**Table 3** Replication capacity and susceptibility to adefovir of the HBV mutants

| HBV mutants | HBV DNA ($\times 10^7$ log copies/mL)* | Fold replication† | $IC_{50}$ ($\mu$M)* | Fold resistance† |
|---|---|---|---|---|
| Wild type | 13.60 ± 3.50 | 1 | 0.42 ± 0.06 | 1 |
| M204I | 2.17 ± 0.38 | 0.16 | 0.87 ± 0.2 | 2.07 |
| L180M+M204V | 4.38 ± 0.77 | 0.32 | 0.73 ± 0.06 | 1.74 |
| L180M+T184S+M204V | 5.98 ± 0.80 | 0.44 | 0.91 ± 0.04 | 2.17‡ |
| L180M+A200V+M204V | 8.90 ± 0.56 | 0.65‡ | 1.09 ± 0.12 | 2.60‡ |
| L180M+S202C+M204V | 4.86 ± 0.19 | 0.36 | 2.19 ± 0.63 | 5.21‡ |
| L180M+M204V+N236T | 0.88 ± 0.68 | 0.07‡ | >10 | >25 |
| L180M+A200V+M204V+N236T | 0.54 ± 0.38 | 0.04‡ | >10 | >25 |

*Values are expressed as means ± SD of experiments performed in triplicate. †(Mean value of the mutant)/(mean value of the wild type). ‡$P < 0.05$ in comparison with the clone with rtL180M+M204V.

with these mutations were not major, because they had no effect in enhancing the replication capacity of HBV.

## DISCUSSION

As clinical and histological improvement accompanies reductions in HBV replication, therapies that reduce HBV replication are expected to limit the progression of liver disease and improve the natural history of chronic HBV infection [10]. Currently, the management of hepatitis B patients with drug resistance is one of the major problems in clinical practice for hepatitis B. A substantial part of 3TC-treated patients has mutant HBV with the YMDD mutation, and several clinical trials to treat 3TC-resistant hepatitis B have been performed. It has been reported first that with ADV alone and in combination with 3TC, the viral and biochemical responses were the same for 3TC-resistant patients in a 1 year study [31]. However, several studies of longer term treatment have shown that adding ADV was superior to switching to ADV monotherapy for patients with 3TC resistance [32–34]. In this study, we demonstrated that the add-on ADV therapy for 3TC-resistant hepatitis B patients effectively suppressed serum HBV DNA for a median of 47 months. Moreover, the biochemical response of ALT normalization was achieved in 77% patients and HBeAg loss in 88% of the HBeAg-positive patients at 48 months. The undetectability of HBV DNA was assessed by the Amplicor HBV monitor test, but recently, this can be assessed by a more sensitive real-time assay such as the Cobas TaqMan HBV test (Roche Diagnostics). The treatment duration to achieve HBV DNA undetectability might be longer if a more sensitive assay was used.

The influence of HBV genotype on the response or resistance to ADV has not been clarified, whereas the efficacy to 3TC was reported to be different between HBV genotypes [24,25]. This study showed that the virological response to 3TC+ADV was significantly earlier in genotype B than in C. However, there were several limitations of the results: the patients with genotype B were fewer, and no multivariate analysis was performed. In addition, all patients with HCC were genotype C, and ALT levels of genotype B tended to be higher, although there were no significant differences. The effect of genotype on the response to 3TC±ADV should be confirmed in larger studies. The baseline HBeAg status in 3TC+ADV combination therapy in 3TC-resistant patients was reported to influence the viral response: HBeAg-negative patients showed better virological and biological response [35]. In this study, the same tendency was observed, but the difference was not significant.

Initial virological suppression by ADV monotherapy was reported to be a good prognostic factor for the treatment of both naïve patients [36] and 3TC-resistant patients [37]. Taking into account the results of this study and previous reports, it is suggested that patients with genotype B HBV might develop resistance to 3TC+ADV less frequently than those with genotype C. In fact, the 3TC+ADV-resistant patient in this study was infected with genotype C HBV. Because the development of resistance to 3TC+ADV combination therapy is rare [22,35], it is difficult to evaluate whether the early virological response or genotype B is associated with the lower frequency of resistance to 3TC+ADV combination therapy. Further long-term study is needed to clarify this issue.

Although the emergence of resistance in this study was rare during the combination therapy as previously reported [22,35], one patient developed virological breakthrough after 4.5 years. We identified a characteristic mutation pattern of HBV in this patient. The mutation of rtA200V rescued the *in vitro* replication capacity that was impaired by rtL180M+M204V and reduced the susceptibility to ADV. In previous reports, rtA200V emerged as an additional mutation with the 3TC-resistant mutation in patients under 3TC monotherapy [38,39]. The effect of this mutation is not as strong as the effect of rtM204I/V±L180M on 3TC susceptibility *in vitro*, which showed >1000-fold resistance [40]. However, the clinical dose of ADV is comparatively low

because of renal toxicity [41], and the weakly resistant profile *in vitro* can explain the great clinical impact. Villet *et al.* reported that rtA200V was observed in a patient with 3TC monotherapy, and it was no longer detected after the combination therapy with ADV and 3TC [39]. The difference of results between the previous study and our study may be because of the emergence of mutations with a potent effect on ADV resistance, such as rtV173L and rtA181V, in the previous study. Because these mutations may have a greater effect on ADV resistance than rtA200V, the HBV clones with rtA200V seemed to disappear in the previous study case.

The known ADV-resistant mutation of rtN236T was found in only 25% clones, exclusively with rtA200V. This may indicate that rtN236T appeared after the emergence of rtA200V. In the active replication of the clones with rtA200V, which restored the replication capacity and enhanced ADV resistance, other mutations including rtN236T might occur more readily.

The rtA200V mutation is the result of nucleotide substitution C728T. This change in the overlapping S region results in an amino acid substitution affecting HBsAg: Leu to Phe at aa192 (sL192F). There is a possibility that sL192F may affect the replication capacity of HBV, but the actual mechanism is unknown.

Interestingly, the ETV-resistant mutations of rtT184S and rtS202C were also detected during 3TC+ADV combination therapy by clonal analysis. These mutations confer ETV resistance in the presence of the 3TC-resistant mutations of rtM204I/V±L180M [21]. This study showed that these mutations also have an ADV-resistance profile. These mutations may not cause viral breakthrough, because the population of these mutants in the patient was minor (4% and 6%, respectively), and their replication capacity was lower than that with rtA200V *in vitro*. The emergence of these mutations suggested that long-term 3TC+ADV therapy has the possibility of leading to multiple drug resistance including ETV resistance.

The combination therapy of 3TC and ADV is very effective with little frequency of viral breakthrough for 3TC-refractory patients. However, some patients do not achieve complete viral suppression of serum HBV DNA to under 2.6 log copies/mL. It was considered that the incomplete suppression of viral replication might favour further selection of drug-resistant mutants [42]. Although there have been a few reports of cases that showed resistance to 3TC+ADV therapy to date, the number of resistant cases will increase along with the increase in cases with long-term therapy. The 3TC- and ADV-resistant patient in this study was treated with 3TC and TDF after the virological breakthrough, and HBV DNA was promptly suppressed. Although TDF was reported to show cross-resistance with ADV *in vitro* [16,40,43], there are several reports that showed the effectiveness of TDF for ADV-refractory patients [44–46]. It is thought that the potency of TDF might result from its higher clinical dose compared to that of ADV [47].

In conclusion, this study showed that the combination therapy of 3TC and ADV effectively suppressed HBV replication in 3TC-resistant patients with chronic HBV infection for 4 years. Especially, patients with genotype B achieved earlier virological response than those with genotype C. However, one of the 28 patients developed virological breakthrough during the combination therapy over 4 years, and the HBV mutation of rtA200V, in addition to 3TC-resistant mutations, was demonstrated to contribute to the ADV resistance. Moreover, ETV-resistant mutations emerged coincidentally in minor HBV clones. The risk of emergence of multiple drug-resistant mutant should be considered in cases with long-term therapy with nucleos(t)ide analogues, especially when serum HBV DNA cannot be suppressed completely. Potent antiviral agents should be administered in such cases to prevent the emergence of multiple drug-resistant HBV mutants that are difficult to treat.

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## REFERENCES

1 Ganem D, Prince AM. Hepatitis B virus infection – natural history and clinical consequences. *N Engl J Med* 2004; 350: 1118–1129.
2 Doong SL, Tsai CH, Schinazi RF, Liotta DC, Cheng YC. Inhibition of the replication of hepatitis B virus in vitro by 2′,3′-dideoxy-3′-thiacytidine and related analogues. *Proc Natl Acad Sci U S A* 1991; 88: 8495–8499.
3 Lai CL, Chien RN, Leung NW *et al.* A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. *N Engl J Med* 1998; 339: 61–68.
4 Ling R, Mutimer D, Ahmed M *et al.* Selection of mutations in the hepatitis B virus polymerase during therapy of transplant recipients with lamivudine. *Hepatology* 1996; 24: 711–713.
5 Lai CL, Dienstag J, Schiff E *et al.* Prevalence and clinical correlates of YMDD variants during lamivudine therapy for patients with chronic hepatitis B. *Clin Infect Dis* 2003; 36: 687–696.
6 Zoulim F. Hepatitis B virus resistance to antivirals: clinical implications and management. *J Hepatol* 2003; 39(Suppl. 1): S133–S138.
7 Tipples GA, Ma MM, Fischer KP, Bain VG, Kneteman NM, Tyrrell DL. Mutation in HBV RNA-dependent DNA polymerase confers resistance to lamivudine *in vivo*. *Hepatology* 1996; 24: 714–717.
8 Delaney IV WE, Yang H, Westland CE *et al.* The hepatitis B virus polymerase mutation rtV173L is selected during lamivudine therapy and enhances viral replication in vitro. *J Virol* 2003; 77: 11833–11841.

9 Ono SK, Kato N, Shiratori Y *et al.* The polymerase L528M mutation cooperates with nucleotide binding-site mutations, increasing hepatitis B virus replication and drug resistance. *J Clin Invest* 2001; 107: 449–455.

10 Dienstag JL. Hepatitis B virus infection. *N Engl J Med* 2008; 359: 1486–1500.

11 Angus P, Vaughan R, Xiong S *et al.* Resistance to adefovir dipivoxil therapy associated with the selection of a novel mutation in the HBV polymerase. *Gastroenterology* 2003; 125: 292–297.

12 Villeneuve JP, Durantel D, Durantel S *et al.* Selection of a hepatitis B virus strain resistant to adefovir in a liver transplantation patient. *J Hepatol* 2003; 39: 1085–1089.

13 Fung SK, Andreone P, Han SH *et al.* Adefovir-resistant hepatitis B can be associated with viral rebound and hepatic decompensation. *J Hepatol* 2005; 43: 937–943.

14 Hadziyannis SJ, Tassopoulos NC, Heathcote EJ *et al.* Long-term therapy with adefovir dipivoxil for HBeAg-negative chronic hepatitis B. *N Engl J Med* 2005; 352: 2673–2681.

15 Hadziyannis SJ, Tassopoulos NC, Heathcote EJ *et al.* Long-term therapy with adefovir dipivoxil for HBeAg-negative chronic hepatitis B for up to 5 years. *Gastroenterology* 2006; 131: 1743–1751.

16 Brunelle MN, Jacquard AC, Pichoud C *et al.* Susceptibility to antivirals of a human HBV strain with mutations conferring resistance to both lamivudine and adefovir. *Hepatology* 2005; 41: 1391–1398.

17 Papatheodoridis GV, Manolakopoulos S, Dusheiko G, Archimandritis AJ. Therapeutic strategies in the management of patients with chronic hepatitis B virus infection. *Lancet Infect Dis* 2008; 8: 167–178.

18 Lau DT, Khokhar MF, Doo E *et al.* Long-term therapy of chronic hepatitis B with lamivudine. *Hepatology* 2000; 32: 828–834.

19 Westland CE, Yang H, Delaney IV WE *et al.* Activity of adefovir dipivoxil against all patterns of lamivudine-resistant hepatitis B viruses in patients. *J Viral Hepat* 2005; 12: 67–73.

20 Chang TT, Gish RG, Hadziyannis SJ *et al.* A dose-ranging study of the efficacy and tolerability of entecavir in Lamivudine-refractory chronic hepatitis B patients. *Gastroenterology* 2005; 129: 1198–1209.

21 Tenney DJ, Levine SM, Rose RE *et al.* Clinical emergence of entecavir-resistant hepatitis B virus requires additional substitutions in virus already resistant to Lamivudine. *Antimicrob Agents Chemother* 2004; 48: 3498–3507.

22 Lampertico P, Vigano M, Manenti E, Iavarone M, Sablon E, Colombo M. Low resistance to adefovir combined with lamivudine: a 3-year study of 145 lamivudine-resistant hepatitis B patients. *Gastroenterology* 2007; 133: 1445–1451.

23 Tenney DJ, Rose RE, Baldick CJ *et al.* Long-term monitoring shows hepatitis B virus resistance to entecavir in nucleoside-naive patients is rare through 5 years of therapy. *Hepatology* 2009; 49: 1503–1514.

24 Orito E, Fujiwara K, Tanaka Y *et al.* A case-control study of response to lamivudine therapy for 2 years in Japanese and Chinese patients chronically infected with hepatitis B virus of genotypes Bj, Ba and C. *Hepatol Res* 2006; 35: 127–134.

25 Kobayashi M, Suzuki F, Akuta N *et al.* Response to long-term lamivudine treatment in patients infected with hepatitis B virus genotypes A, B, and C. *J Med Virol* 2006; 78: 1276–1283.

26 Levey AS, Bosch JP, Lewis JB, Greene T, Rogers N, Roth D. A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of Diet in Renal Disease Study Group. *Ann Intern Med* 1999; 130: 461–470.

27 Takahashi M, Nishizawa T, Gotanda Y *et al.* High prevalence of antibodies to hepatitis A and E viruses and viremia of hepatitis B, C, and D viruses among apparently healthy populations in Mongolia. *Clin Diagn Lab Immunol* 2004; 11: 392–398.

28 Inoue J, Ueno Y, Nagasaki F *et al.* Enhanced intracellular retention of a hepatitis B virus strain associated with fulminant hepatitis. *Virology* 2009; 395: 202–209.

29 Tenney DJ, Rose RE, Baldick CJ *et al.* Two-year assessment of entecavir resistance in Lamivudine-refractory hepatitis B virus patients reveals different clinical outcomes depending on the resistance substitutions present. *Antimicrob Agents Chemother* 2007; 51: 902–911.

30 Melegari M, Scaglioni PP, Wands JR. Hepatitis B virus mutants associated with 3TC and famciclovir administration are replication defective. *Hepatology* 1998; 27: 628–633.

31 Peters MG, Hann HW, Martin P *et al.* Adefovir dipivoxil alone or in combination with lamivudine in patients with lamivudine-resistant chronic hepatitis B. *Gastroenterology* 2004; 126: 91–101.

32 Gaia S, Barbon V, Smedile A *et al.* Lamivudine-resistant chronic hepatitis B: an observational study on adefovir in monotherapy or in combination with lamivudine. *J Hepatol* 2008; 48: 540–547.

33 Fung SK, Chae HB, Fontana RJ *et al.* Virologic response and resistance to adefovir in patients with chronic hepatitis B. *J Hepatol* 2006; 44: 283–290.

34 Rapti I, Dimou E, Mitsoula P, Hadziyannis SJ. Adding-on versus switching-to adefovir therapy in lamivudine-resistant HBeAg-negative chronic hepatitis B. *Hepatology* 2007; 45: 307–313.

35 Yatsuji H, Suzuki F, Sezaki H *et al.* Low risk of adefovir resistance in lamivudine-resistant chronic hepatitis B patients treated with adefovir plus lamivudine combination therapy: two-year follow-up. *J Hepatol* 2008; 48: 923–931.

36 Gallego A, Sheldon J, Garcia-Samaniego J *et al.* Evaluation of initial virological response to adefovir and development of adefovir-resistant mutations in patients with chronic hepatitis B. *J Viral Hepat* 2008; 15: 392–398.

37 Chan HL, Wong VW, Tse CH *et al.* Early virological suppression is associated with good maintained response to adefovir dipivoxil in lamivudine resistant chronic hepatitis B. *Aliment Pharmacol Ther* 2007; 25: 891–898.

38 Zollner B, Petersen J, Puchhammer-Stockl E *et al.* Viral features of lamivudine resistant hepatitis B genotypes A and D. *Hepatology* 2004; 39: 42–50.

39 Villet S, Pichoud C, Villeneuve JP, Trepo C, Zoulim F. Selection of a multiple drug-resistant hepatitis B virus strain in a liver-transplanted patient. *Gastroenterology* 2006; 131: 1253–1261.

40 Shaw T, Bartholomeusz A, Locarnini S. HBV drug resistance: mechanisms, detection and interpretation. *J Hepatol* 2006; 44: 593–606.
41 Marcellin P, Chang TT, Lim SG *et al.* Adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. *N Engl J Med* 2003; 348: 808–816.
42 Santantonio T, Fasano M, Durantel S *et al.* Adefovir dipivoxil resistance patterns in patients with lamivudine-resistant chronic hepatitis B. *Antivir Ther* 2009; 14: 557–565.
43 Qi X, Xiong S, Yang H, Miller M, Delaney WE. In vitro susceptibility of adefovir-associated hepatitis B virus polymerase mutations to other antiviral agents. *Antivir Ther* 2007; 12: 355–362.
44 Tan J, Degertekin B, Wong SN, Husain M, Oberhelman K, Lok AS. Tenofovir monotherapy is effective in hepatitis B patients with antiviral treatment failure to adefovir in the absence of adefovir-resistant mutations. *J Hepatol* 2008; 48: 391–398.
45 van Bommel F, Zollner B, Sarrazin C *et al.* Tenofovir for patients with lamivudine-resistant hepatitis B virus (HBV) infection and high HBV DNA level during adefovir therapy. *Hepatology* 2006; 44: 318–325.
46 Choe WH, Kwon SY, Kim BK *et al.* Tenofovir plus lamivudine as rescue therapy for adefovir-resistant chronic hepatitis B in hepatitis B e antigen-positive patients with liver cirrhosis. *Liver Int* 2008; 28: 814–820.
47 Lok AS, McMahon BJ. Chronic hepatitis B. *Hepatology* 2007; 45: 507–539.

## 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 $1.25 \times 10^5$ 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 $5.0 \times 10^5$ 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.

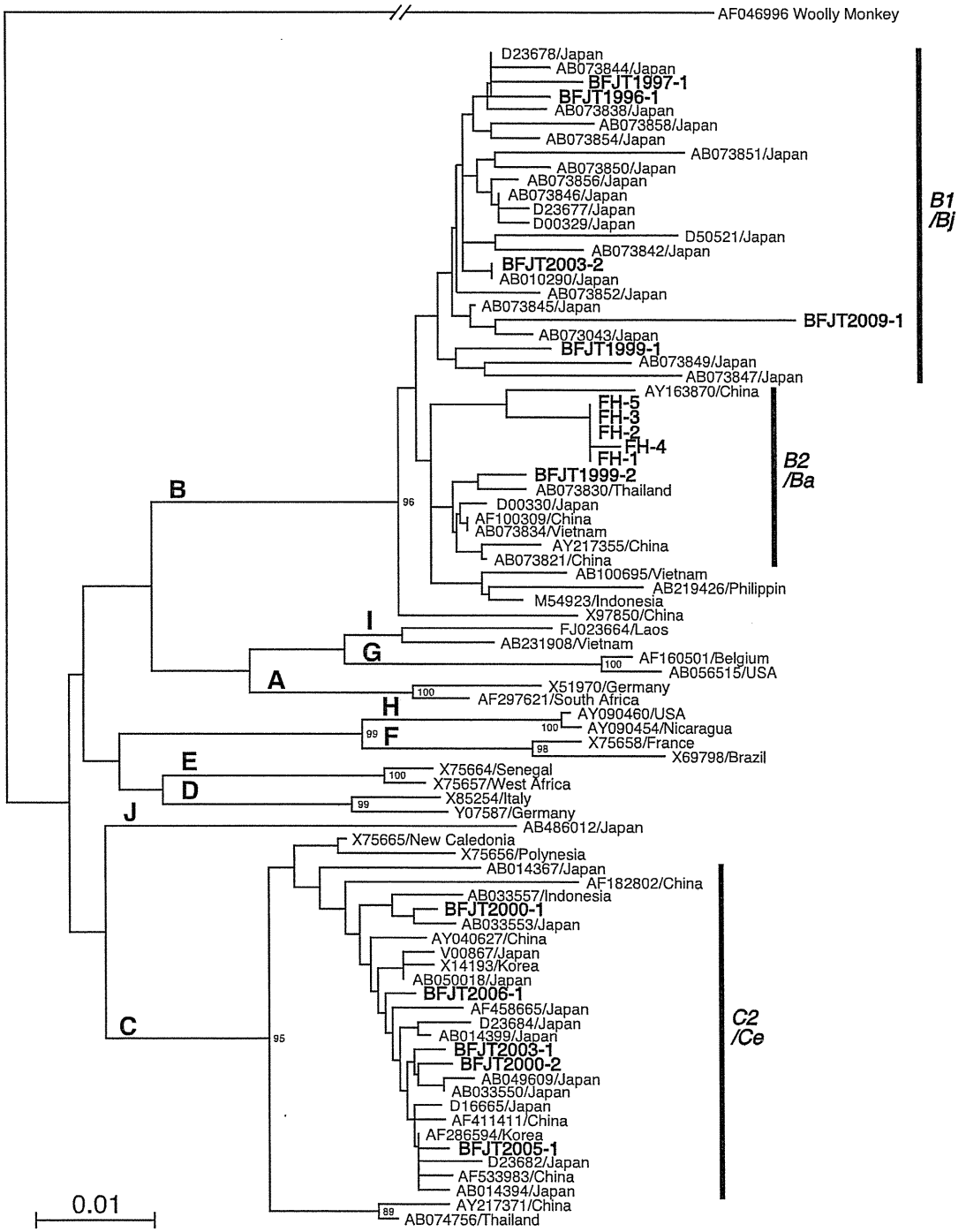



**Figure 1.** Phylogenetic tree constructed by the neighbor-joining method based on the partial 396-nucleotide sequences in the S region of 84 hepatitis B virus isolates. In addition to the 16 isolates found in this study, which are indicated in bold type for visual clarity, 68 reported isolates of genotypes 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].

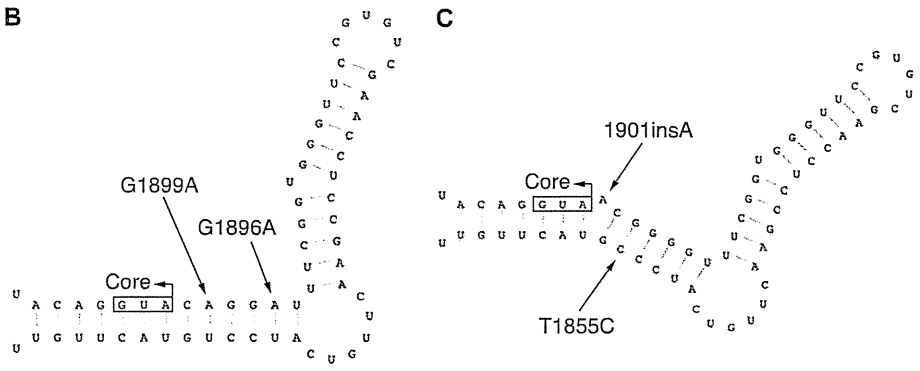
sequences of the single nucleotide insertions/deletions in this study are shown in Figure 2. The insertions in this region make a termination codon at nucleotide 1909 or 1915 in a frame of the precore protein, and the deletion makes a termination codon at nucleotide 1993. Therefore, these single nucleotide 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

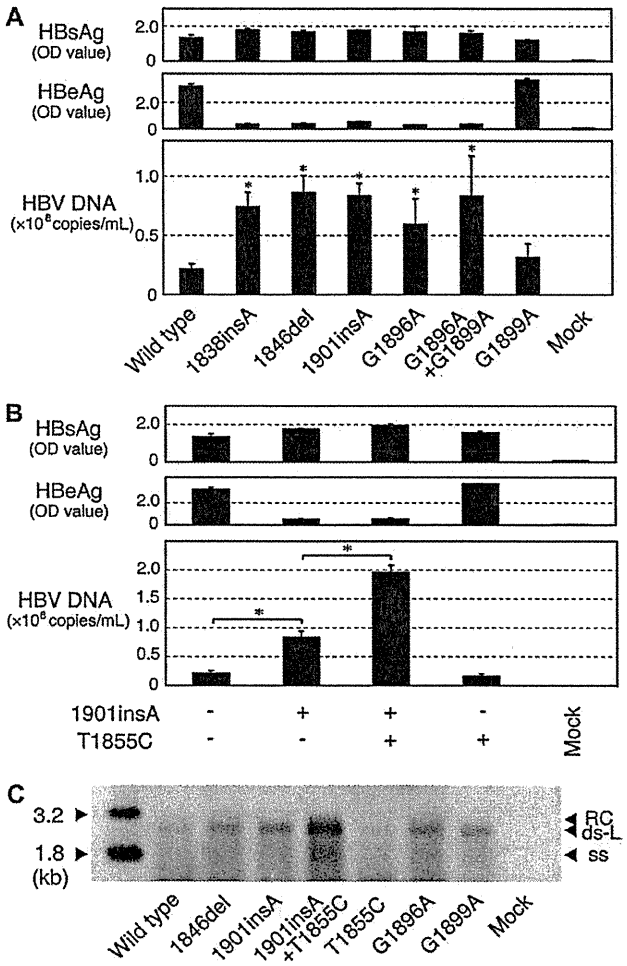



**Figure 3.** *A*, Level of hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), and hepatitis B virus (HBV) DNA in the culture supernatant of HepG2 cells that were transfected with several HBV constructs with a frameshift insertion/deletion or mutations in the precore region. *, $P < .05$ 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.

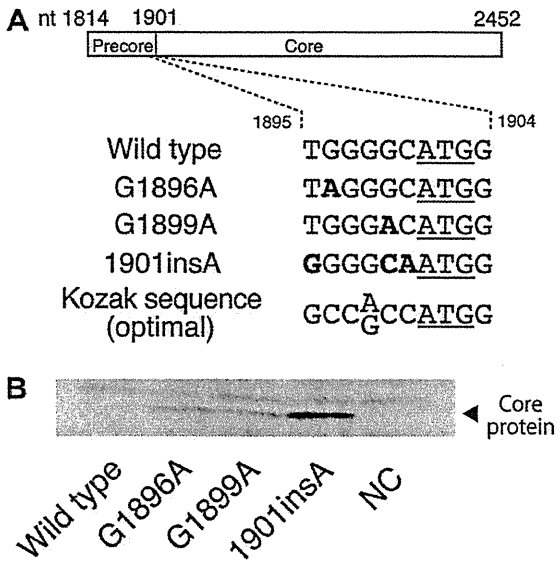



**Figure 4.** *A*, Schema of the Kozak sequence around the initiation codon of the core protein. The changed nucleotides in the Kozak sequene, which were found in hepatitis B virus (HBV) with G1896A, G1899A, or 1901insA, are shown in bold type. *B*, Results of Western blot analysis of the expressed HBV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


1. Hasegawa K, Huang J, Rogers SA, Blum HE. Liang TJ. Enhanced replication of a hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. J Virol **1994**; 68:1651–9.
2. Baumert TF, Rogers SA, Hasegawa K, Liang TJ. Two core promotor mutations identified in a hepatitis B virus strain associated with fulminant hepatitis result in enhanced viral replication. J Clin Invest **1996**; 98:2268–76.
3. Rivero M, Crespo J, Fabrega E, et al. Apoptosis mediated by the Fas system in the fulminant hepatitis by hepatitis B virus. J Viral Hepat **2002**; 9:107–13.
4. Okamoto H, Tsuda F, Sakugawa H, et al. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. J Gen Virol **1988**; 69:2575–83.
5. Norder H, Courouce AM, Coursaget P, et al. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. Intervirology **2004**; 47:289–309.
6. Olinger CM, Jutavijittum P, Hubschen JM, et al. Possible new hepatitis B virus genotype, southeast Asia. Emerging Infect Dis **2008**; 14: 1777–80.
7. Tatematsu K, Tanaka Y, Kurbanov F, et al. A genetic variant of hepatitis B virus divergent from known human and ape genotypes isolated from a Japanese patient and provisionally assigned to new genotype. J Virol **2009**; 83:10538–47.
8. Kao JH, Chen PJ, Lai MY, Chen DS. Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. Gastroenterology **2000**; 118:554–9.
9. Imamura T, Yokosuka O, Kurihara T, et al. Distribution of hepatitis B viral genotypes and mutations in the core promoter and precore regions in acute forms of liver disease in patients from Chiba, Japan. Gut **2003**; 52:1630–7.
10. Ozasa A, Tanaka Y, Orito E, et al. Influence of genotypes and precore mutations on fulminant or chronic outcome of acute hepatitis B virus infection. Hepatology **2006**; 44:326–34.
11. Kusakabe A, Tanaka Y, Mochida S, et al. Case-control study for the identification of virological factors associated with fulminant hepatitis B. Hepatol Res **2009**; 39:648–56.
12. Hou J, Lin Y, Waters J, et al. Detection and significance of a G1862T variant of hepatitis B virus in Chinese patients with fulminant hepatitis. J Gen Virol **2002**; 83:2291–8.
13. Kosaka Y, Takase K, Kojima M, et al. Fulminant hepatitis B: induction by hepatitis B virus mutants defective in the precore region and incapable of encoding e antigen. Gastroenterology **1991**; 100:1087–94.
14. Liang TJ, Hasegawa K, Rimon N, Wands JR, Ben-Porath E. A hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. N Engl J Med **1991**; 324:1705–9.
15. Omata M, Ehata T, Yokosuka O, Hosoda K, Ohto M. Mutations in the precore region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. N Engl J Med **1991**; 324:1699–704.
16. Scaglioni PP, Melegari M, Wands JR. Posttranscriptional regulation of hepatitis B virus replication by the precore protein. J Virol **1997**; 71: 345–53.
17. Fattovich G, Bortolotti F, Donato F. Natural history of chronic hepatitis B: special emphasis on disease progression and prognostic factors. J Hepatol **2008**; 48:335–52.
18. Matsuura K, Tanaka Y, Hige S, et al. Distribution of hepatitis B virus genotypes among patients with chronic infection in Japan shifting toward an increase of genotype A. J Clin Microbiol **2009**; 47:1476–83.
19. Takahashi M, Nishizawa T, Gotanda Y, et al. High prevalence of antibodies to hepatitis A and E viruses and viremia of hepatitis B, C, and D viruses among apparently healthy populations in Mongolia. Clin Diagn Lab Immunol **2004**; 11:392–8.
20. Inoue J, Ueno Y, Nagasaki F, et al. Enhanced intracellular retention of a hepatitis B virus strain associated with fulminant hepatitis. Virology **2009**; 395:202–9.
21. Liu Y, Hussain M, Wong S, Fung SK, Yim HJ, Lok AS. A genotype-independent real-time PCR assay for quantification of hepatitis B virus DNA. J Clin Microbiol **2007**; 45:553–8.
22. Inoue J, Ueno Y, Kanno N, et al. Living related liver transplantation for acute fulminant hepatitis B: experience from two possible hyper-acute cases. Tohoku J Exp Med **2005**; 205:197–204.
23. Nagasaki F, Ueno Y, Niitsuma H, et al. Analysis of the entire nucleotide sequence of hepatitis B causing consecutive cases of fatal fulminant hepatitis in Miyagi prefecture Japan. J Med Virol **2008**; 80: 967–73.
24. Sugauchi F, Orito E, Ohno T, et al. Spatial and chronological differences in hepatitis B virus genotypes from patients with acute hepatitis B in Japan. Hepatol Res **2006**; 36:107–14.
25. Santantonio T, Jung MC, Miska S, Pastore G, Pape GR, Will H. High prevalence and heterogeneity of HBV preC mutants in anti-HBe-positive carriers with chronic liver disease in southern Italy. J Hepatol **1991**; 13(Suppl 4):S78–81.
26. Okamoto H, Yotsumoto S, Akahane Y, et al. Hepatitis B viruses with precore region defects prevail in persistently infected hosts along with seroconversion to the antibody against e antigen. J Virol **1990**; 64: 1298–303.
27. Shin IT, Tanaka Y, Tateno Y, Mizokami M. Development and public release of a comprehensive hepatitis virus database. Hepatol Res **2008**; 38:234–43.
28. Lok AS, Akarca U, Greene S. Mutations in the pre-core region of hepatitis B virus serve to enhance the stability of the secondary structure of the pre-genome encapsidation signal. Proc Natl Acad Sci U S A **1994**; 91:4077–81.
29. Kozak M. At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. J Mol Biol **1987**; 196:947–50.
30. Inoue J. Factors involved in the development of fulminant hepatitis B: are the mutations of hepatitis B virus implicated? Hepatol Res **2009**; 39:1053–5.
31. Milich D, Liang TJ. Exploring the biological basis of hepatitis B e antigen in hepatitis B virus infection. Hepatology **2003**; 38: 1075–86.
32. Sainokami S, Abe K, Sato A, et al. Initial load of hepatitis B virus (HBV), its changing profile, and precore/core promoter mutations correlate with the severity and outcome of acute HBV infection. J Gastroenterol **2007**; 42:241–9.
33. Ogawa M, Hasegawa K, Naritomi T, Torii N, Hayashi N. Clinical features and viral sequences of various genotypes of hepatitis B virus compared among patients with acute hepatitis B. Hepatol Res **2002**; 23:167–77.
34. Chu CJ, Hussain M, Lok AS. Hepatitis B virus genotype B is associated with earlier HBeAg seroconversion compared with hepatitis B virus genotype C. Gastroenterology **2002**; 122:1756–62.
35. Sugauchi F, Kumada H, Sakugawa H, et al. Two subtypes of genotype B (Ba and Bj) of hepatitis B virus in Japan. Clin Infect Dis **2004**; 38: 1222–8.
36. Ryu DK, Kim S, Ryu WS. Hepatitis B virus polymerase suppresses translation of pregenomic RNA via a mechanism involving its interaction with 5' stem-loop structure. Virology **2008**; 373:112–23.
37. Ahn SH, Kramvis A, Kawai S, et al. Sequence variation upstream of precore translation initiation codon reduces hepatitis B virus e antigen production. Gastroenterology **2003**; 125:1370–8.