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Fatal exacerbation of type B chronic hepatitis triggered by changes in relaxed circular viral DNA synthesis and virion secretion

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ABSTRACT

Virological features of fulminant liver disease-causing hepatitis B virus (HBV) have not been fully elucidated. We studied longitudinally the viruses obtained before and after fulminant liver disease in a patient with chronic HBV infection showing fatal exacerbation. HBV strains were obtained before and after exacerbation (designated as FEP1 and FEP2). Their virological features were investigated by *in vitro* transfection. FEP1 and FEP2 possessed higher activity of overall HBV DNA synthesis than the wild-type. FEP1 lacked competence for relaxed circular (RC) HBV DNA synthesis and RC HBV DNA-containing virion secretion, but FEP2 maintained it. Chimeric analysis revealed that the preS/S gene, where FEP1 had a considerable number of mutations and deletions but FEP2 did not, was responsible for impaired RC HBV DNA synthesis and virion secretion. Furthermore, incompetence of FEP1 strain was transcomplemented by the preS/S protein of wild-type strain. In conclusion, the viral strain after exacerbation showed resurgent RC HBV DNA synthesis and virion secretion, which was caused by conversion of the preS/S gene from a hypermutated to hypomutated state. This may have been responsible for disease deterioration in the patient. This is a novel type of HBV genomic variation associated with the development of fulminant liver disease.

1. Introduction

Type B fulminant hepatitis develops in approximately 1% of patients with acute hepatitis B virus (HBV) infection and results in a high rate of mortality [1]. Serious disease exacerbation like fulminant hepatitis can also occur during chronic HBV infection. The virological characteristics of fulminant hepatitis-causing HBV strains have been widely studied. An A1896 mutation in the precore gene, and T1762/A1764 and V1753/V1754 mutations (V = not T) in the core promoter have been shown to be detected more frequently in fulminant hepatitis-related strains than in non-fulminant hepatitis-related ones [2–4], although these viral mutations do not completely account for the pathogenesis of fulminant hepatitis. A few investigators have conducted detailed studies on the strain-specific virological feature of an individual fulminant hepatitis-causing HBV strain in comparison with the representative wild-type HBV strain [5–8]. Baumert et al. [5,6] reported that a fulminant hepatitis-causing HBV strain with rare types of mutations in the core promoter showed a robust increase of viral encapsidation and strong induction of cellular apoptosis. Pult et al. [7] also revealed that the strain isolated from a patient with heart transplantation-associated fulminant hepatitis had the 11-bp insertion in the core promoter and revealed the elevated viral transcription via generation of a novel binding site of hepatocyte nuclear factor 1. In addition, Kalinina et al. [8] reported that the strain derived from a fulminant hepatitis patient after liver transplantation was secretion-defective due to several mutations in the surface (S) gene. According to these observations, in fulminant hepatitis-causing HBV strains, both frequent mutations and strain-specific viral genomic variations may contribute to the development of the disease. However, there have been no longitudinal virological studies of HBV strains obtained before and after the onset of fulminant liver disease in chronic HBV carriers showing serious disease exacerbation such as fulminant hepatitis. Such investigations may lead to better understanding of the role of viral genomic changes on the pathogenesis of HBV-related fulminant liver disease.

HBV is a double-stranded circular DNA virus approximately 3.2 kb long and has four open reading frames, preS/S, precore/core,

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polymerase and X genes. HBV replicates its genome via reverse transcription of pregenome RNA. Reverse transcription conducted by viral polymerase takes place after encapsidation of pregenome RNA, resulting in minus-strand DNA synthesis. The RNA template is simultaneously degraded by RNaseH activity, and single-stranded (SS) HBV DNA is constructed. Subsequently, viral polymerase mediates production of plus-strand DNA from minus-strand DNA. In this process, appropriate template switches for primer translocation and circularization lead to the formation of relaxed circular (RC) HBV DNA, whereas the extension *in situ* without template switches results in the formation of duplex linear (DL) HBV DNA [9–11]. The virion containing RC viral DNA has been shown to be more infectious than those containing DL viral DNA in duck hepatitis B virus (DHBV) [12].

In this study, we examined an elderly patient with chronic HBV infection showing fatal exacerbation accompanied by an increase in the viral replicative level. The viruses obtained before and after exacerbation were longitudinally studied. The virus before exacerbation lacked competence for RC HBV DNA synthesis and virion secretion, but the virus after exacerbation had recovered it. Resurgent RC HBV DNA synthesis and virion secretion as the prime causes of disease deterioration originated in conversion of the preS/S gene from a hypermutated to a hypomutated state. This is a novel type of the HBV genomic variation associated with the development of HBV-related fulminant liver disease.

2. Materials and methods

2.1. Case presentation

The patient described in this study was a 83-year-old Japanese male with type B chronic hepatitis. At the initial phase, he was free of symptoms. His alanine aminotransferase (ALT) fluctuated over a range of 50–160 IU/l. Hepatitis B surface antigen (HBsAg) was weakly positive (2^5 according to reversed passive hemagglutination assay), and HBV DNA was below the detection limit based on the slot–blot hybridization assay on day 29. However, after 1 year, he suddenly became fatigued and anorexic. ALT rose to 729 IU/l accompanied by an increase of HBsAg titer (>2^{12}) and the detectable level of HBV DNA on day 374. He was hospitalized due to diagnosis of acute exacerbation of chronic HBV infection. After hospitalization, ALT declined to 150–250 IU/l, but HBV DNA continued to be detectable. The total bilirubin reached up to 5.0 mg/dl on day 418 and increased thereafter. On day 508, he died of hepatic failure despite intensive treatment. Liver histology at autopsy showed features of massive liver necrosis superimposed on chronic non-cirrhotic liver disease. During follow-up, he tested negative for both hepatitis B e antigen (HBeAg) and antibody to HBeAg by radioimmunoassay. Antibody to HBsAg was also negative according to passive hemagglutination assay. The clinical course of the patient is summarized in Fig. 1. Informed consent was obtained from his family members. Serum samples collected on day 29 (1 year before exacerbation, designated as P1) and day 407 (after exacerbation, designated as P2) were stored at −80 °C and used for this study. HBV DNA levels as measured by the PCR-based assay (Amplicor HB Monitor, Roche Diagnostics) using stored sera at P1 and P2 were $10^{5.4}$ and $10^{8.6}$ copies/ml.

2.2. PCR, sequencing and subcloning

To obtain the full-length HBV DNA strains before and after exacerbation, PCR reaction was carried out using stored sera at P1 and P2. After DNA extraction, the DNA was amplified for 35 cycles using Taq/Pwo DNA polymerase (Roche Diagnostics) according to the method described by Günther et al. [13]. The primers were BF1 (5′-CCGGAAAGCTTGAGCTCTTCTTTTTCACCTCTGCCTAATCA-3′, nt 1821–1841) and BR1 (5′-CCGGAAAGCTTGAGCTCTTCAAAAAG TTGCATGGTGCTGG-3′, nt 1825–1806), both of which had a SapI recognition site at the 5′ end. After brief incubation with Taq polymerase to create A overhang, the PCR product was cloned into the plasmid pCR-TOPO4 (Invitrogen). To avoid misreading in the PCR reaction, nucleotide sequences of six independent full-length HBV DNA clones were determined. Nucleotides that were detected in only one clone at the corresponding nucleotide position were excluded. If necessary, the interchange in the portion of HBV DNA using plural number of clones, or site-directed mutagenesis,

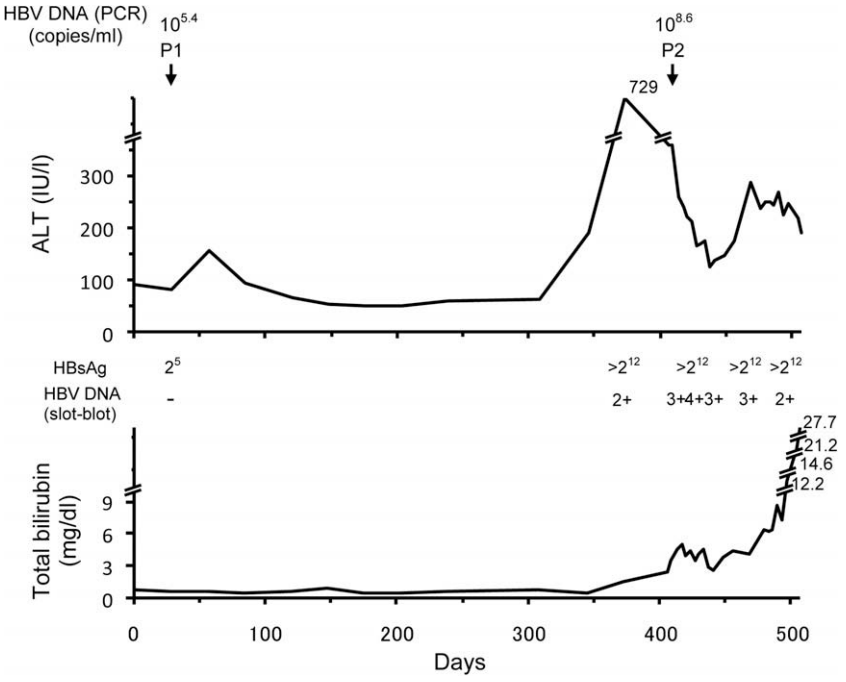

Fig. 1. Clinical course of a patient with type B chronic hepatitis showing fatal exacerbation.

was conducted. Finally, the HBV strains, FEP1 and FEP2 (GenBank Accession Nos. AB485809 and AB485810), were obtained as predominant viruses at P1 and P2. These two HBV strains were subjected to phylogenetic tree analysis along with representative HBV strains of various genotypes.

2.3. Plasmid constructs

Plasmid pHBC had 1.2 times the genomic length of HBV DNA and expressed the wild-type genotype C HBV strain adr4 (GenBank Accession No. X01587) [14]. As for the HBV strains FEP1 and FEP2, the full-length HBV DNA fragment was removed from the pCR-TOPO4 by SapI digestion, followed by synthesis of circular double-stranded HBV DNA by T4 ligase treatment. Based on this, the HBV-expressing plasmids pFEP1 and pFEP2, which carried the 1.2-fold HBV genome, were constructed. pFEch1 and pFEch2, made by swapping the BstEII/BstBI fragment between pFEP1 and pFEP2, expressed the chimeric HBV strains FEch1 and FEch2. For trans-complementation analysis, another plasmid pHBV1.5, which expressed wild-type genotype A HBV strain adw2 (GenBank Accession No. X02763) [15], was used. pHBV1.5Δpol and pHBV1.5ΔS lacked production of the polymerase protein and all surface proteins, respectively, due to insertion of the in-frame stop codon. pCMV-SEAP expressed a secreted alkaline phosphatase.

2.4. Examination for mixed viral population

To examine the mixed viral population in the preS/S gene, PCR-subcloning analysis was performed. The DNA fragment encompassing the whole preS2/S gene was amplified by PCR using the primers BF5 (5′-AAGAGACAGTCATCCTCAGG-3′ nt 3183–3202) and BR7 (5′-GGGTTCAAATGTATACCCAA-3′, nt 839–820). The PCR

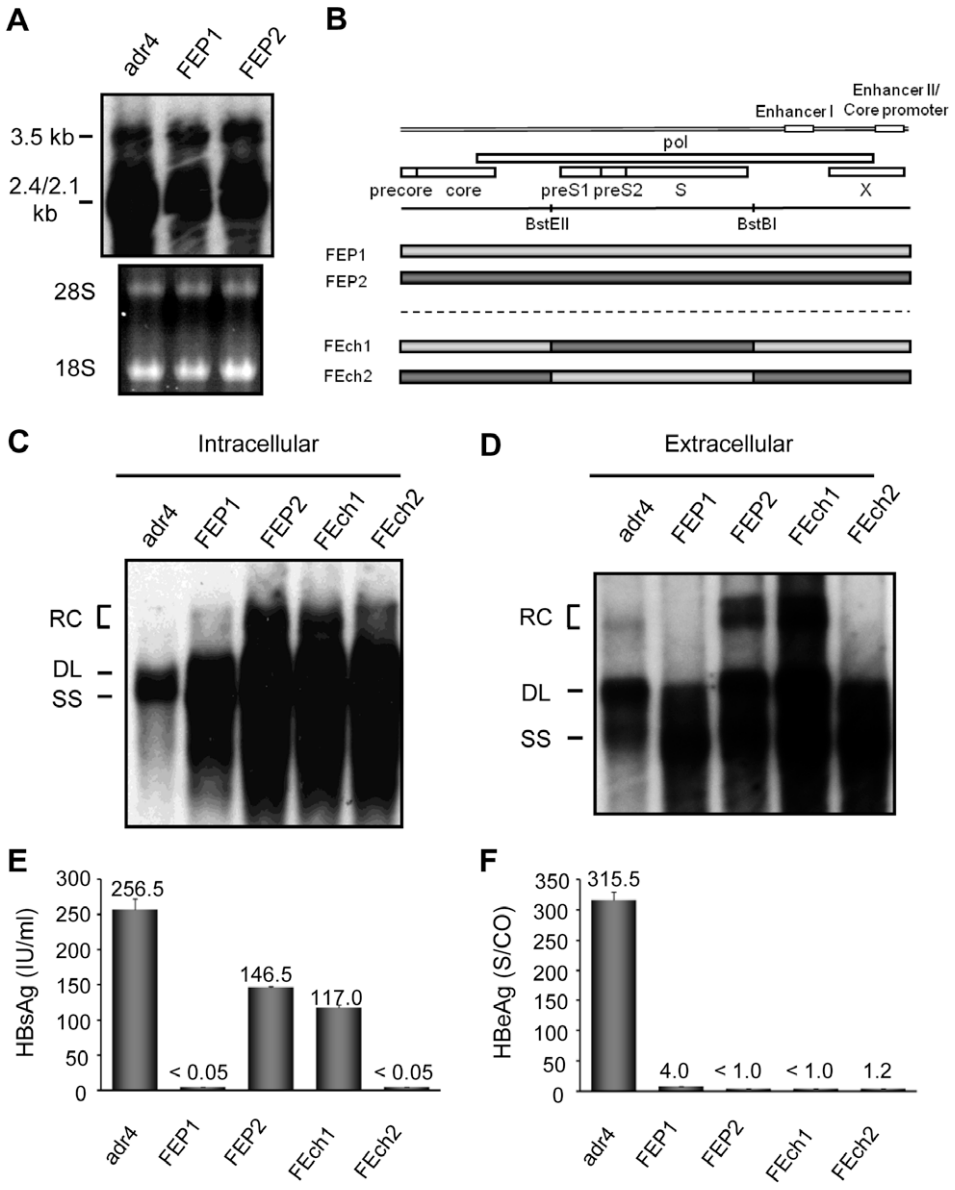

Fig. 2. Viral transcription, replication, virion secretion and antigen production of FEP1 and FEP2, and their chimeric constructs in transfected cells. (A) HBV transcripts in transfected cells examined by Northern blot analysis. The lower panel indicates ethidium bromide staining as a loading control. (B) A scheme of chimeric HBV strains, FEch1 and FEch2. (C) Intracellular progeny HBV DNA in transfected cells examined by Southern blot analysis. (D) Extracellular progeny HBV DNA in transfected cells examined by Southern blot analysis. (E) Secreted HBsAg in the culture supernatant of transfected cells. Data were expressed as IU/ml. (F) Secreted HBeAg in the culture supernatant of transfected cells. Data were expressed as the ratio of optical density of the sample to the cut-off value (S/CO). SS, single-stranded HBV DNA; DL, duplex linear HBV DNA; RC, relaxed circular HBV DNA.

product was cloned into the plasmid pCR-TOPO4, and inserted HBV DNA sequences of independent clones were determined. As another examination of the mixed viral population, PCR was done to obtain the short HBV DNA fragment including the preS2 deletion site using the primers BF-11 (5′-AAGAGACAGTCATCCTCAGG-3′, nt 3183–3202) and BR-11 (5′-AACTGGAGCCACCAGCAGGA-3′, nt 74–55). Next, the product was electrophoresed on polyacrylamide gel. In this assay, the various ratios of the mixture of plasmids pFEP1 and pFEP2 were used as templates of the PCR reaction. Consequently, a minor population of the virus could be detected if it was present at approximately one-tenth of the total population.

2.5. Transfection study

Huh7 cells (7×10^5 cells) were seeded on a 60-mm-diameter culture dish and transfected with 2 μg of various HBV-expressing plasmids and 0.1 μg of pCMV-SEAP using the FuGENE6 reagent (Roche Diagnostics). In transcomplementation analysis, 1 μg of pFEP1 was cotransfected with 1 μg of pHBV1.5Δpol, pHBV1.5ΔS or pBluescriptIISK$^+$ (mock). The cellular nucleic acid and culture supernatant were collected on day 5 after transfection. The alkaline phosphatase activity in the culture supernatant was measured to evaluate transfection efficiency.

For Northern blot analysis to detect HBV transcripts, the total RNA was extracted using an TRIzol reagent (Invitrogen), followed by RNase-free DNaseI treatment, phenol/chloroform extraction and ethanol precipitation. The sample was then electrophoresed in a formaldehyde–agarose denaturing gel, transferred onto a nylon membrane, hybridized with alkaline phosphatase-labeled HBV DNA probe and detected with the chemiluminescent substrate CDP-star (GE Healthcare Life Sciences). Southern blot analyses to detect intracellular and extracellular progeny HBV DNAs were carried out as described elsewhere [16]. Secreted HBsAg and HBeAg in the culture supernatant were measured by chemiluminescent immunoassay. All transfection experiments were done at least three times, and the representative results are shown.

3. Results

3.1. Differences in viral sequences between FEP1 and FEP2 strains were most prominent in the preS/S gene

The HBV strains FEP1 and FEP2, obtained before and after fatal exacerbation of the patient with chronic HBV infection, were classified as genotype C2, the most prevalent genotype in Japan, according to the phylogenetic tree analysis (data not shown). FEP1 and FEP2 had sequence divergences of 3.1% and 2.8% from the representative wild-type genotype C2 HBV strain adr4 [14] and differed by 0.8% from each other. Amino acid substitutions in the preS/S, precore/core, X and polymerase gene in adr4, FEP1 and FEP2 strains are shown in the Supplementary Tables 1 and 2. FEP1 had 20 amino acid substitutions in the preS/S gene, 6 in the precore/core gene, 5 in the X gene and 34 in the polymerase gene, whereas FEP2 had 10 in the preS/S gene, 7 in the precore/core gene, 5 in the X gene and 30 in the polymerase gene compared with adr4. As for comparison between FEP1 and FEP2, substitutions were noted at 15 amino acid residues in the preS/S gene, one in the precore/core gene, none in the X gene and 14 in the polymerase gene. In addition, FEP1, but not FEP2, had a 12-bp deletion in the preS2 gene, a 9-bp deletion in the S gene, and the in-frame stop codon in the distal S gene, which caused truncation of preS/S and polymerase proteins. As for other peculiarities of the FEP1 and FEP2 strains, both had the A1896, T1762/A1764 and C1753 mutations, which have been shown to be frequently detected in fulminant hepatitis [2–4], and disruption of the start codon of the preS2 gene, which has been reported to be commonly found in chronic HBV infection [17]. Thus, FEP1 and FEP2 strains differed considerably in nucleotide sequences from the wild-type adr4 strain. Further-

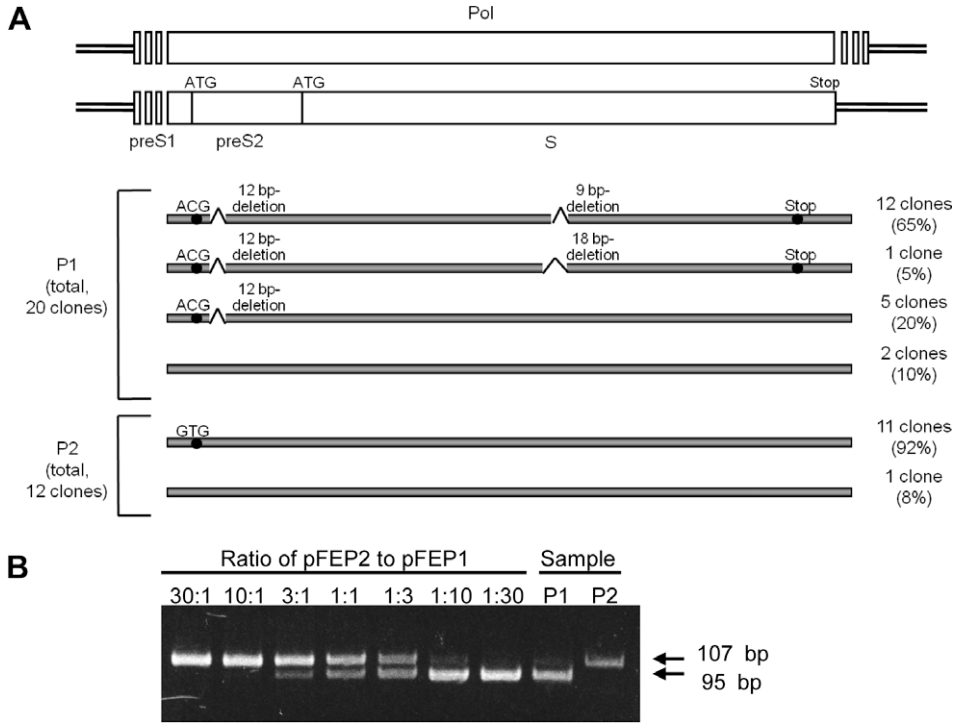

Fig. 3. Mixed viral population in the preS/S gene in a patient with type B chronic hepatitis showing fatal exacerbation. (A) A scheme of the result of PCR-subcloning analysis. The HBV fragment encompassing the whole preS2/S gene was amplified by PCR and subcloned. Next, the independent clones were used for sequencing analysis. A total of 20 and 12 independent clones derived from serum samples P1 and P2 were examined. (B) A representative result of PCR amplification of short HBV DNA fragment including the preS2 deletion site. The product derived from HBV DNA without the deletion was 109 bp long, whereas that derived from HBV DNA with the deletion was 95 bp long.

more, the differences between FEP1 and FEP2 in the sequences were the most prominent in the preS/S gene.

3.2. FEP1 lacked competence for RC HBV DNA synthesis and RC HBV DNA-containing virion secretion but FEP2 possessed it

Next, we investigated viral transcription, DNA synthesis, virion secretion and antigen production in the wild-type adr4 strain, and the patient-derived FEP1 and FEP2 strains using *in vitro* transfection analysis. The levels of HBV transcripts did not differ among the three strains (Fig. 2A). When the levels of intracellular and extracellular progeny HBV DNAs were compared (Fig. 2C and D), both FEP1 and FEP2 revealed more synthetic activity of HBV DNA than adr4 in the intracellular HBV DNA assay, but the differences in the levels of extracellular HBV DNA among adr4, FEP1 and FEP2 were modest. According to these findings, FEP1 and FEP2 strains may possess increased activity of viral encapsidation and/or minus-strand DNA synthesis compared with adr4, whereas wild-type adr4 strain may be superior to FEP1 and FEP2 in the efficient virion secretion. When the differences between FEP1 and FEP2 were examined with respect to the intracellular and extracellular HBV DNA assays, RC HBV DNA synthesis and RC HBV DNA-containing virion secretion were seriously impaired in FEP1, compared with FEP2 (Fig. 2C and D). Regarding the levels of secreted HBsAg and HBeAg, HBsAg was not detected in FEP1 but detectable in adr4 and FEP2 (Fig. 2E). Both FEP1 and FEP2 could not synthesize HBeAg (Fig. 2F), because they harbored the precore-defective A1896 mutation.

3.3. Inability of RC HBV DNA synthesis and of RC HBV DNA-containing virion secretion in FEP1 were responsible for the preS/S gene

The most remarkable difference in the viral genome between FEP1 and FEP2 was observed in the preS/S gene. Therefore, chimeric HBV strains, FEch1 and FEch2, constructed by swapping the entire preS/S region between FEP1 and FEP2 (Fig. 2B), were examined. As shown in Fig. 2C and D, RC HBV DNA synthesis and RC HBV DNA-containing virion secretion were seen in FEch1 but had been prevented in FEch2. Also, HBsAg was detected in FEch1 but not in FEch2 (Fig. 2E). Both FEch1 and FEch2 did not produce HBeAg (Fig. 2F). Thus, incompetence of RC HBV DNA synthesis and of virion secretion in FEP1 were responsible for the preS/S gene.

3.4. Wild-type-like HBV strain coexisted with FEP1 strain

We further examined the detailed viral population at P1 and P2. PCR-subcloning assay was done for the preS2/S gene, which showed the regions with the most differences in sequences between FEP1 and FEP2 (Fig. 3A). Thirteen of 20 clones derived from P1 had the two short deletions in the preS2 and S genes, the in-frame stop codon in the distal S gene and disruption of the preS2 start codon, as was the case for FEP1 strain. Five clones possessed only the deletion in the preS2 gene and disruption of the preS2 start codon. The remaining two clones had none of these mutations and deletions and were similar to the wild-type strain. As for clones derived from P2, 11 of the 12 clones showed disruption of the preS2 start codon, whereas the remaining one clone did not. In PCR analysis for the short region containing the preS2 deletion site (Fig. 3B), the virus with preS2 deletion was predominant, but approximately one-tenth of the virus without deletion was also detected at P1. Only the virus without the deletion was seen at P2. According to these observations, the wild-type-like HBV strain with minimal viral genomic variations in the preS/S gene coexisted as a minor population at P1.

3.5. RC HBV DNA synthesis and virion secretion were transcomplemented by the preS/S protein

Finally, we investigated whether the wild-type-like virus coexisting as a minor population can complement the inability of RC HBV DNA synthesis and virion secretion in FEP1 strain *in trans*. As shown in Fig. 4A, transfection with pHBV1.5 yielded synthesis of RC, DL and SS HBV DNAs, whereas transfection with pHBV1.5ΔS resulted in less amounts of RC HBV DNA than that with pHBV1.5. Transfection with pHBV1.5Δpol showed complete absence of HBV DNA synthesis. As for transcomplementation analysis (Fig. 4B and C), cotransfection of pHBV1.5Δpol with pFEP1 did not augment RC HBV DNA synthesis in the intracellular HBV DNA assay, but led to enhanced secretion of RC HBV DNA-containing virion in the extracellular HBV DNA assay. By contrast, cotransfec-

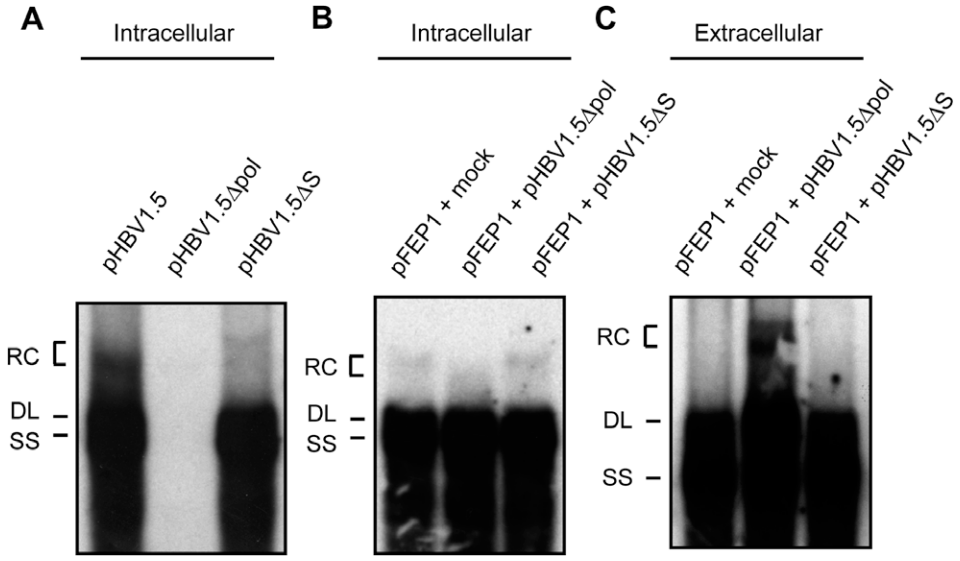

Fig. 4. Transcomplementation of insufficient viral secretion in FEP1 strain by HBV-expressing constructs, pHBV1.5Δpol and pHBV1.5ΔS. (A) Intracellular progeny HBV DNA in cells transfected with pHBV1.5, pHBV1.5Δpol and pHBV1.5ΔS by Southern blot analysis. (B) Intracellular progeny HBV DNA in cells cotransfected of pFEP1 with mock, pHBV1.5Δpol and pHBV1.5ΔS by Southern blot analysis. (C) Extracellular progeny HBV DNA in cells cotransfected of pFEP1 with mock, pHBV1.5Δpol and pHBV1.5ΔS by Southern blot analysis. SS, single-stranded HBV DNA; DL, duplex linear HBV DNA; RC, relaxed circular HBV DNA.

tion of pHBV1.5ΔS with pFEP1 could not compensate for the inability of RC HBV DNA synthesis and virion secretion. These results suggest that insufficiency of RC HBV DNA synthesis and virion secretion in FEP1 may be transcomplemented not by the polymerase protein but by the preS/S protein of the wild-type-like HBV strain.

4. Discussion

The present study describes a patient with type B chronic hepatitis who showed fatal exacerbation accompanied by more than 10^3-fold increment of viral replicative activity. The predominant HBV strains FEP1 and FEP2, obtained before and after exacerbation, were investigated to clarify what viral genomic alterations triggered disease deterioration. FEP1 and FEP2 possessed considerably different nucleotide sequences including A1896, T1762/A1764 and C1753 mutations, that have been reported to be frequently detected in the fulminant hepatitis-related HBV strain, compared with the wild-type strain [2–4]. Not only FEP2 and but also FEP1 revealed more overall synthetic activity of HBV DNA than the wild-type strain. Thus, both FEP1 and FEP2 were potent highly-replicative strains. As for the most significant difference between FEP1 and FEP2, FEP1 lacked competence for RC HBV DNA synthesis and RC HBV DNA-containing virion secretion, whereas FEP2 maintained it. Because of the difference in the virological feature between them, FEP1 was not very pathogenic, but FEP2 became a highly virulent strain. These results indicate that resurgent RC HBV DNA synthesis and virion secretion may lead to the onset of fulminant liver disease in this patient.

Chimeric analysis revealed that the preS/S gene, where FEP1 had a considerable number of mutations and deletions but FEP2 did not, accounted for the difference in the ability of RC HBV DNA synthesis and virion secretion between FEP1 and FEP2. In addition, insufficient RC HBV DNA synthesis and virion secretion in FEP1 were transcomplemented not by the polymerase protein but by the preS/S protein of the wild-type virus. Although mutations and deletions in the preS/S gene observed in FEP1 certainly affect the properties of both preS/S and polymerase proteins, disability of the preS/S protein rather than the polymerase protein may be responsible for the inability of RC HBV DNA synthesis and virion secretion in FEP1. By contrast, FEP2 may produce the competent preS/S protein to accomplish RC HBV DNA synthesis and virion secretion because of fewer mutations in the preS/S gene. Taken together, conversion from a hypermutated to a hypomutated state in the preS/S gene may have been the strain-specific viral genomic change serving as the primitive cause of lethal disease deterioration in our patient.

Examination for viral population showed that the wild-type-like strain carrying no mutations in the preS/S gene coexisted as a minor population with the predominant strain epitomized by FEP1 before exacerbation. Transcomplementation analysis also revealed that the wild-type-like HBV strain may function as a helper virus that compensates for impaired synthesis of RC HBV DNA and virion secretion in FEP1 strain. This may be a reason why the patient already had a certain degree of viral replication and chronic liver inflammation before exacerbation.

A few investigators reported secretion-defective HBV strains with the mutations in the S gene derived from patients with fulminant hepatitis and those with chronic HBV infection [8,18]. Regarding the former strain, the secretion-defective strain was isolated after the onset of fulminant hepatitis, suggesting the pathogenic importance of the deficiency in the viral antigen secretion itself [8]. This does not agree with our findings because the virus lacking the ability of RC HBV DNA-containing virion secretion was not very pathogenic, but the virus having it triggered fulminant liver disease in our study. Further work should be done to clarify the involvement of the secretion-defective HBV in various clinical manifestations.

In the process of plus-strand DNA synthesis, the presence or absence of template switches for primer translocation and circularization determines the preference for RC HBV DNA synthesis. Appropriate template switches and subsequent RC HBV DNA synthesis require donor and acceptor sites for template switches and several other *cis*-acting sequences [10,11]. FEP1 is, to our knowledge, the first naturally-occurring HBV strain displaying incompetence of RC HBV DNA synthesis and virion secretion. Such incompetence in FEP1 was compensated not by the polymerase protein but by the preS/S protein of the wild-type HBV. This lets us hypothesize that the preS/S protein may play a pivotal role in the secretion of RC HBV DNA-containing virus and possibly, the synthesis of RC HBV DNA. The preS/S protein, as well as the polymerase protein and *cis*-acting sequences within the viral genome, may be an important component for efficient RC HBV DNA formation. The hypothesis is also supported by our other finding that the synthetic activity of RC HBV DNA was lower in cells transfected with pHBV1.5ΔS than in those transfected with pHBV1.5.

In summary, we identified a novel type of the viral genomic variation associated with the development of fulminant liver disease in the longitudinal virological study of a type B chronic hepatitis patient showing fatal exacerbation. The virus before exacerbation revealed insufficiency of RC HBV DNA synthesis and virion secretion, but the virus after exacerbation had the ability for both. The change in the virological character was based on conversion from a hypermutated to a hypomutated status in the preS/S gene, which may be the main cause for disease deterioration in the patient. Our findings offer a new insight into the pathogenesis of HBV-related fulminant liver disease.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.02.114.

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