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Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Krausslich HG, Mizokami M, Bartenschlager R, Liang TJ.

Production of infectious hepatitis C virus in tissue culture from a cloned viral genome.

Nat Med. 2005 Jul;11(7):791-6. Epub 2005 Jun 12. Erratum in: Nat Med. 2005 Aug;11(8):905.

Hepatitis C virus (HCV) infection causes chronic liver diseases and is a global public health problem. Detailed analyses of HCV have been hampered by the lack of viral culture systems. Subgenomic replicons of the JFH1 genotype 2a strain cloned from an individual with fulminant hepatitis replicate efficiently in cell culture. Here we show that the JFH1 genome replicates efficiently and supports secretion of viral particles after transfection into a human hepatoma cell line (Huh7). Particles have a density of about 1.15-1.17 g/ml and a spherical morphology with an average diameter of about 55 nm. Secreted virus is infectious for Huh7 cells and infectivity can be neutralized by CD81-specific antibodies and by immunoglobulins from chronically infected individuals. The cell culture-generated HCV is infectious for chimpanzee. This system provides a powerful tool for studying the viral life cycle and developing antiviral strategies.

Watashi K, Ishii N, Hijikata M, Inoue D, Murata T, Miyanari Y, Shimotohno K.

Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase.

Mol Cell. 2005 Jul 1;19(1):111-22.

Viruses depend on host-derived factors for their efficient genome replication. Here, we demonstrate that a cellular peptidyl-prolyl cis-trans isomerase (PPIase), cyclophilin B (CyPB), is critical for the efficient replication of the hepatitis C virus (HCV) genome. CyPB interacted with the HCV RNA polymerase NS5B to directly stimulate its RNA binding activity. Both the RNA interference (RNAi)-mediated reduction of endogenous CyPB expression and the induced loss of NS5B binding to CyPB decreased the levels of HCV replication. Thus, CyPB functions as a stimulatory regulator of NS5B in HCV replication machinery. This regulation mechanism for viral replication identifies CyPB as a target for antiviral therapeutic strategies.

Zhang J, Yamada O, Sakamoto T, Yoshida H, Araki H, Shimotohno K.

Exploiting cis-acting replication elements to direct hepatitis C virus-dependent transgene expression.

J Virol. 2005 May;79(10):5923-32.

We describe here a novel targeting gene therapy strategy to direct gene expression responsive to hepatitis C virus (HCV). The goal was approached by engineering a construct containing the antisense sequence of the transgene and internal ribosome entry site of encephalomyocarditis virus flanked by 5'- and 3'-end sequences of HCV cDNA that contain cis-acting replication elements. Thus, expression of the transgene is only promoted when the minus-strand RNA has been synthesized by the functional replication machinery present in infected cells. Reporter assay and strand-specific reverse transcription-PCR showed selective transgene expression in Huh-7 cells harboring an autonomously replicating HCV subgenome but remaining silent in uninfected cells. Furthermore, using the cytosine deaminase suicide gene as a transgene coupled with recombinant adenovirus delivery, we demonstrated that cytosine deaminase was specifically expressed in replicon cells, resulting in marked chemosensitization of replicon cells to the cytotoxic effects of flucytosine. This new targeting strategy could be extended to other single-stranded RNA viruses encoding the unique RNA-dependent RNA polymerase that has no parallel in mammalian cells.

HBV

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Novel Type of Hepatitis B Virus Mutation: Replacement Mutation Involving a Hepatocyte Nuclear Factor 1 Binding Site Tandem Repeat in Chronic Hepatitis B Virus Genotype E

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The genetic diversity of hepatitis B virus (HBV) strains has evolved through mutations such as point mutations, deletions or insertions, and recombination. We identified and characterized a novel type of mutation which is a complex of external insertion, deletion, and internal duplication in sequences from one of six patients with chronic hepatitis B virus genotype E (HBV/E). We provisionally named this mutation a "replacement mutation"; the core promoter upstream regulatory sequence/basic core promoter was replaced with a part of the S1 promoter covering the hepatocyte nuclear factor 1 (HNF1) binding site, followed by a tandem repeat of the HNF1 site. A longitudinal analysis of the HBV population over 6 years showed the clonal change from wild-type HBV/E to replacement-mutant type, resulting in a lower hepatitis B (HB) e antigen titer, a high HBV DNA level in serum, and progression of liver fibrosis. In an in vitro study using a replication model, the replacement-mutant HBV showed higher replication levels than the wild-type HBV/E replicon, probably mediated by altered transcription factor binding. Additionally, this HNF1 site replacement mutation was associated with excessive HB nucleocapsid protein expression in hepatocytes, in both in vivo and in vitro studies. This novel mutation may be specific to HBV genotype E, and its prevalence requires further investigation.

Viral genetic diversification occurs, in general, through mutation, recombination, and reassortment (44). Since reassortment does not occur in the hepatitis B virus (HBV) genome, HBV strains diversify through mutations and recombinations. The viral mutations are divided into point mutations, deletions, and insertions (8). Furthermore, the insertions in the HBV genome can be of two types. The first one is the insertion of a nucleotide sequence which does not exist close to the affected sequence; examples include the 36-bp insertion observed in HBV genotype G (4, 15, 34) and the insertion of the hepatocyte nuclear factor 1 (HNF1) binding site (10, 19, 20, 30). The second type is the insertion of a nucleotide sequence located close to the inserted portion, which is termed internal duplication or tandem repeat (10). The traditional concept is that these mutations occur independently.

HBV genotypes are determined by nucleotide differences of more than 8% (25). Each genotype has its distinct geographical distribution. The accumulating evidence suggests that there are correlations between different HBV genotypes and specific viral mutations. For example, HBV subgenotype Aa (HBV/Aa), which is distributed in Asia and Africa, has a subgenotype-specific mutation just prior to the precore open reading frame (ORF) start which was shown to reduce HBV e antigen (HBeAg) expression (2, 35). HBV genotypes B, C, and D are prone to develop precore stop codons at position 1896, based on the nucleotide base-pairing of the stem-loop structure (16). Genotype A has a genotype-specific insertion of six nucleotides in the core gene. HBV genotype E (HBV/E) is restricted to West Africa, and its virological and clinical characteristics are not well defined.

The HNF1 binding site is a necessary part of the S1 promoter for maximal transcriptional activity (32). Cases with an HNF1 site insertion in the basic core promoter (BCP) have been reported in patients with various clinical statuses (10, 19, 20, 30). An HNF1 site insertion in the BCP elevates core/pregenomic mRNA transcription activity and excessive HBcAg deposition in the nuclei and cytoplasm of infected hepatocytes (30). The well-known double mutation in the core promoter, G1762T/G1764A (24), is thought to create an HNF1 site in the BCP and enhanced pregenomic mRNA transcription in an in vitro study (21). These studies have shown that the presence of an HNF1 site in the BCP affects pregenomic RNA transcription.

In the present study we discovered and describe the characteristics of a novel type of HBV genome rearrangement, which is a combination of external insertion, deletion, and internal duplication of a single sequence in a patient with chronic hepatitis due to HBV/E. This novel mutation was provisionally given the name "replacement mutation," and its uniqueness is that a part of the S1 promoter covering the HNF1 site is tandemly repeated in the core promoter upstream regulatory

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TABLE 1. Characteristics of six patients with HBV/E in the United Kingdom

Patient	Age (yr)	Sex	Ethnicity and country of origin	HBeAg	ALT (IU/liter)	Liver histology
1	44	Male	African, Nigeria	Negative	135	Grade 6, stage 3
2	35	Male	African, Nigeria	Positive	429	Grade 6, stage 2
3	34	Female	African, Nigeria	Positive	170	Grade 4, stage 1
4	35	Male	African, Ghana	Positive	81	Grade 4, stage 3
5	52	Male	African, Nigeria	Negative	111	Grade 4, stage 3–4
6	45	Male	African, Nigeria	Negative	74	Grade 5, stage 3

sequence (CURS)/BCP. Additionally, functional analyses of this mutation are conducted in vivo and in vitro.

MATERIALS AND METHODS

Patients. Six patients with chronic hepatitis B, all infected with HBV genotype E, were included in this study (Table 1). The presence of HBV/E was determined by the HBV genotyping assay (Innogenetics) and further confirmed by sequencing and a phylogenetic analysis. All six patients were originally from West Africa but were all residing in the United Kingdom at the time of the study. The longitudinal data of one patient (patient 2) were investigated later.

Serological testing. Levels of HBeAg and the antibody to HBeAg (anti-HBe) were determined semiquantitatively using a commercially available chemiluminescent-enzyme immunoassay (Lumipulse *f*; Fujirebio Inc., Tokyo, Japan).

RTD-PCR. Serum HBV DNA was quantitatively detected by real-time detection PCR (RTD-PCR) based on TaqMan chemistry as reported previously (1), with some modification (9). The lower limit of detection for this system was as little as 5 DNA copies/assay, and the linear standard curve was obtained from 5 to 10^6 DNA copies/assay.

PCR amplification and sequencing of HBV. The serum samples were stored at −80°C until use. Total DNA was extracted from 100 μl of serum using microspin columns (QIAamp blood kit; QIAGEN K.K., Tokyo, Japan). Purified DNA was resuspended in 80 μl of distilled water. PCR was carried out as described previously (36). The digest was run by electrophoresis on 3% or 5% (wt/vol) agarose, stained with ethidium bromide, and observed in UV light. The nucleotide sequences of the amplicons were determined directly by the dideoxy method, using the ABI Prism BigDye Terminator cycle sequencing ready reaction kit with a fluorescent model 3100 DNA sequencer (Applied Biosystems, Foster City, CA).

Molecular cloning for sequencing. To detect the CURS/BCP sequence changes in patient 2, sequences including the CURS/BCP sequence were determined in serial samples. Six samples from October 1999 to 2002 could be sequenced directly from PCR products; however, the samples from 1997 and 1998 could not. The amplified products were cloned into TA cloning vector (TOPO TA cloning kit; Invitrogen, Carlsbad, CA) and sequenced.

Plasmid construction for replication model (replicon). Using DNA extracted from patient 2, two overlapping fragments, fragment A and fragment B, covering the full HBV genome of approximately 1,700 bp, were amplified by nested PCR. The primers used for fragment A were E0010S (nucleotides [nt] 10 to 39, 5′-ATTCCACCAAGCTCTGCTAGATCCCAGAGT-3′) and E1813R (nt 1783 to 1813, 5′-GGTGCTGGTGCGCAGACCAATTTATGCCTA-3′) for the first PCR and B0046S-C (nt 10 to 39, 5′-ATTCCACCAAGCTCTGCTAGATCC CAGAGT-3′) and B1760R-C (nt 1755 to 1731, 5′-TAATCTCCTCCCCCA ACTCCTCCCA-3′) for the second PCR. The primers used for fragment B were E1601S (nt 1601 to 1630, 5′-ACGTCGCATGGAGACCACCGT-3′) and E0266R (nt 262 to 232, 5′-ATGGCGTCTCAGATCTGAGCACCACCT GAA-3′) for the first PCR and E1601S and B0207R-C (nt 207 to 178, 5′-CCC GCCTGTAATACGAGCAGGGGTCCTAGG-3′) for the second PCR. These fragments were then ligated into pGEM-T vector (Promega, Madison, WI) and cloned in DH5α cells. Ten clones each (pGEM-fragA-1 to -10 and pGEM-fragB-1 to -10) were obtained, and the nucleotide sequences were determined. Of these, pGEM-fragA-3 and pGEM-fragB-2, with consensus sequences and without core deletion or replacement mutation (not major clones), were used as templates to construct HBV replicons. To produce fragment C, pGEM-fragA-3 and pGEM-fragB-2 were mixed and amplified with primers E1039F-HindIII and E2168R. The PCR product was digested with HindIII and AvrII, and fragment C-B-HindIII-AvrII was produced. The PCR product was also cloned, and pGEM-fragA-C was produced. Finally, the fragments C-B-HindIII-AvrII and pGEM-fragA-C, cut with AvrII and SacI, were cloned into pUC19 without promoters (Invitrogen) cut with HindIII and SacI, and a pUC19-HBV/E wild-type replicon encoding a replication-competent 1.35-unit-length HBV genome was produced. In addition, the pUC19-HBV/E wild-type replicon was digested by RsrIII and XbaI, the fragment with the replacement mutation from patient 2 (strain UK2), cut with RsrIII and XbaII, was ligated, and a pUC19-HBV/E replacement replicon was produced.

Cell culture and DNA transfection. HuH-7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. For the standard replication assay, 10-cm-diameter dishes were seeded with 1×10^6 HuH-7 cells per dish. Sixteen hours postseeding, cells were transfected with 5 μg of DNA construct using the Fugene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) and harvested 3 days later. Transfection efficiency was measured by cotransfection of 1 μg of reporter plasmid expressing secreted alkaline phosphatase and determination of secreted alkaline phosphatase enzymatic activity in the cell culture supernatant.

Isolation of core-associated HBV DNA from transfected cells. HBV DNA was purified from intracellular core particles by a method described by Turelli et al. (41), with minor modifications. Briefly, cells were suspended in 1.5 ml of lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 1% NP-40. Nuclei were pelleted by centrifugation at 4°C and 15,000 rpm for 5 min. The supernatant was adjusted to 6 mM Mg acetate and treated with 200 μg/ml of DNase I and 100 μg/ml of RNase A for 2 h at 37°C. The reaction was stopped by the addition of EDTA to a final concentration of 10 mM, and then the mixture was incubated for 10 min at 65°C. Proteins of the sample were digested with 200 μg/ml of proteinase K, 1% sodium dodecyl sulfate, and 100 mM NaCl for 2 h at 37°C. Nucleic acids were purified by phenol-chloroform (1:1) extraction and ethanol precipitation after the addition of 20 μg of glycogen.

Preparation of total RNA. Transfected cells were lysed by ISOGEN (Nippon Gene, Japan). After the addition of 500 μl of chloroform and 15 min of incubation on ice, the lysates were centrifuged for 15 min at 15,000 rpm. The aqueous phase was precipitated with isopropanol. Total RNA was pelleted by centrifugation, washed with ethanol, and dissolved in water.

Southern and Northern blot hybridization. Isolated core-associated HBV DNAs were separated on a 1.2% agarose gel. Twenty micrograms of total RNA was separated on a 1% agarose-formaldehyde gel. DNAs and RNAs were transferred to a positively charged nylon membrane (Roche Diagnostics, Germany) and hybridized with either an alkaline phosphatase-labeled full-length HBV fragment or a 1.3-kb GAPDH (glyceraldehyde-3-phosphate dehydrogenase) cDNA fragment generated with a Gene Images AlkPhos direct labeling system (Amersham Biosciences, United Kingdom). The detection was performed with CDP-Star (Amersham Biosciences, United Kingdom). The signals were analyzed by using a LAS-1000 image analyzer (Fuji Photo Film, Japan).

Hepatic expression of HBcAg. A liver biopsy was performed as part of a routine diagnostic assessment, and the grade of inflammation and fibrosis stage were scored according to established criteria (14). The expression of HBcAg in hepatocytes was determined by immunoperoxidase staining using rabbit polyclonal anti-HBc as a primary antibody (22) and an EnVision detection kit (Dako Ltd., Ely, England). A semiquantitative assessment of the immunoreactivity was carried out by scoring the proportion of positive cells in four microscopic fields at a magnification of ×250 (23).

Immunofluorescence assay for HBV core protein. At 3 days posttransfection, monolayer cultures on coverslips were washed with phosphate-buffered saline three times before fixation. The cells attached to the coverslips were fixed in ice-cold acetone-methanol (1:1) for 10 min. After blocking using antibody diluent (Dako Co., Carpinteria, CA), hepatitis B core antigen (HBcAg) was stained with a diluted mouse monoclonal antibody (Hyb-3120; Institutes of Immunology, Tokyo, Japan) which recognizes a capsid conformation-specific epitope (6). Goat anti-mouse immunoglobulin G-fluorescein isothiocyanate was used as a secondary antibody for the experiments. The nuclei of cells were counterstained with 10 μg of 4′,6′-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO). The results were visualized under an ECLIPSE E800M fluorescence

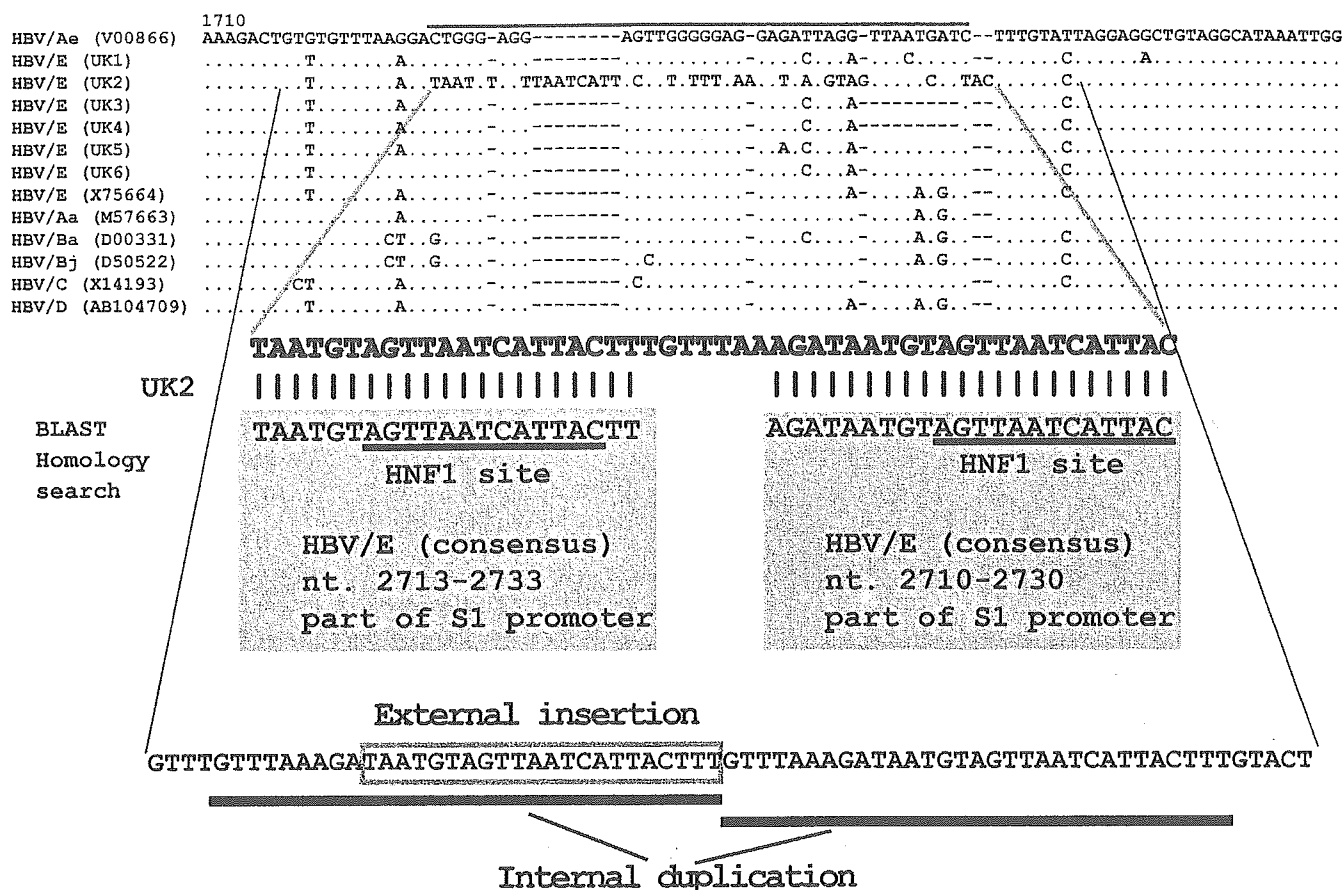

FIG. 1. Nucleotide alignment of the six HBV genotype E sequences of the core upstream regulatory region to the basic core promoter region, with references to other genotypes and subtypes. The sequence enlarged under the alignment shows the UK2-specific 50-nucleotide sequences which were analyzed by BLAST homology search. The 1st to 21st nucleotides and 29th to 50th nucleotides matched the conserved sequences of HBV genotype E, nt 2713 to 2733 and nt 2710 to 2730, shown in shaded rectangles. Furthermore, the consequences of mutations which occurred in the UK2 sequence are shown. The normal CURS/BCP sequences are deleted, the S1 promoter, including the HNF1 site, is externally inserted, and internal duplication occurs.

microscope (Nikon, Tokyo, Japan) and a Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss, Germany).

Molecular evolutionary analysis. The complete sequences of the HBV/E strains isolated from six patients (strains UK1 to UK6) were aligned along with the complete HBV genome strains of different genotypes by use of the CLUSTAL W software program (40), and the alignment was confirmed by visual inspection. A homology search for the UK2 unique partial sequence was carried out using NCBI BLAST 2.2.6 (3). The HBV genome database search was conducted with the Hepatitis Virus Database (http://s2as02.genes.nig.ac.jp/).

Nucleotide sequence accession number(s). The sequences reported in this paper have been deposited in GenBank/DDBJ/EMBL databases (accession numbers AB219529 to AB219534).

RESULTS

HBV genome alignment and identification of a specific CURS/BCP sequence. Six complete HBV/E genomes (for strains UK1 to UK6) were aligned with reference sequences of other genotypes (DNA data bank accession no. V00866, X75664, M57663, D00331, D50522, X14193, and AB104709). The alignment of the CURS/BCP region of the HBV genome is shown in Fig. 1. A new 50-nucleotide sequence was identified in UK2, starting from nt 1720.

To clarify whether the region of 50 nucleotides is part of a particular structure or whether it represents a random accumulation of nucleotide substitutions, the 50-nucleotide sequence was examined by NCBI BLAST 2.2.6 (3). The result showed that the first 21 nucleotides match completely the well-conserved HBV/E nt 2713 to 2733, and the last 21 nucleotides completely matched well-conserved HBV/E nt 2710 to 2730. In HBV/E, nt 2706 to 2806 comprise the conserved S1 promoter region where the HNF1 binding site AGTTAA TCATAC is located. Therefore, the insertion of the UK2-specific 50 nucleotides in the HBV genome consists of an HNF1 site (S1 promoter) tandem repeat (Fig. 1).

Replacement mutation. The mechanism of the aforementioned HNF1 site (S1 promoter) tandem repeat was sought next. A detailed inspection of the HBV DNA sequence showed that the mutation comprises three genomic variations. These include a deletion of the normal CURS/BCP sequence, insertion of part of the S1 promoter, and internal duplication of the sequence GTTTAAAGATAATGTAGTTAATCATTACTTT (Fig. 1). The internal duplication starts upstream from the 50-nucleotide BLAST-searched sequence. We therefore named this novel genetic rearrangement a "replacement mutation."

Clinical characteristics of the HBV-infected patient with this HNF1 site replacement mutation. Three of six patients

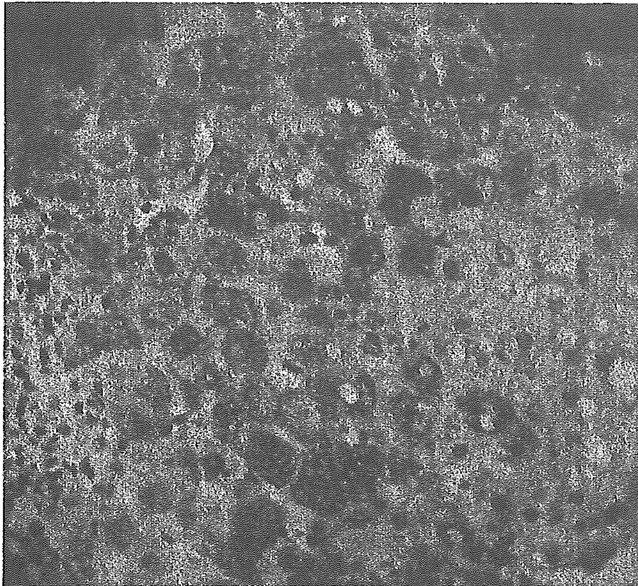

FIG. 2. Immunohistochemical detection of HBcAg in the liver of the patient 2. Strong staining is observed in both nuclei and cytoplasm.

(patients 2, 3, and 4) were seropositive for HBeAg, and the other three (patients 1, 5, and 6) were anti-HBe positive. All three anti-HBe-positive patients had sequences with a double mutation, in nt 1762 and 1764, as well as the precore stop codon mutation (G1896A). Core deletions were observed in sequences from HBeAg-positive patients, and pre-S deletions were noted in anti-HBe-positive patients in this study. Strain UK2 had deletions from nt 2135 to 2308, UK3 from nt 2132 to 2229, and UK4 from nt 1989 to 2051 and nt 2118 to 2219. Among strains from anti-HBe-positive patients with pre-S deletions, UK1 had deletions from nt 44 to 55, UK5 from nt 3168 to 3170, and UK6 from nt 1 to 30, although these patients also had wild-type clones without any deletions, suggesting that these deletions are not always associated with HBeAg production. The patient with the replacement mutation had high HBV DNA levels (1.3×10^8 copies) in serum and active hepatitis (grade 6, stage 2, with an alanine aminotransferase [ALT] level of 429 IU/liter). Immunohistochemical analyses of liver specimens showed that this patient had hepatic HBcAg expression patterns in both nuclei and cytoplasm (Fig. 2) which were quite distinct from those of the other patients (Table 2).

Longitudinal analysis of the replacement mutation. We investigated the evolution of the replacement mutation at nine time points from 1997 to 2003 with the corresponding clinical data. Patient 2 demonstrated the HNF1 site replacement mutation since October 1998, confirmed by direct sequencing of PCR products; however, the results for three earlier samples during 1997 and 1998 were not able to be determined by direct sequencing of PCR products, due to mixed viral populations. Sequencing analysis of 18 clones from the sample from 1997 showed 11 clones (61.1%) with wild-type sequences in CURS/BCP and 7 (38.9%) with the replacement mutation. Of nine clones in May 1998, 5 (55.6%) showed the wild type, 3 (33.3%) the replacement type, and 1 (11.1%) the BCP deletion type. Of an additional 19 clones from October 1998, 4 (21.1%) showed the wild type, 13 (68.4%) showed the replacement type, and 2 (10.5%) showed the BCP deletion type (Fig. 3). No other clinical events, except for initial interferon (IFN) therapy, were observed before 1997. In addition, the progression of the fibrosis stage occurs after the initial IFN therapy and correlates with a viral sequence change from a wild-type and replacement sequence mixed status to a replacement-dominant pattern. The titration of HBeAg decreased and the ratio of replacement increased during the time course.

In vitro study using replication model (replicon). Southern blot analyses of total cellular DNA isolated from HuH-7 cells 72 h after transfection revealed that the pUC19-HBV/E replicon with the replacement mutation, which had the HBV/E construct with an HNF1 site tandem repeat in the CURS/BCP, replicated much more than the pUC19-HBV/E wild-type replicon (Fig. 4A). Northern blot analyses indicated that the replacement mutant with increased replication had higher pregenomic and pre-S/S RNA levels (Fig. 4B).

Immunofluorescence experiments were performed to investigate the intracellular localization of the viral protein products. After transfection of the pUC19-HBV/E wild-type replicon, homogenous cytoplasmic staining for HBcAg was evident, while no nuclear localization could be detected (Fig. 5A and B). In contrast, transfection of the pUC19-HBV/E replacement replicon indicated that HBcAg was localized in the nucleus and perinucleus, and only a faint cytoplasmic staining was found (Fig. 5C and D). These results were confirmed with a confocal laser scanning microscope.

DISCUSSION

We report here an HNF1 site replacement mutation observed in a sequence from a chronic hepatitis patient with HBV/E. This report has dual impacts: one is that this mutation

TABLE 2. HBV DNA levels and HBV genome mutations of the six patients

Patient	HBeAg[b]	HBV DNA levels (copies/ml)	Mutation at nt[a]:		Core deletion[b]	preS deletion[b]	% HBc staining[c]	
			1762 and 1764	1896			Nucleus	Cytoplasm
1	−	2.4×10^4	Variant	Variant	−	+	NT	NT
2	+	1.3×10^8	Rep	No mutation	+	−	75	75
3	+	4.2×10^8	Deletion	No mutation	+	−	5	40
4	+	2.2×10^8	Deletion	No mutation	+	−	1	10
5	−	5.4×10^4	Variant	Variant	−	+	NT	NT
6	−	5.2×10^4	Variant	Variant	−	+	20	0

[a] Rep, replacement mutation.
[b] +, presence; −, absence.
[c] NT, not tested.

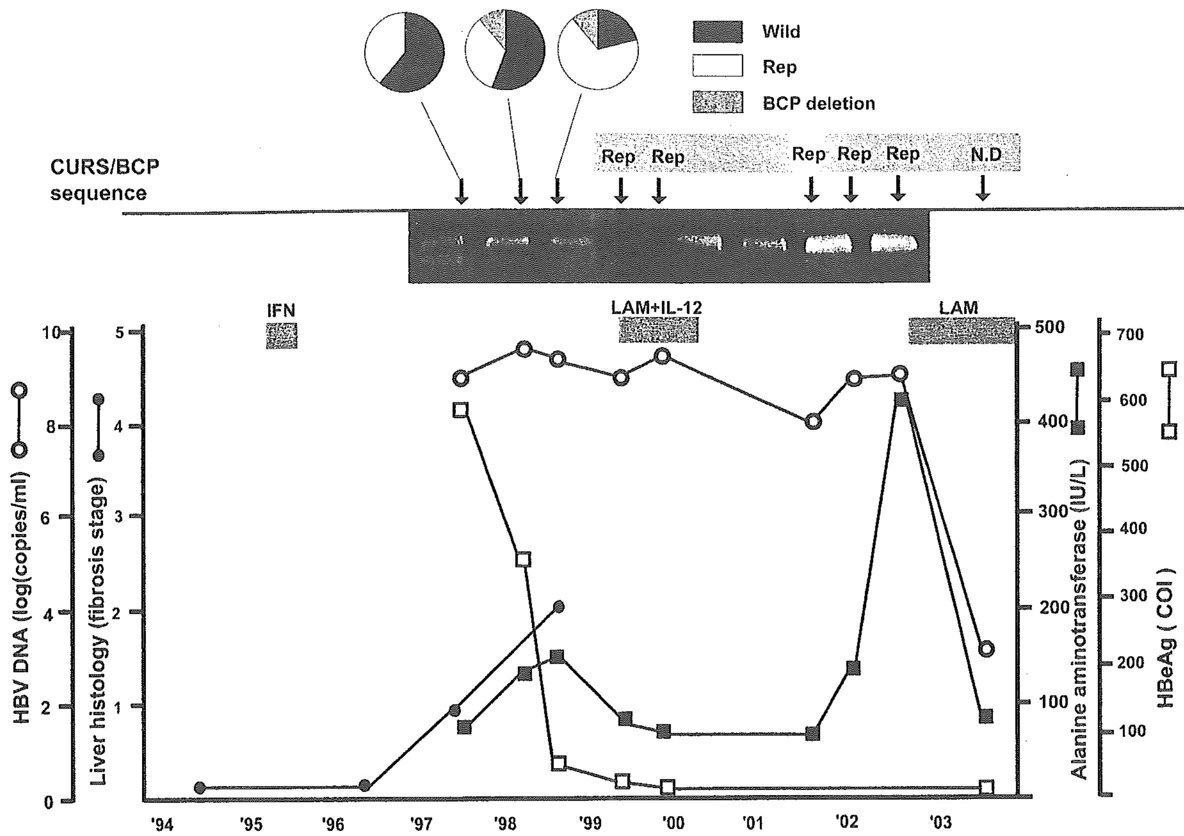

FIG. 3. Clinical course of patient 2 based on HBV DNA levels, fibrosis stage, HBeAg titer, and ALT levels. The three circular charts show how the proportions of wild type, replacement mutation (Rep) type, and BCP deletion type changed during the time course. The electropherogram (5%) shows that mixed types (wild type and Rep) are found during the first three points. ○, HBV DNA levels; ●, fibrosis stage; □, HBeAg titer; ■, ALT levels. The patient was treated with IFN monotherapy and then with the combination therapy of lamivudine (LAM) and interleukin-12 (IL-12). The patient has been treated with LAM since 2003. COI, cutoff index.

is a novel mode of viral mutation arising as a complex of known types of mutations, and the other is that an HNF1 site tandem repeat due to replacement mutation affects not only the RNA transcription and DNA replication efficiency of HBV but also accumulation of hepatitis B core protein in nuclei and cytoplasm of hepatocytes.

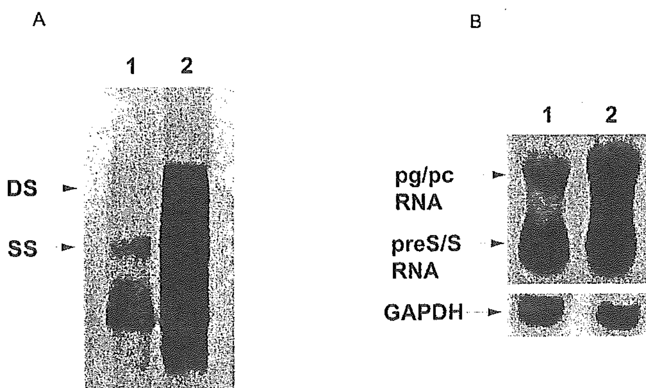

FIG. 4. (A) Southern blot analysis of intracellular replication competence of replacement mutation with HNF1 site. Double-stranded (DS) DNA and single-stranded (SS) DNA are indicated by arrows. The pUC19-HBV/E replacement replicon (lane 2) replicates at a much higher rate than the pUC19-HBV/E wild-type replicon (lane 1). (B) Northern blot analysis of HBV transcripts. Lane 1, pUC19-HBV/E wild-type replicon; lane 2, pUC19-HBV/E replacement replicon. pg/pc RNA, pregenomic/precore RNA. A GAPDH probe was used to quantify RNA in each lane.

There are no previous reports that deletion, remote insertion, and internal duplication were observed in identical portions of the sequence. We provisionally named the novel mode of mutation "replacement mutation," which is defined as a complex of insertion of remote sequence, deletion, and internal duplication in a single sequence portion. We examined 839 HBV strains with CURS/BCP nucleotide sequences which were deposited in the Hepatitis Virus Database (http://s2as02.genes.nig.ac.jp/); however, no similar mutations were found. In addition, we searched 530 complete genomes for this complex mutation in the HCV database; however, no similar mutations were found in any part of the complete genome sequences. This is the first report of replacement mutation as a viral mutation. Though it might be a rare mutation, researchers might have ignored the strange sequences as errors, especially if the strange sequences were seen in otherwise quite conserved parts of nucleotide sequences.

One of the impacts of this replacement mutation on viral mutation is that the virus will undergo large nucleotide changes in a relatively short period, and they are drastic changes compared with other mutations. Accumulation of point mutations, which occurs frequently, accounts for intragenotypic differences. One replacement mutation could cause a drastic change

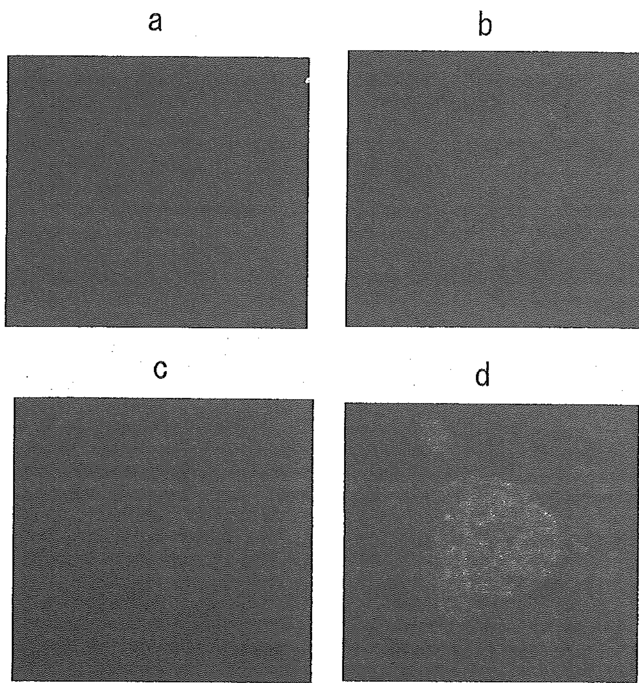

FIG. 5. Intracellular localization of HBcAg (magnification, ×400). Immunofluorescence staining of HBcAg in HuH-7 cells transfected with (a) pUC19-HBV/E wild-type replicon and (c) pUC19-HBV/E replacement replicon. (b and d) Nuclear staining for the same set of cells was performed by use of DNA staining. DAPI stain shows as red.

to the genomic sequence, which might explain the evolutionary events.

In this report, CURS/BCP was replaced with a part of the S1 promoter covering the HNF1 site. The S1 promoter originally is a large surface antigen promoter of approximately 100 bp, and this promoter includes the binding site for the liver-specific transcriptional factor HNF1 (28, 33). Without the HNF1 site, S1 promoter activity is reduced 10- to 20-fold (31, 32), indicating that the HNF1 site is accepted as the key part of the S1 promoter. There have been reports of insertion of an HNF1 site in BCP among HBV-infected patients with various clinical statuses (10, 19, 20, 30). One study has shown that HNF1 site insertion caused enhanced viral replication in a fulminant-hepatitis patient, and the accumulation of massive amounts of cytoplasmic and nuclear HBcAg was observed in infected hepatocytes (30). These data support our results that the HNF1 site replacement mutation causes accumulation of massive amounts of cytoplasmic and nuclear HBcAg in liver immunostaining and enhances viral replication on the basis of an in vitro replication model.

In our results, core deletions in sequences from three HBeAg-positive patients and pre-S deletions in sequences from three anti-HBe-positive patients were observed. Core deletions are reported to be related to the presence of HBeAg and seroconversion in the near future (43). In an in vitro experiment, the core deletion type can replicate more efficiently than the wild type when complemented with wild-type core protein, and the replication enhancement by core deletion is not through the enhancement of transcription (11). Although a minor wild-type clone might enhance the replication of a core deletion clone, HNF1 binding seems to be the major cause of replication enhancement. It is because only strain UK2 has a distinctively high percentage of both nuclear and cytoplasmic core protein expression, though all three HBeAg-positive patients demonstrated core deletions in the middle of core ORFs. Furthermore, the in vitro experiment shows that an HNF1 site tandem repeat enhances core/pregenomic RNA transcription (Fig. 4) when a part of the CURS/CP of the wild-type HBV/E replication clone was replaced by the HNF1 binding tandem repeat of UK2. Pre-S deletion is observed in cases of long-lasting HBV chronic infection and advanced liver diseases (27, 37). As the positions and lengths of pre-S deletions differ in each case, their effects on HBV replication have not been clearly elucidated. Additionally, the HNF1 tandem repeat causes X protein truncation, i.e., 27 amino acids of the C terminus are truncated. The role of X protein in HBV replication is controversial (5, 39). However, X protein truncation is reported to be related to hepatocellular carcinoma (13, 29). Further investigation into the relationship between the HNF1 tandem repeat and hepatocellular carcinoma is needed.

The analysis of serial samples revealed that the HBV population included both CURS/BCP wild-type and CURS/BCP replacement clones from 1997 to October 1998, and the proportion of the replacement type gradually rose and then became predominant. Insertions at the HNF1 site were observed in sequences from patients with immunosuppressive therapy (10, 19, 30); no therapy except IFN was included in this case. Although sequences for many patients undergoing IFN therapy have been reported (7, 12, 18, 42), the HNF1 replacement has not been reported, as mentioned in the database research results. However, most of the database-deposited HBV genome sequences for patients with IFN therapy are genotypes HBV/A, -B, -C, and -D. There have been no reports of HBV/E-related chronic hepatitis patients who were treated with IFN therapy. One possible explanation could be that HBV/E has specific sequences which allow the HNF1 site insertion and the BCP deletion from the wild type. As is known, HBV genotypes are determined by nucleotide differences of more than 8% (25). HBV genotypes reflect not only distinct geographical distributions but also genotype-specific mutation patterns. HBV/C has a higher G1762T/G1764A double mutation rate and a lower G1896A mutation rate than does HBV/B (26). A subgenotype of HBV/A, HBV/Aa (17, 35), which is distributed in Asia and Africa, has subgenotype-specific substitutions just prior to precore ORF start codons (Kozak sequences), and the mutation causes reduction of HBeAg (2, 38).

Though patient 2 has not had a very severe clinical course, probably due to the recent advancement of antiviral therapy, this HNF1 tandem repeat potentially could cause a severe form of hepatitis, as shown in the fulminant-hepatitis case with a single HNF1 site insertion (30). In addition, the HNF1 tandem repeat is correlated with not only HBeAg reduction but also viral replication and progression of liver fibrosis, as shown in our data. Further epidemiological and clinical studies will reveal the impact of the HNF1 tandem repeat on HBV infection.

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T1653 Mutation in the Box α Increases the Risk of Hepatocellular Carcinoma in Patients with Chronic Hepatitis B Virus Genotype C Infection

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Background. Most patients with chronic hepatitis B virus infection become carriers of inactive virus after hepatitis B e antigen seroconversion; however, a subgroup of patients have persistent abnormal transaminase levels and develop hepatocellular carcinoma after seroconversion.

Methods. In an age-matched case-control study, 40 carriers of inactive virus (mean age ± standard deviation [SD], 50.9 ± 11.1 years), 40 patients with chronic hepatitis (mean age ± SD, 50.2 ± 8.9 years), and 40 patients with hepatocellular carcinoma (mean age ± SD, 50.7 ± 9.4 years) who were infected with hepatitis B virus genotype C and had test results positive for antibody to hepatitis B e antigen were analyzed.

Results. The prevalence of T1653 in the box α was significantly higher among patients with hepatocellular carcinoma than among carriers of inactive virus who did not have hepatocellular carcinoma (70% vs. 25%; $P<.0001$) or chronic hepatitis (70% vs. 35%; $P = .003$). Mutations in the basic core promoter region (T1762/A1764) were frequently found in all groups, regardless of clinical status (in 77.5% of carriers of inactive virus, 77.5% of patients with chronic hepatitis, and 90% of patients with hepatocellular carcinoma). In the multivariate analysis, the presence of T1653, an alanine aminotransferase level of ⩾37 U/L, and a platelet count of $<18 \times 10^4$ platelets/mm^3 were independent predictive values for hepatocellular carcinoma (odds ratio [95% confidence interval], 5.05 [1.56–16.35], 12.56 [3.05–51.77], and 11.5 [3.47–38.21], respectively). High α-fetoprotein level was the only independent predictive value for T1653 in patients with hepatocellular carcinoma (odds ratio, 12.67; 95% confidence interval, 1.19–134.17]). Among patients with test results positive for antibody to hepatitis B e antigen who had hepatocellular carcinoma and were infected with different genotypes of hepatitis B virus, the prevalence of T1653 was 40%, 15%, 25%, 25%, 67%, and 23% in patients infected with hepatitis B virus genotypes Aa, Ae, Ba, Bj, C, and D, respectively ($P<.05$ for genotype C vs. genotypes Ae, Ba, Bj, or D).

Conclusions. Our data indicate that the addition of T1653 mutation in the box α to the basic core promoter mutation increases the risk of hepatocellular carcinoma in patients with hepatitis B virus genotype C.

Hepatocellular carcinoma (HCC) is the fifth most frequent cancer and the third leading cause of cancer-related death in the world, with an estimated prevalence of >500,000 cases worldwide per year [1]. It is now accepted that hepatitis B virus (HBV) has a carcinogenic potential in humans. Several mutations in the HBV genome have been reported to occur during the course of persistent viral infection, and there has been increasing evidence of an association between these molecular alterations and the development of HCC in patients with HBV infection.

During persistent HBV infection, carriers frequently undergo seroconversion from hepatitis B e antigen (HBeAg) to the corresponding antibody (anti-HBe). Most patients who acquire chronic HBV infection with HBV genotype C (which is a common genotype in East

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Asian countries) by perinatal transmission become carriers of inactive virus after seroconversion. A subgroup of patients have persistent abnormal serum transaminase levels and develop HCC in the anti-HBe–positive phase. Many of these patients have active viral replication and are infected with several mutant viruses. The association between different clinical events after seroconversion and specific HBV genomic mutations has not been clearly defined.

Mutations in the basic core promoter (BCP) region at nucleotides (nt) 1762/1764 (T1762/A1764) and mutation in the precore (preC) region at nt 1896 (A1896) are associated with seroconversion and persistent viral replication. It is noteworthy that both BCP and preC mutations are often found in patients with advanced liver disease, (e.g., HCC) [2–8]. The T1762/A1764 mutation alters HBeAg production at the transcription level, and the A1896 in the preC region terminates translation of the precursor protein, abrogates HBeAg production, and results in seroconversion. A1896 was also reported previously to be associated with severe forms of chronic liver disease [7,8].

HBV has been classified into 8 major genotypes with use of the complete nucleotide sequence of the viral genome [10]. HBV genotypes not only have distinct geographical distributions [7, 11, 12] but also have different clinical manifestations and responses to therapy (e.g., IFN therapy). Furthermore, HBeAg positivity and levels of HBV DNA, which are controlled by specific mutations, differ between HBV genotypes (e.g., the BCP double mutation is more prevalent among strains of HBV genotype C, followed by HBV genotype A, and the A1896 mutation is frequently found in HBV genotypes B and D) [13–16].

There have been many studies involving viral mutations associated with clinical features, but most previous studies have ignored age, sex, HBeAg status, and HBV genotypes. In Japan, most patients with HCC experience seroconversion (i.e., they are anti-HBe positive) and have HBV genotype C; therefore, we performed an age-matched case-control study among anti-HBe–positive patients infected with HBV genotype C (including carriers of inactive virus, patients with chronic hepatitis, and patients with HCC) to determine the specific HBV genome mutations associated with disease progression.

PATIENTS AND METHODS

Serum samples. Serum samples were obtained from 211 patients from different regional areas worldwide. A total of 120 patients from Japan who were infected with HBV genotype C (40 carriers of inactive virus, 40 patients with chronic hepatitis, and 40 patients with HCC) were matched with control subjects according to age and HBe status. Control serum samples were obtained from patients with HCC who were positive for anti-HBe and who were infected with HBV genotype Aa (10 subjects), Ae (13), Ba (20), Bj (20), C (15), and D (13). Control subjects were from Hong Kong (19 subjects), Japan (36), and the United States (36). The majority of patients infected with HBV genotypes Aa, Ba, Bj, and C were Asian, and the majority of patients infected with HBV genotypes Ae and D were white and black. None of the subjects had serological test results positive for markers of infection with hepatitis C virus or HIV-1.

The study protocol was approved by ethics committees of the participating institutions in accordance with the 1975 Helsinki declaration. Informed consent was obtained from each patient.

Serological assays for HBV markers. HBeAg and anti-HBe were detected by chemiluminescent EIA (Lumipulse *f*, Fujirebio). HBV genotypes were determined by the restriction fragment–length polymorphism method on the S gene sequence amplified by PCR [29] and ELISA with monoclonal antibodies directed to distinct epitopes on the preS2 region products [18], with use of commercial kits (HBV genotype EIA; Institute of Immunology). The genotypes were also confirmed with use of a phylogenetic tree analysis. α-Fetoprotein and serum protein induced by the absence of vitamin K (antagonist II) were examined with use of chemiluminescent EIA.

Amplification and sequencing of the core promoter and the precore region plus core gene. HBV DNA sequences bearing the core promoter and preC or core regions were amplified by PCR with heminested primers by the method described elsewhere [19]. Thereafter, PCR products were sequenced directly with Prism Big Dye (Applied Biosystems) in the ABI 3100 DNA automated sequencer (Applied Biosystems). Accession numbers for all strains are AB236515–AB236634.

Case-control study. A carrier of inactive virus was defined as an HBsAg-positive individual with normal alanine aminotransferase (ALT) levels for a 2-year period (with at least 4 evaluations at 3-month intervals) and without the presence of portal hypertension. Chronic hepatitis was defined as persistent elevation of ALT levels (> 1.5 × upper limit of normal [35 U/L]) during a 6-month period (with at least 3 evaluations at 2-month intervals) without a decrease in platelet count or albumin level, and hypersplenism (splenomegaly on ultrasonographic examination). Twenty-one patients were confirmed to have chronic hepatitis by means of a fine-needle biopsy of the liver. Staging and grading (expressed as mean value ± SD [95% CI]) were 1.24 ± 0.64 (0.99–1.58) and 1.36 ± 0.58 (1.07–1.59), respectively, as previously described [30]. None had received antiviral treatment during the follow-up period. Of 40 patients with HCC, 23 patients received a diagnosis of HCC on the basis of a pathologic examination, and 17 patients received a diagnosis of HCC on the basis of results of abdominal ultrasonography, angiography, CT, or MRI, as well as an elevated serum α-fetoprotein level ($\geqslant$400 ng/mL).

Statistical evaluation. Data were expressed as mean ±

SD. Statistical analyses were performed using χ^2 test and Fisher's exact test for categorical variables. Mann-Whitney U test or 1-way analysis of variance were used for continuous variables, as appropriate. Mantel-Haenszel χ^2 test was used to analyze the trend of frequencies of viral mutations. Multivariate analyses with logistic regression were used to determine the independent factors associated with HCC and T1653. Differences were considered to be significant for P values <.05. The statistical analysis software used was Stata software, version 8.0 (StataCorp).

RESULTS

Table 1 compares ALT level, platelet count, and HBV DNA level, as well as mutations in the box α (enhancer II), core promoter, and preC region, among 40 carriers of inactive virus, 40 patients with chronic hepatitis, and 40 patients with HCC who were infected with HBV genotype C in an age-matched case-control study. ALT and HBV DNA levels were significantly lower among carriers of inactive virus than among patients with chronic hepatitis or patients with HCC ($P<.0001$ and $P = .001$, respectively). Platelet count was lower among patients with HCC than among carriers of inactive virus or patients with chronic hepatitis ($P<.0001$).

The frequency of the T1653 mutation in the box α was significantly higher among patients with HCC (70%) than among carriers of inactive virus (25%) or patients with chronic hepatitis (35%; $P<.0001$) (table 1). Of interest, the T1653 mutation had an opposite correlation with the M1753 mutation. The prevalence of T1762/A1764 was high in all clinical status groups, with no statistically significant difference between groups (table 1). The trend of the frequency of T1653, increasing from carriers of inactive virus to patients with chronic hepatitis to patients with HCC, was analyzed by Mantel-Haenszel χ^2 test (OR, 2.48; 95% CI, 1.59–3.85; $P = .0001$) (figure 1). The trend of the frequency of T1762/A1764 was not statistically significant ($P = .1502$) (figure 1).

The attributable risk of multiple factors, including sex, HBV DNA level, ALT level, platelet count, and the presence of the T1653, M1753, T1762/A1764, and A1896 mutations for HCC in the HBV carriers was determined by multiple logistic regression analysis (table 2). There was a statistically significant association between development of HCC and ALT level >37 U/L (OR, 12.56; 95% CI, 0.55–6.21; $P<.0001$) and platelet count $<18 \times 10^4$ platelets/mm^3 (OR, 11.5; 95% CI, 3.47–38.21; $P<.0001$). The T1653 mutation was still significantly associated with the development of HCC (OR, 5.05; 95% CI, 1.56–16.35; $P = .007$).

The attributable risk of multiple factors, including HBV DNA level, ALT level, platelet count, α-fetoprotein level, protein in-

Table 1. Demographic, clinical, and virologic characteristics of patients infected with hepatitis B virus (HBV) genotype C who were matched for age and hepatitis B e antigen (HBeAg) status.

	Clinical status			
Variable	Carriage of inactive virus (n = 40)	Chronic hepatitis (n = 40)	Hepatocellular carcinoma (n = 40)	P
Male sex	31 (77.5)	37 (92.5)	36 (90)	.171
Age, years	50.9 ± 11.1	50.2 ± 8.9	50.7 ± 9.4	Matched
HBeAg positive	0 (0)	0 (0)	0 (0)	Matched
Anti-HBeAg positive	40 (100)	40 (100)	40 (100)	Matched
HBV genotype C	40 (100)	40 (100)	40 (100)	Matched
Alanine transaminase level, U/L[a]	20.8 ± 7.6	102 ± 108.7	83.2 ± 84.8	.0001
Platelet count, $\times 10^4$ platelets/mm^3[b]	20.7 ± 3.1	17.4 ± 4.1	12.8 ± 5.7	.0001
HBV DNA level, LGE/mL[c]	4.3 ± 0.8	5.9 ± 1.5	5.4 ± 1.5	<.0001
Mutation in the box α: T1653[d]	10 (25)	14 (35)	28 (70)	<.0001
Mutation in the core promoter				
M1753	10 (25)	6 (15)	9 (22.5)	.609
T1762/A1764	31 (77.5)	31 (77.5)	36 (90)	.289
Mutation in the precore region: A1896	25 (62.5)	26 (65)	25 (62.5)	1.0

NOTE. Data are no. (%) of patients or mean value ± SD. Anti-HBeAg, antibody to HBeAg; LGE, log genome equivalents.

[a] $P<.0001$ for carriers of inactive virus vs. patients with chronic hepatitis; $P = .002$ for carriers of inactive virus vs. patients with hepatocellular carcinoma.

[b] $P<.0001$ for patients with hepatocellular carcinoma vs. carriers of inactive virus or patients with chronic hepatitis; $P = .002$ for carriers of inactive virus vs. patients with chronic hepatitis.

[c] $P<.0001$ for carriers of inactive virus vs. patients with chronic hepatitis; $P = .001$ for carriers of inactive virus vs. patients with hepatocellular carcinoma.

[d] $P<.0001$ for carriers of inactive virus vs. patients with chronic hepatitis; $P = .001$ for carriers of inactive virus vs. patients with hepatocellular carcinoma.

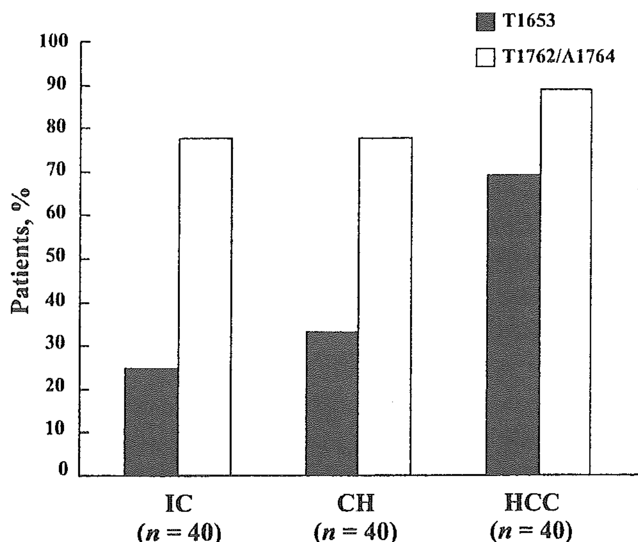

Figure 1. Prevalence of T1653 box α and T1762/A1764 basic core promoter mutations among patients with chronic hepatitis B virus infection, stratified by clinical status. The trend of the frequency of the T1653 mutation was analyzed by Mantel-Haenszel χ^2 test. The OR estimate is an approximation of the OR for carriers of inactive virus (IC), patients with chronic hepatitis (CH), and patients with hepatocellular carcinoma (HCC) having a strain with the mutation (OR, 2.48; 95% CI, 1.59–3.85; $P = .0001$). The trend of the frequency of the T1762/A1764 mutation was not statistically significant according to the Mantel-Haenszel χ^2 test ($P = .1502$).

duced by the absence of vitamin K (antagonist II) level, for T1653 in patients with HCC with HBV genotype C infection was determined by multiple logistic regression analysis (table 3). An α-fetoprotein level >300 ng/mL was the only independent predictive value for the presence of the T1653 mutation in patients with HCC with HBV genotype C infection (OR, 12.67; 95% CI, 1.19–134.17; $P = .035$).

Table 4 compares sex, age, and mutations in the box α, core promoter, and preC region among patients infected with HBV genotypes Aa (10 patients), Ae (13), Ba (20), Bj (20), C (15), and D (13) with the same variables among patients with HCC. Mean age was significantly higher among patients with HBV genotype Bj infection, compared with patients with HBV genotype Ba, genotype C, and genotype D infection ($P < .05$). The prevalence of T1653 among patients with HBV genotype C infection (66.7%) was significantly higher than it was among patients infected with other genotypes (15%–25%; $P < .05$), excluding patients infected with HBV genotype Aa. The prevalence of T1762/A1764 among patients with HBV genotype Ba infection (85%) and HBV genotype C infection (86.7%) was also significantly higher than it was among patients infected with other genotypes (20%–50%; $P < .05$). The prevalence of A1896 among patients with HBV genotype Aa infection and HBV genotype Ae infection was significantly lower than it was among patients infected with other genotypes ($P < .05$).

DISCUSSION

Many previous studies have reported that the clinical course of chronic HBV infection may be modified by several specific viral mutations [5, 20, 21], although the significance of such specific mutations in patients with chronic hepatitis B remains controversial. Because most studies have not controlled for different variables, such as age, HBV genotype, and HBe status, it is unknown whether the mutations were associated with disease progression, greater age of the patient, the specific HBV genotype or subtype, or HBe status. In this study, to exclude any biases, we performed an age-matched case-control study involving only anti-HBe–positive patients infected with HBV genotype C.

In the present case-control study, the prevalence of T1653 was found to be significantly higher among patients with HCC, compared with carriers of inactive virus and patients with chronic hepatitis with HBV genotype C infection; however, the prevalence of T1762/A1764 was high in all clinical status groups. During the anti-HBe–positive phase of infection, T1653 was more reliable than T1762/A1764 as a predicting factor for

Table 2. Multivariate analysis of variables with independent predictive value for development of hepatocellular carcinoma among a group of 120 patients with hepatitis B virus infection.

Variable	OR (95% CI)	*P*
Sex		
Female	1	
Male	5.06 (0.85–30.15)	.075
HBV DNA level		
<4.8 LGE/mL	1	
⩾4.8 LGE/mL	0.34 (0.09–1.21)	.096
Alanine transaminase level		
<37 U/L	1	
⩾37 U/L	12.56 (3.05–51.77)	.0001[a]
Platelet count		
⩾18 × 10^4 platelets/mm^3	1	
<18 × 10^4 platelets/mm^3	11.51 (3.47–38.21)	.0001[a]
T1653 mutation		
No	1	
Yes	5.05 (1.56–16.35)	.007[a]
M1753 mutation		
No	1	
Yes	1.23 (0.31–5.04)	.770
T1762/A1764 mutation		
No	1	
Yes	2.67 (0.57–12.54)	.214
A1896 mutation		
No	1	
Yes	0.96 (0.29–3.11)	.943

NOTE. Each OR was adjusted for age and other variables in the analysis. LGE, log genome equivalents.
[a] Statistically significant.

Table 3. Multivariate analysis of variables with independent predictive value for the presence of the T1653 mutation among 40 patients with hepatocellular carcinoma.

Variable	OR (95% CI)	P
HBV DNA level		
<4.9 LGE/mL	1	
≥4.9 LGE/mL	0.89 (0.16–4.79)	.899
ALT level		
<53 U/L	1	
≥53 U/L	1.72 (0.29–9.96)	.541
Platelet count		
≥12 × 10^4 platelets/mm^3	1	
<12 × 10^4 platelets/mm^3	1.39 (0.28–7.02)	.683
α-Fetoprotein level		
<300 ng/mL	1	
≥300 ng/mL	12.67 (1.19–134.17)	.035[a]
PIVKA-2 level		
<50 mAU/mL	1	
≥50 mAU/mL	0.25 (0.05–1.43)	.120

NOTE. Each OR was adjusted for age and other variables in the table. PIVKA-2, protein induced by the absence of vitamin K (antagonist II).

[a] Statistically significant.

the development of HCC. In fact, in the multivariate analysis, the presence of T1762/A1764 was not an independent predictor of HCC, but ALT level >37 U/L, platelet count <18 × 10^4 platelets/mm^3, and the presence of T1653 were independent predictors of HCC. The T1653 mutation had also been reported by Takahashi et al. [17]; they reported that this specific mutation was prevalent among Japanese patients with HCC, although their study was not a case-control study. These results do not deny that T1762/A1764 is associated with hepatocarcinogenesis, because poor prognosis associated with HBV genotype C infection, compared to that associated with HBV genotype B (Ba and Bj) infection, correlated with a high prevalence of T1762/A1764 [2, 9, 16], indicating that the BCP double mutation is associated with a high potential for hepatocarcinogenesis. The appearance of the T1653 mutation after the occurrence of the T1762/A1764 mutation (the T1762/A1764 mutation usually occurs earlier than the T1653 mutation) could indicate that the virulence of HBV is increasing, which could result in the development of HCC. In the multivariate analysis, however, HBV DNA level was no longer a predicting factor for HCC. One of the reasons for this is that the HBV DNA data used in this study were obtained at the time of diagnosis of HCC. A recent prospective study from Taiwan has indicated that high HBV DNA levels at baseline and infection with HBC genotype C were independent predictors for HCC, but the mean viral load at the time of diagnosis of HCC was significantly lower than at baseline [27]. Although our data could not indicate an association between HBV DNA level and hepatocarcinogenesis, if we could measure the HBV DNA level before diagnosis of HCC, it might found to be be a predicting factor for HCC. Furthermore, an examination of the characteristics of patients with HCC who had the T1653 mutation showed that an elevated α-fetoprotein level (≥300 ng/mL) was the only predictor for the development of HCC in patients with the T1653 mutation. It has been reported that α-fetoprotein level is useful not only for diagnosis but also as a prognostic indicator for patients with HCC [22, 23], suggesting that the T1653 mutation might be associated with poor prognosis for patients with HCC.

The prevalence of several mutations among patients with HCC differed from that among patients with different HBV genotypes (Aa, Ae, Ba, Bj, C, and D) (table 4). The prevalence

Table 4. Demographic and virological characteristics of patients with hepatocellular carcinoma who were positive for antibody to hepatitis B e antigen (anti-HBe), by hepatitis B virus (HBV) genotype.

	HBV genotype						
Variable	Aa (n = 10)	Ae (n = 13)	Ba (n = 20)	Bj (n = 20)	C (n = 15)	D (n = 13)	P
Male	10 (100)	12 (92.3)	18 (90)	15 (75)	15 (100)	13 (100)	.10
Age, years[a]	54.4 ± 7.7	55.3 ± 4.4	54.4 ± 14.8	64.9 ± 9.6	47.9 ± 7.6	53.5 ± 8.3	.0002
HBeAg positive	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	Matched
Anti-HBe positive	10 (100)	13 (100)	20 (100)	20 (100)	15 (100)	13 (100)	Matched
Mutation in the box α: T1653[b]	4 (40)	2 (15.4)	5 (25)	5 (25)	10 (66.7)	3 (23.1)	.039
Mutations in the core promoter region							
M1753	3 (30)	3 (23.1)	5 (25)	4 (20)	2 (13.3)	1 (7.7)	.759
T1762/A1764[c]	5 (50)	6 (46.2)	17 (85)	4 (20)	13 (86.7)	5 (38.5)	<.0001
Mutation in the precore region: A1896[d]	0 (0)	0/13 (0)	9/20 (45)	15/20 (75)	9/15 (60)	8/13 (61.5)	<.0001

NOTE. Data are no. (%) of patients or mean value ± SD. HBeAg, hepatitis B e antigen.

[a] $P<.05$ for Bj vs. Ba or D; $P<.0001$ for Bj vs. C.

[b] $P<.05$ for C vs. Ba or Bj or D; $P<.01$ for Ae vs. C.

[c] $P<.05$ for Ae vs. Ba or C; $P<.01$ for D vs. Ba or C; $P<.0001$ for Bj vs Ba or C.

[d] $P<.05$ for Ba vs. Aa or Ae; $P<.005$ for Aa vs. C or D and for Ae vs. Ba or C or D; $P<.0001$ for Bj vs Aa or Ae.

of T1653 was the highest among patients with HBV genotype C infection, followed by those with HBV genotype Aa infection, although the number of patient with HBV genotype Aa infection was too small for any conclusions to be drawn. The prevalence of T1762/A1764 was higher among patients with HBV genotype Ba and HBV genotype C infection than among patients infected with other genotypes. HBV genotype Ba has a sequence that closely resembles that of HBV genotype C in the core promoter region, because it is recombinant HBV between HBV genotype Bj and HBV genotype C from nucleotides 1740 to 2485. Although A1896 was not found in HBV genotype Aa and HBV genotype Ae, as has been reported elsewhere [15], HBV genotype Aa had some specific mutations upstream of the preC initiation codon and encapsidation signal site. Therefore, several HBV genotype–specific mutations would be associated with different mechanisms on seroconversion or HBV replication for each genotype or subtype.

Buckwold et al. [24] reported that T1762/A1764 can no longer bind liver-enriched transcription factors and that the transcription of precore RNA and the expression of HBeAg were reduced. Thereafter, Li et al. [25] reported that this mutation not only removed the nuclear receptor–binding site but also created a hepatic nuclear factor 1 transcription factor–binding site. As for a factor correlated with BCP, the core upstream regulatory sequence, which has a strong stimulation effect on the BCP, was reported. In an earlier article by Yu et al. [28], the box α elements (nucleotides 1646–1668) individually stimulated promoter activity >100-fold. The T1653 mutation converts the box α binding site for CCAAT/enhancer-binding protein and related factors into the perfect palindromic sequence 1648-TCTTATATAAGA, which might enhance binding affinity and core promoter/enhancer II activity. Therefore, it is possible that the mutation in the box α influenced the HBe production and viral replication through the BCP activity. In addition, the T1653 mutation corresponds to an amino acid change from histidine to tyrosine at aa 94 of the X protein, so this alteration of X protein might be hepatocarcionogenesis. Gunther et al. [26] analyzed T1653, T1762, and A1764 mutations in the context of an in vitro study involving wild-type HBV (genotype D, AF043594), and they reported that the preC mRNA and HBeAg secretion was reduced, but the amount of progeny virus DNA in the cells and in the culture medium increased only marginally (if at all), as determined by Southern blot analysis. However, because the genotype was different from that in our study (genotype D vs. genotype C) and the mutant type included not only T1653, T1762, and A1764 mutations but also other mutations in the core promoter, it is possible that some other mutation influenced the results in the earlier study.

In conclusion, the addition of the T1653 mutation in the box α to the BCP mutation increases the risk of HCC in patients with HBV genotype C infection, suggesting that HBV with both the T1653 mutation and the BCP double mutation in patients with chronic hepatitis B should be eradicated by antiviral therapy. Functional analyses of HBV strains with the T1653 mutation are needed in vitro and in vivo.

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A new subtype (subgenotype) Ac (A3) of hepatitis B virus and recombination between genotypes A and E in Cameroon

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Blood samples ($n=544$) from two different populations (Pygmies and Bantus) in Cameroon, West Africa, were analysed. Serological tests indicated that the anti-hepatitis C virus (HCV) prevalence in Bantus (20·3 %) was higher than that in Pygmies (2·3 %, $P<0·0001$), whereas the distribution of hepatitis B virus (HBV) serological markers was equally high in both populations: in total, 9·4, 17·3 and 86·8 % for HBsAg, anti-HBs and anti-HBc, respectively. HBV genotype A (HBV/A) and HBV/E were predominant (43·5 % each) in both populations, and HBV/D was found in a minority (13 %). The preS/S region was sequenced in nine cases (five HBV/A and four HBV/E) and the complete genome in six cases (four HBV/A and two HBV/E). Subsequent phylogenetic analysis revealed that the HBV/A strains were distinct from the subtypes (subgenotypes) described previously, Ae (A2) and Aa (A1), and in the preS/S region they clustered with previously reported sequences from Cameroon. Based on the nucleotide difference from Aa (A1) and Ae (A2), more than 4 % in the complete genome, the Cameroonian strains were suggested to represent a new subtype (subgenotype), designated HBV/Ac (A3). A high (3·9 %) nucleotide divergence in HBV/Ac (A3) strains suggested that the subtype (subgenotype) has a long natural history in the population of Cameroon. One of the HBV/Ac (A3) strains was found to be a recombinant with an HBV/E-specific sequence in the polymerase reverse transcriptase domain. Further cohort studies will be required to assess detailed epidemiological, virological and clinical characteristics of HBV/Ac (A3), as well as its recombinant form.

INTRODUCTION

According to the World Health Organization, hepatitis B virus (HBV) infection is one of the major global public health problems. Of the two billion people who have been infected with HBV worldwide, more than 350 million are at risk of developing cirrhosis and hepatocellular carcinoma due to chronic infection (Kane, 1995).

The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences determined in this study are AB194947–AB194955.

Based on a genomic sequence divergence in the entire genome exceeding 8 %, HBV strains have been classified into seven genotypes, denoted A (HBV/A) to G (HBV/G) (Norder *et al.*, 1994; Okamoto *et al.*, 1988; Stuyver *et al.*, 2000). A possible eighth genotype has been proposed with the tentative designation 'H' (Arauz-Ruiz *et al.*, 2002), which is, however, closely related to genotype F phylogenetically, with a complete genome difference of around 8 % (Kato *et al.*, 2005).

Research on HBV genotypes during the last decade has