**Y**	**G**	A/G	G	**A**	**A**	...
BFJT1999-2	B2/Ba	T	T	A/G	G	**A**	G	...
FH-1,2,3,4,5[c]	B2/Ba	T	T	**T/A**	**T**	**A**	G	...
BFJT2000-1	C2/Ce	T	T	**T/A**	G	**A**	G	...
BFJT2000-2	C2/Ce	T	T	**T/A**	G	**A**	G	...
BFJT2003-1	C2/Ce	T	T	A/**A**	G	**A**	G	...
BFJT2005-1	C2/Ce	**G**	T	**T/A**	G	G	G	...
BFJT2006-1	C2/Ce	T	T	A/G	G	G	G	...
Frequency (%)		17	33	42	8	67	25	25

NOTE. 1846del, a single nucleotide deletion at nucleotide 1846; 1901insA, an insertion of A between nucleotide 1900 and 1901; 1838insA, an insertion of A between nucleotide 1837 and 1838.

[a] The nucleotides of mutation are indicated in bold type.

[b] Insertion/deletion that causes a frameshift in the precore protein.

[c] These isolates are indicated as a single strain because of the high identity [23].

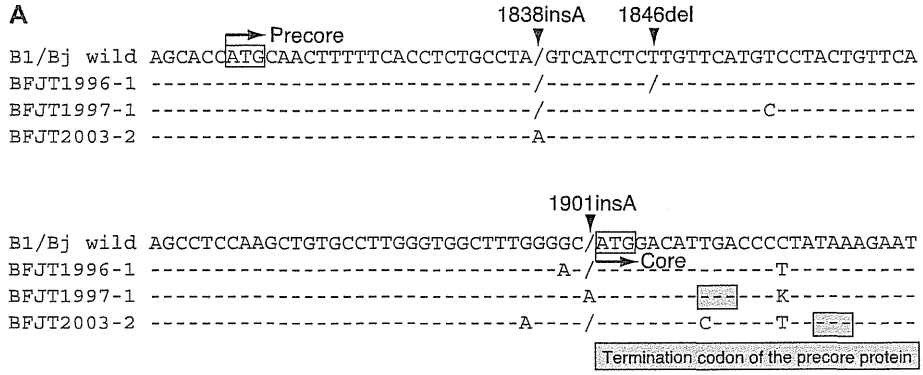
sequences of the single nucleotide insertions/deletions in this study are shown in Figure 2. The insertions in this region make a termination codon at nucleotide 1909 or 1915 in a frame of the precore protein, and the deletion makes a termination codon at nucleotide 1993. Therefore, these single nucleotide insertions/deletions resulted in frameshifts of the precore protein, and they were thought to abrogate HBeAg expression. These precore frameshift mutants were previously found in HBeAg-negative HBV carriers [25, 26]. As for self-limited acute hepatitis B patients, we found that only 1 of 96 (1%) patients had the frameshift mutant (data not shown).

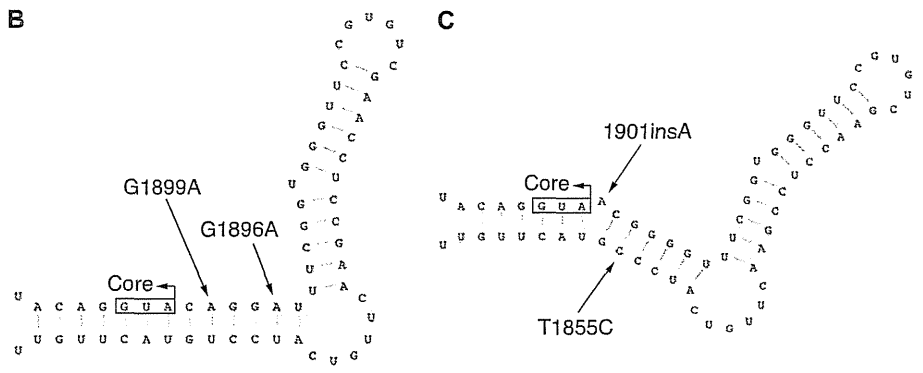
With the aim of clarifying the distribution of the precore frameshift insertion/deletion in the general population, the HBV isolates whose entire sequences were known were retrieved from the Hepatitis Virus Database [27]. In November 2010, a total of 3457 full-length sequences of HBV were registered, and of these, 3391 sequences were proved to belong to genotypes A–I based on a phylogenetic tree analysis. In total, 11 (0.3%) isolates with the precore frameshift were found in genotypes A, B, C, and D isolates (Table 3). Therefore, the frameshift seemed to be rare in general, but can occur in several genotypes other than genotype B.

Of the mutations, insertions, and deletion in the precore region found in the FH patients, G1896A, G1899A, and 1901insA can affect the ϵ signal of HBV. The ϵ signal, which forms a secondary structure of pregenomic RNA, is highly conserved among HBV strains and is essential for the initiation of the encapsidation of pregenomic RNA [28]. Figure 2 depicts the structure of the ϵ signal with G1896A and G1899A, which stabilize the nucleotide pair in the lower stem. The stabilized ϵ signal has an advantage for pregenomic RNA encapsidation [28] and is considered to lead to heightened replication efficiency of HBV. However, 1901insA distorted the secondary structure of the lower stem and seemed to make the ϵ signal wobble (Figure 2). The mutation of T1855C, which might compensate for the instability, was present along with 1901insA in the BFJT1997-1 isolate. The change of the ϵ signal stability was evaluated by calculating the minimum free energy (Figure 2): the lower energy value indicates higher stability of the structure. It was indicated that the ϵ signal with G1896A and/or G1899A had higher stability than the wild type. The structure with 1901insA has lower stability, and it was confirmed that the mutation of T1855C restores the stability, which was still lower than that of the wild type.

Effect of the Precore Frameshift on HBV Replication In Vitro

To confirm whether the frameshift in the precore region of subgenotype B1/Bj HBV has significance in the development of FH, the replication capacity of the HBV clones with the frameshift insertion/deletion (1838insA, 1846del, and 1901insA) was evaluated in vitro using plasmids containing the 1.3-fold HBV genome (Figure 3). As expected, the level of HBsAg in the culture supernatant was almost equal, and the HBeAg level of the clones with the frameshift was significantly lowered to the same level as the clones with G1896A, which is known to abrogate HBeAg. When the amount of HBV DNA in the culture supernatant was assayed, it was revealed that the clones with the frameshift had significantly higher HBV DNA levels than did the wild type. The HBV DNA level of the clone with G1896A ± G1899A was significantly higher, also.





D

Mutation/insertion in the ε signal	Minimum free energy (kcal/mol)
B1/Bj wild type (D00329)	-24.9
G1896A	-25.5
G1899A	-26.1
G1896A+G1899A	-26.7
1901insA	-21.0
1901insA+T1855C	-24.1

Figure 2. *A*, Partial sequences around the precore region of hepatitis B virus (HBV) with frameshift insertion/deletion obtained in this study. The sequence (nucleotides 1808–1925) of a subgenotype B1/Bj wild-type isolate (D00329) is shown on the first line for comparison. The white boxes indicate the initiation codons of the precore/core protein, and the gray boxes indicate the termination codons of the precore protein resulting from the frameshift insertions. *B*, Secondary structure of the ϵ signal of HBV pregenomic RNA with the mutations of both G1896A and G1899A, which are found commonly in fulminant hepatitis patients and hepatitis B e antigen (HBeAg)–negative carriers. *C*, Secondary structure of the ϵ signal with both 1901insA and T1855C found in the BFJT1997-1 isolate. *D*, Stability of the ϵ signal evaluated with minimum free energy, which was calculated using Genetyx Mac. The mutation/insertion was assumed to be present in the B1/Bj wild-type sequence (D00329) and analyzed.

Because the BFJT1997-1 isolate with 1901insA had T1855C in the precore region, the effect of the mutation on HBV replication was evaluated (Figure 3). Interestingly, T1855C increased significantly the HBV DNA level of the clone with 1901insA. The clone with T1855C without 1901insA did not increase the HBV DNA level in comparison with that of the wild type. Therefore, the effect of T1855C was considered to be a restoration of the ϵ signal instability with 1901insA as described above.

The amount of the intracellular replicative intermediates of HBV was evaluated with Southern blot analysis (Figure 3). The result was concordant with that of the HBV DNA level in the culture supernatant. This indicated that the precore frameshift had an effect in the replication cycle before the release step of virion, such as the encapsidation of pregenomic RNA.

Change of the Core Protein Expression Level With the Frameshift Insertion

Although it is considered that the stability of the ϵ signal is necessary for efficient replication [28], the structure of the ϵ signal with 1901insA and T1855C seemed not to be more stable than the wild type as shown in Figure 2. Therefore, another mechanism by which the HBV replication is enhanced was assumed

Table 3. Distribution of Insertions and Deletions That Cause Frameshift of the Precore Protein Among HBV Genotypes A–I, Based on the Isolates Whose Full-length Sequences Were Known

			Subgenotype									
Genotype[a]	A	B	B1	B2	C	D	E	F	G	H	I	Total
No.	427	856	40	659	1191	499	249	77	26	30	36	3391
Insertion	2	1	1	0	1	1	0	0	0	0	0	5
Deletion[b]	1	2	0	2	3	0	0	0	0	0	0	6
Total (%)	3	3	1	2	4	1	0	0	0	0	0	11
	(0.7)	(0.4)	(2.5)	(0.3)	(0.3)	(0.2)	(0)	(0)	(0)	(0)	(0)	(0.3)

NOTE. HBV, hepatitis B virus.

[a] If the recombination of the genome among different genotypes was present, the genotype of HBV was determined by the phylogenetic tree analysis based on the full-length HBV sequences.

[b] The isolates that had deletions including the precore initiation codon were not counted as having the frameshift deletion.

to act. We focused on the change of the Kozak sequence around the initiation codon of the core protein. The Kozak sequence includes the 6-nucleotide sequence just before the initiation codon and 1-nucleotide after that (optimal sequence, GCCA/GCCATGC), and it affects the translation efficiency [29]. Figure 4 shows the altered Kozak sequence of the core protein with G1896A, G1899A, and 1901insA. Whereas G1896A and G1899A make a 1-nucleotide change in the Kozak sequence, 1901insA makes a 3-nucleotide change. A cell-free protein expression system was used to clarify whether the Kozak sequence alteration affects the core protein expression. Western blot analysis of the expressed core protein showed that the Kozak sequence with G1896A or G1899A increased the expression slightly in comparison with the wild-type sequence and, notably, that the Kozak sequence with 1901insA increased the protein level greatly (Figure 4). This increment of the core protein may enhance the replication of HBV particles and may lead to the development of FH.

DISCUSSION

It has been considered that FHB results from the rapid increase of HBV and the vigorous host immune response to HBV-infected hepatocytes [30, 31]. Several mutations found in HBV of FH patients were reported to enhance the HBV replication in vitro [1, 10]. We previously reported that a FH strain caused intracellular retention of HBV, which was thought to be associated with pathogenesis [20]. Here, we described that the single nucleotide insertion/deletion in the precore region leading to a frameshift, which abrogates HBeAg, was found frequently in our FH patients with subgenotype B1/Bj HBV. The frameshift mutants had never been reported in self-limited acute hepatitis patients, whose HBeAg-positive rate is high (56%–84%) [10, 11, 32, 33]. The number of patients in this study was small, but the significance of the frameshift could be confirmed using an in vitro HBV replication system. Although HBV isolates with the frameshift were rarely found in general, patients with acute infection with these isolates may be at risk of developing FH.

This study showed that genotype B HBV was found frequently (69%) in the FHB patients in our hospital in northeast Japan. Recently, the frequency of genotype B in chronic hepatitis B patients in northeast Japan was reported to be higher than that in all Japan (44% vs 14%, respectively) [18]. Although the percentage of genotype B in FHB patients in the area had not reported, this study confirmed that the genotype B percentage was higher. Whereas genotype B HBV frequently leads to FH [9–11], it causes less progressive chronic liver disease than genotype C. This phenomenon was considered to link to earlier HBeAg/Ab seroconversion in the natural course of genotype B compared with that of genotype C [34].

In this study, the precore frameshift was found exclusively in subgenotype B1/Bj strains. The database search showed that the frameshift could occur in several genotypes, at least genotypes A–D, but not frequently (0.3% in total). Interestingly, a previous report by Sugauchi et al showed that 7 of 275 (2.5%) chronically infected patients with subgenotype B1/Bj had the frameshift insertion of 1838insA [35]. Because the number of genotype B1/Bj isolates in that report is larger than that from the database search of full-length HBV sequences, this frequency is more convincing. This may be one of the reasons why subgenotype B1/Bj HBV frequently causes FH.

Previous reports described that HBV with G1896A had a high replication capacity [10], and the present study showed that the precore frameshift insertion/deletion also enhanced the HBV replication to the same level as G1896A. It was reported that p22, the N-terminal-processed p25 precore protein, inhibited the formation of nucleocapsids and regulated the HBV replication [16]. The protein of p22 is further modified at the C-terminal region to secrete p17 HBeAg. HBV with G1896A or the frameshift insertion/deletion in the precore region does not express p25 and the resulting p22. If there is no p22, the nucleocapsids are formed efficiently and the replication of HBV particles can be accelerated. However, this contravenes the general course of HBV carriers, whose seroconversion of HBeAg/HBeAb leads to the reduction of serum HBV DNA

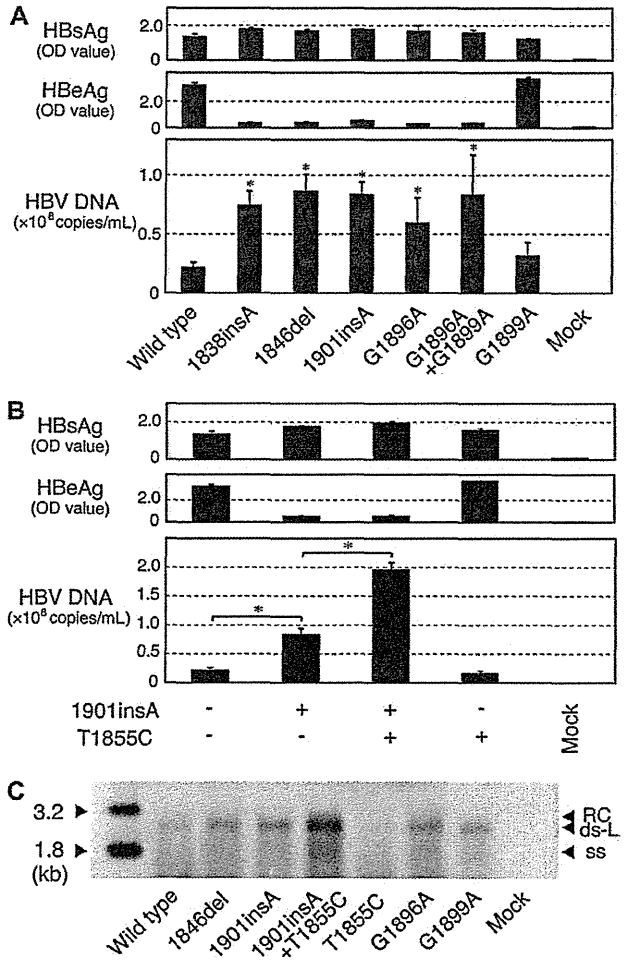


Figure 3. *A*, Level of hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), and hepatitis B virus (HBV) DNA in the culture supernatant of HepG2 cells that were transfected with several HBV constructs with a frameshift insertion/deletion or mutations in the precore region. *, $P < .05$ in comparison with the wild type. *B*, Level of HBsAg, HBeAg, and HBV DNA in the culture supernatant of HepG2 cells transfected with HBV constructs with 1901insA and/or T1855C. *, $P < .05$. *C*, Representative data of the intracellular replicative intermediates of HBV detected with Southern blot analysis. ds-L, double-stranded linear HBV DNA; OD, optical density; RC, relaxed-circular HBV DNA; ss, single-stranded HBV DNA.

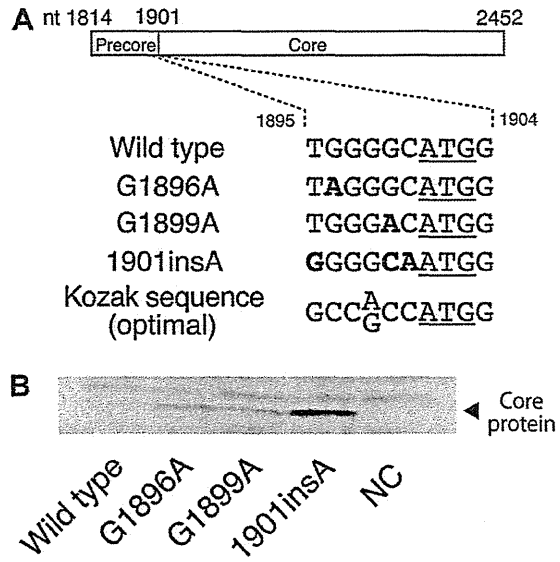


Figure 4. *A*, Schema of the Kozak sequence around the initiation codon of the core protein. The changed nucleotides in the Kozak sequene, which were found in hepatitis B virus (HBV) with G1896A, G1899A, or 1901insA, are shown in bold type. *B*, Results of Western blot analysis of the expressed HBV core protein in a cell-free protein expression system. NC, negative control.

[17]. The discrepancy may be due to the adaptive immune response in HBV carriers. Under the suppression by cytotoxic T lymphocytes, HBeAg-negative HBV clones, which have an advantage in the replication cycle, may be barely persistent in the late phase of HBV infection.

The ϵ signal of HBV pregenomic RNA is recognized by HBV polymerase, and both of them are encapsidated into the core particle [36]. Stability of the ϵ signal favors replication [28] and, therefore, G1896A and G1899A may easily occur in the natural course of HBV infection. However, 1901insA, which was found in an FH patient, degrades the stability. It was compensated by a distinct mutation of T1855C, but seemed not to be so stable based on the secondary structure of the ϵ signal. This in vitro study revealed that the enhancement of HBV replication by the novel insertion of 1901insA resulted from the change of the Kozak sequence of the core protein. It was also interesting that G1896A and G1899A increased the core protein expression level slightly. As for the Kozak sequence in HBV, the sequence just upstream of the precore initiation codon was described previously [37]: it affects the expression of HBeAg, and associates with the seroconversion of HBeAg/Ab. There is a possibility that the Kozak sequence of other HBV proteins such as polymerase, HBsAg, and X protein may alter the HBV replication capacity or the disease outcome.

In conclusion, the frameshift insertion/deletion in the precore region was found frequently in subgenotype B1/Bj HBV from FH patients in northeast Japan. The frameshift was shown to enhance the HBV replication in vitro and, in particular, the insertion of 1901insA heightened the replication capacity via the novel mechanism of the changed Kozak sequence of the core protein. Therefore, the precore frameshift may have significance in the development of FH.

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ウイルス性肝炎における最新の治療法の標準化を目指す研究

平成22年度～平成24年度　総合研究報告書

研究代表者　　熊田　博光

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Ⅲ．平成24年度 研究成果の刊行に関する一覧表

Ⅲ．研究成果の刊行に関する一覧表

書　籍

太字のみ研究成果の刊行物として収載

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